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Liste de publications et participation aux conférences

1) **Liste des publications réalisées dans le cadre du projet de thèse :**

- **Articles publiés :**

- Zerrouki H, **Hamieh A**, Hadjadj L, Rolain JM, Baron SA. The effect of combinations of a glyphosate-based herbicide with various clinically used antibiotics on phenotypic traits of Gram-negative species from the ESKAPEE group. Sci Rep. 2024 Sep 9;14(1):21006. doi: 10.1038/s41598-024-68968-6. PMID: 39251613; PMCID: PMC11383965.

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- **A. Hamieh**, **H. Zerrouki**, L. Hadjadj, J-M. Rolain and S. A. Baron. Does 5-enolpyruvylshikimate-3-phosphate synthase class correlate with bacterial sensitivity to glyphosate formulations in vitro, or are other mechanisms involved?

- H. Djahoudi, **A. Hamieh**, R. M. Wasfy, L. Naoun, H. Athamnia, H. Djadi, S. M Diene, K. Amoura, J-M. Rolain. Metagenomic Analysis of Gut Microbiota Diversity Across Treatment Stages in Algerian Patients with Colorectal Cancer.

- T. Vo, **A. Hamieh**, M. Levy, P. Pontarotti, J-M Rolain, and V. Merhej. Genomic features of carbapenem-resistant *Klebsiella pneumoniae* isolated in French Polynesia and novel transposon responsible for $bla_{\text{NDM-1}}$ dissemination.

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- T. Vo, **A. Hamieh**, M. Levy, P Pontarotti, J-M. Rolain, and V. Merhej. Poster. Genomic features of carbapenem-resistant *Klebsiella pneumoniae* isolated in French Polynesia and novel transposon responsible for blaNDM-1 dessimination, 34ème ECCMID Global, 27-30 Avril 2024, Barcelone, Espagne.

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- H. Zerrouki, **A. Hamieh**, L. Hadjadj, J-M. Rolain, S. A. Baron. Poster. Effet des combinaisons de glyphosate et d'antibiotiques sur les bactéries à Gram-négatif du groupe ESKAPEE ». 43ème RICAI, 18-19 Décembre 2023, Paris, France.

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- **A. Hamieh**. Présentation orale. Séminaire intitulé « Glyphosate and bacterial tolerance ». 16 Février 2024, Marseille, France.

- Participation aux réunions « work in progress » chaque mardi à l'IHU Méditerranée Infection, Marseille, (2021-2024).

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Résumé

Le glyphosate est un herbicide largement utilisé et efficace contre de nombreuses plantes indésirables. Cependant, son usage intensif suscite des préoccupations concernant son impact potentiel sur la santé humaine, animale et sur l'environnement. Malgré ces inquiétudes, l'Union européenne a renouvelé l'autorisation de son utilisation en 2023 pour une durée de 10 ans. Le glyphosate est un antimicrobien qui perturbe le métabolisme des bactéries et les conduit à s'adapter pour survivre. Cette thèse de recherche visait à : (i) résumer les connaissances actuelles sur les mécanismes de tolérance au glyphosate chez les microorganismes décrits dans la littérature, (ii) décrire les mécanismes de tolérance au glyphosate chez les bactéries à Gram négatif et (iii) évaluer le rôle du glyphosate dans la résistance aux antibiotiques. On retrouve les trois grands types de mécanismes de tolérance des bactéries aux agents antimicrobiens : des mécanismes d'efflux et d'imperméabilité, un mécanisme de modification de la cible (ici l'*aroA*) et un mécanisme de modification de l'antibiotique (dégradation du glyphosate). Si les mécanismes majeurs ont été identifiés, d'autres mécanismes restent inconnus à ce jour. Les mécanismes de tolérance peuvent être d'origine chromosomique ou médiés par des éléments génétiques mobiles. Durant nos travaux, nous avons identifié un gène *aroA* de classe II plasmidique chez des souches de *Klebsiella* spp. tolérantes à un herbicide à base de glyphosate (HBG) et isolées d'échantillons humains et alimentaires provenant d'une zone géographique utilisant des herbicides et des biocides. L'analyse de ce gène et de son environnement génétique suggère que ce plasmide a été selectionné par l'usage excessif de ces biocides. Nous avons également mené une étude préliminaire afin d'identifier un lien entre tolérance au glyphosate et résistance aux antibiotiques. Bien que nous n'ayons pas mis en évidence de lien entre les deux phénomènes, nous avons identifié certains gènes de résistance uniquement chez les souches isolées de patients exposés au HBG. Des études supplémentaires sont nécessaires pour identifier le rôle du glyphosate dans la résistance aux antibiotiques. Cette thèse démontre l'importance d'étudier la problématique de la résistance aux antibiotiques selon un approche « One Health ».

Mots-clés: Glyphosate, Herbicide à base de glyphosate, CMI-glyphosate, EPSPS, ESKAPEE, Bactérie Gram-negative ; One Health

Abstract

Glyphosate is a widely used herbicide effective against many unwanted plants. However, its intensive use raises concerns about its potential impact on human, animal, and environmental health. This herbicide contaminates various ecosystems and may exert selective pressure on antibiotic resistance genes. Despite these concerns, the European Union renewed its authorization for use in 2023 for a period of 10 years. Glyphosate is an antimicrobial agent that disrupts bacterial metabolism, leading bacteria to adapt for survival. This research aimed to: (i) summarize the current knowledge on glyphosate tolerance mechanisms in microorganisms as described in the literature, (ii) describe glyphosate tolerance mechanisms in Gram-negative bacteria (GNB), and (iii) assess the role of glyphosate in antibiotic resistance. The three major types of bacterial tolerance mechanisms to antimicrobial agents were observed: efflux and impermeability mechanisms, a target modification mechanism (in this case, *aroA*), and a mechanism involving the modification of the antibiotic (glyphosate degradation). Although the main mechanisms have been identified, other mechanisms remain unknown to date. The tolerance mechanisms may be chromosomal in origin or mediated by mobile genetic elements. During our research, we identified a plasmid-encoded class II *aroA* gene in *Klebsiella* spp. strains tolerant to a glyphosate-based herbicide (GBH), isolated from human and food samples originating from a geographical area where herbicides and biocides are widely used. The analysis of this gene and its genetic environment suggests that this plasmid was selected due to the excessive use of these biocides. We also conducted a preliminary study to identify a potential link between glyphosate tolerance and antibiotic resistance. Although we did not find a direct link between these two phenomena, we did identify certain resistance genes exclusively in strains isolated from patients exposed to GBH. Further studies are needed to elucidate the role of glyphosate in antibiotic resistance. This thesis underscores the importance of addressing the issue of antibiotic resistance using a "One Health" approach.

Keywords: Glyphosate, Glyphosate-based formulation, Glyphosate-MIC, EPSPS, ESKAPEE, Gram-negative bacteria, One Health.

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Introduction

Les herbicides sont des produits agrochimiques conçus pour contrôler et éliminer les mauvaises herbes. Ils sont fréquemment utilisés dans les cultures agricoles, les jardins, les rails de train etc…. Ces pesticides jouent un rôle essentiel dans la protection des cultures et l'optimisation des rendements. Ils peuvent être sélectifs, ciblant certaines espèces de mauvaises herbes, ou non sélectifs, détruisant toutes les plantes avec lesquelles ils entrent en contact. Ils sont des piliers de l'agriculture moderne, facilitant la gestion des terres en réduisant la main-d'œuvre nécessaire pour le désherbage manuel.

Ces agents sont utilisés depuis plus d'un siècle (1874-présent) (1), d'abord avec des herbicides inorganiques tels que l'acide sulfurique (H_2SO_4) (2) et le sulfate de cuivre (Cu_2SO_4) (3). Avec le temps, l'introduction sur le marché de nombreux herbicides synthétiques comme le glyphosate, le paraquat, le glufosinate, l'atrazine, etc.., a marqué une avancée significative dans l'agriculture moderne. Ces herbicides synthétiques sont non seulement plus efficaces mais aussi plus faciles d'utilisation, ce qui explique leur succès mondial (4).

Le glyphosate (C3H8NO5P; N-(phosphonomethyl)glycine) est l'herbicide le plus largement utilisé à l'échelle mondiale (5). Cette molécule, synthétisée en 1950 par le chimiste Henri Martin (6), a été brevetée en tant qu'herbicide en 1970 par la société Monsanto (Etats-Unis) (Patent No. : US 3850608A), qui l'a commercialisé sous le nom de Roundup® en 1974 (7). Utilisé depuis plus de cinq décennies, il a révolutionné les pratiques agricoles en raison de son efficacité à éliminer une large gamme de mauvaises herbes tout en augmentant les rendements des cultures (8), devenant ainsi omniprésent sur le marché des produits herbicides, en tant que composant actif de plusieurs formulations commerciales (9). C'est également un puissant chelateur des métaux, qui se lie facilement aux cations divalents (Ca, Cd, Mg, Mn, Fe) et forme des complexes stables (10,11). Cette propriété a été la première à être découverte et brevetée en 1964 par la société Stauffer chemical (Patent No.: US 3160,632 A) (12).

Le glyphosate, en tant qu'un herbicide ciblant les plantes, a été largement adopté en raison de son effet supposé nul sur la santé humaine. Cette perception d'innocuité a encouragé son utilisation massive et généralisée dans les zones agricoles et urbaines, conduisant à l'accumulation de ce produit dans l'environnement. Depuis plusieurs années, des préoccupations ont émergé concernant ses véritables effets sur la santé humaine. Aujourd'hui, son caractère supposé inoffensif est remis en question, suscitant un débat intense sur les risques potentiels associés à son utilisation à grande échelle.

L'utilisation mondiale de glyphosate, estimée à 700 000 tonnes par an (5), est de plus en plus controversée (13). Plusieurs études ont suggéré un lien entre l'exposition au glyphosate et divers problèmes de santé, notamment les cancers (14), les troubles endocriniens (15), et les anomalies congénitales (16). L'ogranisation mondiale de la santé a décidé de classer cette molécule en tant que cancérogène probable de classe 2A en 2015 (14). De plus, le glyphosate peut contaminer les sols, les eaux souterraines et les cours d'eau, menaçant la biodiversité et les écosystèmes aquatiques (17,18).

La société Monsanto a également breveté le glyphosate en 2010 en tant qu'antibiotique (Patent No.: US 7,771,736 B2). L'effet antimicrobien de cette molécule, tout comme son effet herbicide, est médié par l'inhibition de la voie shikimate, une voie métabolique essentielle aux plantes, bactéries, champigons et parasites, qui permet la synthèse des acides aminés aromatiques (tyrosine, tryptophane, et phénylalanine) (19). Plus spécifiquement, c'est un analogue structural du phosphoénolpuryvate (PEP) qui agit en inhibant de manière spécifique une enzyme clé appelée 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19), responsable de la conversion du PEP et du shikimate-3-phosphate (S3P) en 5-enolpyruvylshikimate-3-phosphate (EPSP) (19). L'EPSPS, aussi appellée 3-phosphoshikimate-1-carboxyvinyltransferase, est codée par le gène *aroA*. Chez les champignons, cette enzyme fait partie d'un domaine protéique multifonctionnelle appelé AroM (20).

Il existe quatre classes connues de cette enzyme (classes I, II, III et IV) et une classe non déterminée (21). Ces classes se différencient par des variations dans les séquences d'acides aminés, celles concernant la structure du site actif entraînant des différences dans l'affinité de l'enzyme pour le glyphosate. Plusieurs études ont démontré que l'affinité de l'EPSPS de classe I pour le glyphosate est supérieure à celle de l'EPSPS de classe II (22). Par ailleurs, la faible affinité de la classe II la rend plus tolérante au glyphosate que la classe I (22). Cette caractéristique de la classe II lui a conféré un grand intérêt dans le domaine de la bio-ingénierie pour le développement de plantes génétiquement modifiées (23). Ainsi, la littérature scientifique s'est concentrée sur les enzymes de classe II, en particulier sur celles provenant de souches des genres *Agrobacterium* et *Pseudomonas*, dont certaines ont été brevetées comme séquences transgéniques pour conférer une résistance au glyphosate chez les plantes (23–25). La CP4-EPSPS est la première enzyme de cette classe découverte par Monsanto à partir d'une souche d'*Agrobacterium sp.* CP4. Cette enzyme a été utilisée pour développer plusieurs cultures transgéniques, telles que le maïs, le soja, le coton, l'alfalfa, etc… (https://www.isaaa.org/gmapprovaldatabase/). Pendant de nombreuses années, la plupart des travaux scientifiques dans le domaine se sont limités à l'étude des activités enzymatiques des EPSPS pour comprendre leur interaction avec leur substrat et leur inhibiteur, le glyphosate. Plusieurs constantes sont ainsi calculées, telles que la constante de Michaelis-Menten (Km) qui indique l'affinité de l'EPSPS pour ses substrats (PEP et S3P), la constante d'inhibition (Ki) qui mesure l'affinité du glyphosate pour l'EPSPS et permet d'évaluer l'efficacité de l'inhibition, ainsi que la vitesse maximale (Vmax) représentant la capacité catalytique maximale de l'EPSPS (26,27). L'identification des microorganismes capables de détoxifier les sols du glyphosate dans le cadre de la bioremédiation de l'environnement a également été l'une des préoccupations des études sur le glyphosate. En revanche, le rôle du glyphosate en tant qu'antimicrobien, ainsi

que les études portant sur son effet sur le microbiote et la résistance aux antibiotiques sont restées confidentielles.

Les effets du glyphosate sur les microorganismes et plus largement sur les microbiotes sont complexes. Cet herbicide peut influencer le microbiote des humains et des animaux, qui régule des fonctions fondamentales telles que la digestion (28), la respiration (29) ou encore l'immunité (15). Une perturbation du microbiote peut entrainer des effets indésirables sur la santé humaine telles que les infections, en favorisant la colonisation par des agents pathogènes. Des études récentes ont montré que le glyphosate impacte non seulement le microbiote intestinal humain (30), des abeilles (31), et des rats (32), mais également le microbiome du sol (33–35) et de l'environnement domestique (36). La tolérance des microorganismes au glyphosate, peut être soit intrinsèque, soit acquise. La tolérance intrinsèque se manifeste par le fait que certains microorganismes possèdent des enzymes EPSPS naturellement tolérantes au glyphosate (classes II, III et IV) (21,37), ou bien des gènes qui sont capables de coder pour des enzymes qui dégradent le glyphosate (38). En revanche, la tolérance acquise est due à des mutations affectant l'EPSPS, généralement celles appartenant à la classe I, ou à des mutations au niveau des pompes d'efflux, entraînant une régulation accrue de l'élimination du glyphosate et réduisant son effet inhibiteur sur l'EPSPS. Cette tolérance est mesurée notamment pour comprendre comment le glyphosate peut affecter la dynamique des populations microbiennes et leur capacité à y résister. Dans la littérature, la détermination de la concentration minimale inhibitrice (CMI) au glyphosate a été la méthode utilisée pour évaluer la sensibilité de chaque microorganisme à cet herbicide (39).

Outre l'impact de cet herbicide sur le microbiote, son influence sur la résistance aux antibiotiques mérite également une attention particulière. Plusieurs études ont émis l'hypothèse que le glyphosate favorise la sélection de bactéries résistantes aux antibiotiques. Cependant, la littérature présente des résultats discordants à ce sujet. Tandis qu'une étude a montré que le

glyphosate n'affecte pas la résistance aux antibiotiques (40), d'autres travaux ont suggéré que le glyphosate pourrait faciliter le transfert latéral de gènes de résistance à médiation plasmidique (41,42) et enrichir les sols en gènes de résistance aux antibiotiques (Antibiotic resistance gene ARGs) ainsi qu'en éléments génétiques mobiles (mobile genetic element MGEs) (41). Une analyse métagénomique des communautés bactériennes dans des mécosomes supplémentés avec un herbicide à base de glyphosate (herbicide-based glyphosate HBG), montre que des doses élevées sélectionnent les ARGs, en particuler les pompes à efflux multidrogue (43). Quant à l'effet mutagène de cette molécule, un effet concentration-dépendant a été observé pour des souches d'*Escherichia coli*. D'une part, le glyphosate, à des grandes concentrations (6.7-0.67 g/L), supprime l'effet mutagène, et d'autre part à des concentrations minimes (0.0067 g/L), l'effet mutagène augmente (44).

Dans ce contexte, l'objectif de ma thèse est de définir les mécanismes de tolérance au glyphosate chez les bactéries et d'évaluer le lien entre tolérance au glyphosate et résistance aux antibiotiques.

La première partie de cette thèse consiste en une revue de la littérature, dont l'objectif était de faire un bilan des connaissances sur les mécanismes de tolérance au glyphosate chez les microorganismes **(Article 1)**.

La deuxième partie de cette thèse porte sur l'étude des mécanismes de tolérance au glyphosate chez des souches bactériennes de différentes origines. En premier lieu, nous avons voulu étudier le mécanisme de tolérance au HBG de souches du genre *Klebsiella* isolées d'échantillons humains et alimentaires. Les échantillons humains ont été prélevés chez des fermiers travaillant dans des fermes utilisant du glyphosate en Thaïlande. L'analyse des génomes de ces souches a révélé la présence d'un gène *aroA* codant pour une EPSPS de classe II, en supplément du gène EPSPS de classe I constitutif présent dans les génomes de *Klebsiella* spp. Cet *aroA* de classe II, trouvé sur un transposon composite au niveau plasmidique était

associé à un gène codant une protéine appartenant à la famille des Major Facilator Superfamily (MFS). La caractérisation de ces gènes a montré que le HBG n'est pas la seule molécule ciblée, ce qui nous a conduit à tester d'autres molécules herbicides et antiseptiques (**Article 2**). En deuxième lieu, il nous paraissait important de redéfinir la notion de résistance au glyphosate dans la littérature. En effet, la définition de tolérance au HBG est basée principalement sur l'activité enzymatique des EPSPs et non sur la CMI. Nous avons voulu évaluer la corrélation entre ces deux méthodes d'évaluation, et utiliser une méthode harmonisée pour la détermination de la CMI au glyphosate. Pour cela, nous avons sélectionné 60 représentants de 57 espèces bactériennes isolées à l'IHU-Méditerranée Infection et avons effectué des analyses *in vitro* et *in silico* pour évaluer leur tolérance et identifier les gènes qui influencent leur réponse à cet herbicide (**Article 3**).

La troisième partie de cette thèse porte sur l'évaluation d'un possible lien entre le glyphosate et la résistance aux antibiotiques chez les bactéries à Gram négatif (BGN). Nous avons d'abord recherché la présence de BGN tolérantes au glyphosate dans le microbiote digestif de fermiers thaïlandais exposés à différents niveaux de glyphosate et évalué leur niveau de résistance à divers antibiotiques, afin de rechercher une corrélation entre résistance aux antibiotiques et tolérance au glyphosate (**Article 4**). Enfin, nous avons évalué l'effet combiné d'un HBG à différents antibiotiques sur des souches bactériennes appartenant au groupe ESKAPEE (**Article 5**).

Chapitre I

Revue de la littérature sur les mécanismes de tolérance au glyphosate chez les microorganismes

Avant-propos

Le développement d'une tolérance au glyphosate constitue un défi croissant pour l'agriculture moderne. Ce phénomène a été largement documenté chez de nombreuses plantes, qui sont les principales cibles de cet herbicide, compromettant ainsi la gestion des cultures et la productivité agricole (45). Cependant, cette tolérance ne se limite pas aux plantes. Divers microorganismes ont également développé des mécanismes de tolérance au glyphosate similaires à ceux observés chez les plantes, partageant ainsi des voies et des mécanismes de défense communs.

Dans ce chapitre, nous présentons les connaissances actuelles concernant les mécanismes de tolérance au glyphosate chez les microorganismes. Ces mécanismes incluent des mutations du gène cible ou de gènes impliqués dans la voie du shikimate, des mutations d'enzymes permettant la dégradation du glyphosate ou encore de mécanismes d'efflux.

Des mutations dans le gène *aroA*, codant pour la cible du glyphosate, l'EPSPS, ont été bien documentées et montrent comment ces altérations permettent à l'enzyme de maintenir sa fonction même en présence de glyphosate. Ces mutations, en modifiant la conformation de l'EPSPS, réduisent l'affinité de l'herbicide pour sa cible, contribuant ainsi à la tolérance (46). D'autres gènes indirectement liés à la voie du shikimate, tels que *ppsR* et *gpmA*, ont également été impliqués dans la variation de la tolérance au glyphosate (47). Un autre mécanisme de tolérance au glyphosate réside dans la capacité de certaines bactéries à dégrader cette molécule en composés moins toxiques (48). Plusieurs gènes, tels que *glpA*, *gox*, et *thiO*, ont été identifiés pour leur rôle dans la dégradation du glyphosate (49–51). Les mécanismes d'efflux jouent également un rôle majeur dans les processus de tolérance au glyphosate et sont rappelés dans ce travail (52). Il est important de souligner qu'il existe des mécanismes encore largement méconnus tel que le rôle de l'opéron *phn*

impliqué dans la dégradation des phosphonates tels que le glyphosate (53). L'étude de ces mécanismes de tolérance sont à la base du développement d'organismes génétiquement modifiés résistants au glyphosate ou encore de la recherche pour la bioremédiation des environnements. Enfin, nous réalisons dans une dernière partie la synthèse des techniques d'évaluation de la tolérance au glyphosate *in vitro*, telles que la détermination de la concentration minimale inhibitrice (CMI), et discutons de la nécessité d'harmonisation ces pratiques.

Article 1

Review: Microbial mechanisms of tolerance to glyphosate

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En cours de préparation

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1- Introduction

2- EPSPS and microbial glyphosate tolerance, glyphosate and its target: Understanding the interaction

3- Mutations in genes encoding proteins indirectly linked to the shikimate pathway increase glyphosate resistance: *ppsR* and *gpmA*

4- Glyphosate degradation

5- Efflux pump, *gltT* and *gltP*

6- Glyphosate-MIC determination : from in silico to in vitro resistance

7- Conclusions and perspectives

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Abstract

Glyphosate is a widely used non-selective herbicide effective against unwanted plants, making it an essential tool in modern agriculture and land management. However, its environmental and health impacts, including potential carcinogenicity and ecological disruptions, have led to greater control of its use. Despite its primary function as an herbicide, glyphosate exhibits antimicrobial properties by disrupting the shikimate pathway, which is crucial for synthesizing aromatic amino acids. This review explores the microbial mechanisms of glyphosate tolerance, focusing on the interactions between glyphosate and the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, which is the primary target of glyphosate. We examine mutations in genes that indirectly impact the shikimate pathway and contribute to glyphosate tolerance. Additionally, we report glyphosate degradation mechanisms and the role of efflux pumps, as well as methodologies for determining the minimum inhibitory concentration (MIC) of glyphosate using both *in silico* and *in vitro* approaches. This comprehensive analysis aims to enhance understanding of microbial glyphosate tolerance mechanisms and inform future research directions in this field.

Keywords: Glyphosate, glyphosate-based herbicide, glyphosate-MIC, EPSPS, glyphosate degradation, Efflux pumps

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1. Introduction

Glyphosate (N-phosphonomethylglycine, $C_3H_8NO_5P$) is an iconic molecule with herbicide properties widely used in modern agriculture and land management practices. It was first synthetized in 1950s by the swiss chemist Henry Martin and used as a chelating agent (1,2). Launched in the 1970s by Monsanto and commercialized in the 1976s by the same company, glyphosate has become one of the most effective weed control applications, used in both agricultural and non-agricultural fields such as forestry, landscaping, and weed control along roadsides and railways (3). Its success is attributed to its broad-spectrum properties, which enable it to target a wide range of undesired plants while allowing valuable crops to thrive. In addition, it is much less expensive than other herbicides on the marketplace. However, this multifaceted herbicide has harmful effects on the environment and human health (4–6). In 2015, the World Health Organization classified glyphosate as probably carcinogenic to humans (class 2A). Studies have suggested links between glyphosate exposure and endocrine disorders, developmental abnormalities, and cancers in human (7–9). Additionally, glyphosate can contaminate soils and aquatic ecosystems, leading to adverse effects on biodiversity and ecological cycles (7,10,11). Regarding bacteria, while glyphosate is primarily designed to target specific metabolic pathways in plants, research has revealed that it can also impact the composition and function of bacterial communities in soil and animal gastrointestinal tracts. Recent studies have highlighted that glyphosate affects honeybees microbiota (12), causes changes in bacterial catabolic activity (13), and induces the transfer of mobile genetic elements between bacteria (14–16). This effect on bacteria had previously been observed by the Monsanto company, which had consequently filed a patent (Patent No.: US 7771736) to utilize glyphosate as an its anti-bacterial, anti-parasitic and anti-fungal agent in 2010 (17–20).

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Glyphosate acts by disrupting the shikimate pathway, a vital biochemical process responsible for the synthesis of essential aromatic amino acids in plants and micro-organisms. It targets the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19) and acts as a competitive inhibitor of the enzyme. Various classes of this enzyme exist in both bacteria and plants, some of them conferring higher levels of tolerance to glyphosate. This property has been exploited to develop glyphosate-resistant plants through the insertion of a gene encoding EPSPS from a glyphosate-tolerant strain into plants. Microorganisms also developed direct and indirect mechanisms that affect glyphosate activity. Of these mechanisms, the modification of its target (EPSPS) and the degradation of glyphosate were the most investigated.

In this review, we will give an overview of the current knowledge of microbial mechanisms associated with tolerance to glyphosate. In a first part, we will present the interaction of the EPSPS enzyme with glyphosate, as well as mutations in genes associated with the shikimate pathway. We will also examine glyphosate degradation processes, highlighting the significance of efflux pumps. Additionally, we will describe in silico and in vitro approaches for determining the minimum inhibitory concentration (MIC) of glyphosate. These comprehensive analyses will provide a more precise understanding of the underlying mechanisms of glyphosate tolerance in microorganisms.

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2. Glyphosate and Its Target, EPSPS: Understanding the Interaction

The 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC2.5.1.19), also known as 3 phosphoshikimate-1-carboxyvinyltransferase, is a fundamental enzyme of the shikimate pathway (21). Encoded by the *aroA* gene, it serves as a nexus for the biosynthesis of vital aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan. The EPSPS catalyzes the formation of 5-enolpyruvylshikimate-3-phosphate (EPSP) from shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP). In plants and bacteria, EPSPS protein (approximatively 450 amino acid length) forms a single domain, whereas in fungi, EPSPS proteins consist of multiple domains (two to eight different domains) and may attain 3,206 amino acids in length (22). This complex protein structure in fungi make the understanding of the glyphosate effect more complex and unclear until now (23). However, it appears that one of the domains of the EPSPS enzyme in fungi is similar to that found in bacteria. This has been demonstrated for instance in S*accharomyces* cerevisiae (strain ATCC 204508), where, among the six domains that compose its EPSPS, one domain is similar to that of *Escherichia coli* K-12 (Figure 1).

EPSPS enzymes are categorized into four distinct known classes (I, II, III, and IV) based on several key factors, including their tolerance level to glyphosate, the degree of similarity in amino acid sequences to *E. coli*, and the Michaelis-Menten kinetic constant (Km) of PEP. The majority of microorganisms predominantly possess class I EPSPS enzymes. Specifically, more than 57% of bacteria, fungi, and archaea are characterized by class I EPSPS, which is susceptible to glyphosate action. In contrast, the remaining EPSPS classes impart varying degrees of tolerance to glyphosate. Among these, class II EPSPS is unique to bacteria, whereas class III is a relatively rare class (less than 3%) found in various phyla such as Bacteria, Archaea, Eukaryotes, and Fungi (24). Class IV EPSPS is a recent discovery, isolated by Griffin *et al*. from

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118 *Streptomyces svecius* (25). In addition, between 7.2% and 16% of existing EPSPS sequences
119 remain unclassified. Across all studies citing EPSPS classes, classes I and II were the most
120 extensively explored. The main difference between these two enzymes, revealed with
121 crystallographic analysis, is that residues at the active site of class II EPSPS do not exhibit the
122 same flexibility as those of class I when binding to glyphosate (26).

123 Based on the existing literature and studies conducted on EPSPS sequences, it seems that each
124 EPSPS class is associated to a specific bacterial genus. We constructed a phylogenetic tree of
125 548 EPSPS protein sequences including 541 reviewed sequences from UniProt
126 (https://www.uniprot.org/), four reference sequences and three plant sequences (*Oryza sativa*,
127 *Glycine max*, and *Zea mays*) using FastTree (Figure 2-A). The EPSPS Class database
128 (https://ppuigbo.me/programs/EPSPSClass/) was used to classify them (Supplementary Table). A
129 Venn diagram (27) was then created to illustrate the common bacterial genera across different
130 EPSPS classes (Figure 2-B). Among the 188 bacterial genera analyzed, the majority (93%,
131 175/188) have EPSPS associated with specific classes, with the exception of 13 genera (7%) that
132 are found across two classes. For instance, classes I and II were found in *Halalkalibacterium*
133 *halodurans* DSM 18197 strain. Class I EPSPS was found in the genus *Streptomyces*, in which a
134 new class IV has also been discovered. Among the unclassified classes, four genera (*Aeromonas*,
135 *Burkholderia*, *Chlamydia*, *Corynebacterium*) also carry a class I EPSPS, and four (*Bartonella*,
136 *Coxiella*, *Helicobacter*, *Streptococcus*) a class II. Up until now, there is no information or data
137 on unclassified EPSPS in terms of level of tolerance to glyphosate, activity of the enzyme and
138 similarity with other distinct classes.

139 The susceptibility of the enzyme to glyphosate became a focal point in herbicide research and the
140 development of genetically modified (GM) crops, highlighting its importance in both basic

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biology and agricultural practices. Extensive research involving crystallography, kinetics, and mutational analyses has been conducted on this enzyme. A CP4 EPSPS from Agrobacterium sp. strain CP4, a class II EPSPS with low glyphosate affinity, was the first bacterial sequence introduced into crops by Monsanto, resulting in glyphosate-tolerant commercial crops (28). Other studies have reported novel class II EPSPS sequences isolated from bacteria which have the ability to confer glyphosate resistance in plants. Dong *et al.* show that a *Japonica* rice cultivar transformed with class II EPSPS from *Pseudomonas fluorescens* G2 can tolerate concentrations of glyphosate 20 times higher than those generally recommended for field use (29). According to the International Service for the Acquisition of Agri-biotech Applications (ISAAA) (https://www.isaaa.org/gmapprovaldatabase/), a second bacterial EPSPS (*epsps grg23*) identified from *Arthrobacter globiformis* was employed for the synthesis of GM *Zea mays L.* with the glyphosate tolerance trait.

3. Bacterial mechanisms of tolerance to glyphosate

3.1 Mutations in the EPSPS

Studies have shown that mutations affecting the glyphosate-targeted protein, specifically those at its active site, alter the affinity of EPSPS for PEP, making the enzyme either more or less sensitive to the inhibitory effect of glyphosate. The affinity of the enzyme was consistently assessed in the literature by measuring the enzymatic activity of EPSPS through the production of inorganic phosphate, using the malachite green dye assay (30). Typically, the reaction takes place in a HEPES buffer (pH 7.0), supplemented with varying concentrations of substrates such as S3P and PEP, and purified enzyme (25). Reactions were initiated by adding the enzyme to the substrate mix, and after three to four minutes of incubation at 28 °C, malachite green and ammonium molybdate were added to develop the color. Sodium citrate was then used to quench

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the reaction, and the absorbance was measured at 660 nm after 30 minutes of incubation. In all cases, a blank reaction without S3P was used as a control. The assays were optimized for temperature, pH, and ionic conditions, with variations in substrate and inhibitor concentrations depending on the study. Kinetic parameters such as Km (Michaelis constant) and IC50 (half maximal inhibitory concentration) were calculated using standard Michaelis-Menten kinetics and fitted data models, allowing for the determination of enzyme efficiency and glyphosate inhibition across different bacterial strains (31).

Usually, bacteria adapt to and tolerate glyphosate concentrations through mutations affecting *aroA* gene coding for the EPSPS. However, mutations have been more reported in class II of this enzyme that increasingly enhance the ability of the bacterium to tolerate glyphosate. This observed tolerance is determined by changes in the activity of the enzyme, demonstrated by a reduced affinity of the enzyme for glyphosate. So far, three mutations (G100A, P101L and T355S) in class II have been reported in *Alcanivorax sp.* L27, *Ochrobactrum haematophilum* P6BS-III and *Halothermothrix orenii* H168 respectively (32–34) (Table 3). Zhang et *al.* demonstrate that the leucine at position 105 of *Alcanivorax sp.* L27 EPSPS is crucial for its enzymatic activity (33). Additionally, five conserved regions (GDKX, SAQXK, RXHXE, NXTR, and RPMXR (35–37), with X represents the non-conserved amino acid, and several conserved residues Arg127, Pro128, Met129, Arg131, Glu-354, and Arg-357 within this class II were described (35,38). These conserved motifs and residues are important because they play a significant role in the PEP/glyphosate binding to EPSPS and mutations affecting these regions may induce the total loss of enzyme activity. Tian *et al.* found two other important residues in class II EPSPS from *Ochrobactrum anthropi* P318R M425T, in addition of N267S residues, which are not involved in PEP-glyphosate biding (39).

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The mutations P101S and G96A in the class I EPSPS sequence were initially documented in *Salmonella typhimurium* and *Klebsiella pneumoniae,* respectively (40,41) (Table 3). *Healy-Fried* et al. shows that P101S mutation induces minor yet significant structural alterations in the active site of the enzyme as well as other mutations at this position (P101G, P101A and P101L) that did not alter the enzyme's catalytic activity, nor lead to the development of glyphosate resistance (42). On the contrary, the G96A mutation induces a substantial structural change in the glyphosate-binding site in *E. coli*, as the Glycine in position 96 is a key site for the efficient binding of glyphosate (43).

The T97I mutation, generally conserved in *Chlamydia* EPSPS and possessing reduced efficacy, is unable to confer resistance to glyphosate (44). But Funke et al have shown that bacteria harboring EPSPS with a P101S mutation can easily acquire the T97I mutation, inducing resistance to glyphosate (45). This double mutation T97I/P101S (TIPS) results in a shift of residue Gly96 onto the glyphosate binding site (44). In addition to the double mutation, a triple mutation (G96A/T97I/P101S) known as GATIPS in *Escherichia coli*, and G97A/T98I/P101S in *Pantoea* sp., confers higher glyphosate tolerance, exceeding 15 mM in an *E. coli* strain where the *aroA* gene has been knocked out, and up to 20 mM in transgenic *Arabidopsis thaliana* plants (46).

In addition, mutations within the highly conserved region (XLGNAGTAXRXL) of the class I EPSPS, which is implicated in PEP biding (47), and within residues located at the junction between the two large globular domains of EPSPS and in the third helix of the core of the N-terminal domain induce glyphosate resistance (32).

Same mutations and mutations closely related to bacterial EPSPS that confer glyphosate resistance have been identified in plants. TIPS double mutation (T97I/P101S) in *E. coli* was

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found also in in *Zea mays* and *Eleusine indica* (T102I/P106S) with a shift of five amino acid in the protein sequence. Furthermore, the residues at the active sites of these EPSPSs are nearly conserved between bacteria and plants (43).

In the other hand, several studies show that EPSPS amplification confer resistance to glyphosate in plants (48). Gaines et al found that glyphosate resistant plants have five to more than 160 copies of the EPSPS gene than susceptible plants (49). Till now, this mechanism conferring resistance to glyphosate was not found in microorganisms, but EPSPS amplification in *E. coli*, generated by recombinant DNA techniques, show that the overexpression of EPSPS increased tolerance to glyphosate (50).

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3.2 Mutations in genes encoding proteins indirectly linked to the shikimate pathway increase glyphosate resistance: *ppsR* and *gpmA*

PpsA (Phosphoenolpyruvate (PEP) synthase) is an enzyme of the glycogenesis pathway that converts pyruvate into PEP, the co-substrate of EPSPS. PpsR (PEP regulatory protein), is a PpsA repressor encoded by the *ppsR* gene (51). It controls PpsA expression through both Pi and ATP/ADP dependences. In a *Burkholderia anthina* isolate subcultured on a minimum medium supplemented with 10 mM of glyphosate, several inactivating mutations in the *ppsR* gene (T68A, E97K, G109D, Q167P and L261P) resulted in an overproduction of PEP. This overproduction enhanced glyphosate tolerance, enabling the mutated strain to tolerate 10 mM of glyphosate compared to the non-mutated strain (52). In the same study, a mutant strain, able to grow in 35 mM of glyphosate, was found to carry a 9 bp deletion in the *gpmA* gene, which encodes Pi-glycerate mutase. This finding suggests that the mutation altered the activity of the enzyme, leading to an overproduction of PEP, as shown by metabolomic analysis, and subsequently preventing the inhibition of EPSPS (52). GpmA is an enzyme responsible for the reversible conversion of 2-phosphoglycerate to 3-phosphoglycerate in both glycolysis and gluconeogenesis, which then leads to the formation of PEP, the key substrate for the EPSPS enzyme.

4. Glyphosate degradation

Glyphosate accumulation in the environment has always been a concern, impacting both health and microbial diversity due to glyphosate's toxicity and endocrine-disrupting effect. Several studies have reported different elimination methods to eliminate glyphosate with minimum harmful side-effects, such as adsorption, different types of oxidation (photolysis-based, Fenton-based, electrochemical and ozonation) and biological treatment also known as bioremediation.

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Bioremediation is a low-cost and eco-friendly process that protects the ecosystem while offering a sustainable solution to reduce the impact of glyphosate on our environment.

Glyphosate degradation pathway varies among bacterial species, and not every bacterium possess the necessary genes to metabolize glyphosate. Various bacterial species can degrade glyphosate using enzymes that contribute to metabolic and catalytic processes. This enables them to use glyphosate efficiently as a source of phosphorus, carbon, and/or nitrogen compounds in soil and water environment.

Glyphosate biodegradation involves three main enzymes, C-P lyase, glyphosate oxidoreductase (GOX) and FAD-dependent glycine oxidase (FAD-GO). C-P lyase, the most abundant enzyme for phosphonate degradation (53), cleaves the carbon-phosphorus (C-P) bond within glyphosate, resulting in the formation of sarcosine. Sarcosine is then catabolized in glycine and formaldehyde via the sarcosine oxidase, which is a heterotetrameric protein encoded by *soxBDAG* operon (54). The GOX (EC 1.5.3.23) and FAD-GO enzymes (EC 1.4.3.19), encoded by *gox* (GenBank accession: GU214711.1) and *thiO* (Genbank accession: KC831746.1) genes respectively, converts glyphosate into aminomethylphosphonic acid (AMPA, CH3NO3P) and glyoxylate. AMPA, an intermediate metabolite with a potential environmental toxicity, can then undergo further metabolic reactions, producing simpler compounds that can be incorporated into the metabolic pathways of the bacterial cell. After undergoing metabolism via C-P lyase, AMPA is also used as a source of phosphate by certain bacterial species. GOX was characterized from from *Ochrobactrum sp. G-1*. The bibliography reports a limited number of bacterial genera that produce glycine oxidase such as *Ochrobactrum*, *Pseudomonas* (*P. putida* KT2440), *Geobacillus* (*G. kaustophilus*), *Bacillus* (*B. subtilis*), *Rhizobium*, and *Bradyrhizobium* (*B. jicamae*, *B. elkanni* and *B. japonicum*) (55). In addition, a recent study show that *thiO* gene coding for this enzyme

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was characterized and transformed in *Escherichia coli* and transgenic rice. The presence of this gene increase the resistance of these transformed organisms to glyphosate (56).

C-P lyase pathway, a prevalent enzyme system across bacterial strains that are proficient in phosphonate metabolism, is encoded by the *phn* operon (*phnCDEFGHIJKLMNOP*) (57). Hove-Jensen *et al.* demonstrate that *this* operon, is involved in glyphosate degradation (58). Within this operon of 14 genes, the functionalities are attributed as follows: *phnCDE* are responsible for encoding an ATP binding cassette transporter, *phnF* operates as a repressor protein and *phnNOP* take on roles related to regulation or auxiliary functions. Central to the C-P lyase pathway are the core components embodied by *phnGHIJKLM*, while the principal C-P cleavage is catalyzed by *phnJ* (59,60). Furthermore, Martinez *et al.* found a new degradation pathway involving *phnY* and *phnZ* genes among uncultured marine bacteria that uses glyphosate and AMPA as phosphate source (61). Generally, a bacterial strain harbors only one operon associated with the C-P lyase pathway. However, there are exceptions, with some strains having between two to four operons involved in phosphonate catabolism. *Pseudomonas stutzeri* is one of these strains with two distincts operons, *htxABCDEFGHIJKLMN* and *phnCDEFGHIJKLMNP* (an operon similar to *Escherichia coli*) (62). In addition three distincts *phn* operon were found in *Mesorhizobium loti*, one is located on the chromosome and the others are located on the same plasmid but on different locus (63). Another strain, *Marinobacter aquaeolei VT8,* with 2 identical C-P lyase-encoding operons located on chromosome and on pMAQU02 plasmid, and 2 others different operons located also on chromosome and another plasmid (pMAQU01) (64).

Most of samples (soil and sludge) used in glyphosate bacterial degradation studies reported are contaminated or treated with glyphosate. Bacterial species degrade glyphosate and use it mainly as a phosphorus source through C-P lyase, GOX and/or glycine oxidase pathways. In addition,

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288 there is some species like *Agrobacterium radiobacter, Sinorhizobium meliloti* (65) and
289 *Geobacillus caldoxylosilyticus T20* (66) with an uncharacterized glyphosate catabolism pathway
290 but are able to use glyphosate as a phosphorus source.

291 In vitro, glyphosate metabolism pathways were characterized by detecting and measuring the
292 secondary and final metabolites of glyphosate degradation present in the culture medium. The
293 presence of a microbial C-P lyase activity is related to the detection of sarcosine and N-
294 methylglycine as degradation metabolites of glyphosate added to culture media. While the
295 detection of AMPA and glyoxylate indicates the presence of a GOX activity. Testing the ability
296 of a microbial strain to degrade glyphosate and to be used in bioremediation strategies is
297 influenced by different experimental parameters such as pH value, temperature, time and the
298 presence of carbon, nitrogen and phosphate sources other than glyphosate.

299 **4.1 GlpA and GlpB:**

300 The GlpA and GlpB, encoded respectively with *glpA* and *glpB* genes, are two anaerobic
301 glycerol-3-phosphate dehydrogenase subunits of *glpABC* operon that are important for the
302 utilization of glycerol as a carbon source by bacteria (67). This enzyme is responsible for
303 catalyzing the conversion of glycerol-3-phosphate (a metabolite derived from glycerol) to
304 dihydroxyacetone phosphate (DHAP), an intermediate in both glycolysis and gluconeogenesis
305 (68). In some bacteria (*Pseudomonas pseudomallei, E. coli*), *glpA* and *glpB* genes have been
306 associated with the glyphosate degradation pathway (69). In addition *glpA* was found to
307 exhibited significant homology with each of *E. coli* and *P. pseudomallei* hygromycin
308 phosphotransferase gene (*hph*), that can confer tolerance to glyphosate by glyphosate
309 phosphorylation (70). The presence of the *glpA* and *glpB* genes in a bacterial species may

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indicate its potential ability to degrade glyphosate, but the complete degradation pathway may involve other enzymes and genes as well.

4.2 GAT

Glyphosate N-acetyltransferase (GAT), an enzyme produced by certain bacterial species and encoded by the gat gene (GenBank accession: AX543338), was first detected in *Bacillus licheniformis* in 2004 (71,72). This enzyme was important, particularly in agriculture, for its ability to detoxify glyphosate. In the presence of the cofactor acetyl-CoA, GAT changes the structure of glyphosate and inhibits its activity by acetylating it. The decrease in the inhibition of the acetylated form of glyphosate is due to the decrease affinity for the active site of EPSPS. Initially, this enzyme, transformed in different organisms, was unable to confer tolerance to glyphosate. But by DNA shuffling, recombinant genes and evolved enzymes, with 4500-fold increase in catalytic efficiency comparing to the native enzyme (73), were generated conferring glyphosate resistance in various organisms transformed by these genes, such as *Escherichia coli*, *Nicotiana tabacum cv. Xanthi* NN and *Zea mays* (introduced by transformation using *Agrobacterium tumefaciens*) (74,75). On the other hand, sequences of the *yitI* gene encoding the putative N-acetyltransferase from *B. subtilis* and *B. cereus* were 58% and 47% respectively identical to the sequence generated from the fourth iteration of the *gat* gene shuffle (GenBank accession: AX543353). In addition, soybeans genetically modified with *gat4601* (the *gat* gene shuffle detected in *B. licheniformis*) have been reported to be tolerant to glyphosate application (76).

5. Changes in cell permeability underlying glyphosate tolerance in microorganisms

Glyphosate tolerance may be associated with changes in cell permeability, resulting from either increased efflux or decreased influx (77). These biochemical systems are regulated by the

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expression of membrane proteins, notably the overexpression of efflux pumps and the reduction of porins (78). Studies have revealed that specific efflux pumps, such as Pdr5, MFS40, and YhhS, found respectively in *Saccharomyces cerevisiae*, *Aspergillus oryzae*, and *E. coli*, can increase resistance to glyphosate (79–82). Microorganisms' efflux pumps do not only target one particular substance; they can also result in cross-resistance to various xenobiotics, such as antibiotics and herbicides (15). Furthermore, experiments have shown that increased efflux pump overexpression simultaneously affects antibiotic and glyphosate sensitivity in *E. coli* and *Salmonella typhimurium* (77,83). When bacterioplankton microbial communities were exposed to GBH, glyphosate and antibiotic resistance increased significantly. Further genomic analysis indicated that this exposure was explicitly selected for multidrug efflux pumps (84).

The authors hypothesized that this mechanism is more widespread in the environment than mutations in the glyphosate target enzyme, allowing bacterioplankton to thrive and survive in the presence of GBH (84). Through growth tests with phenylalanine-arginine beta-naphthylamide (PAβN), an efflux pump inhibitor, Kurenbach *et al.* confirmed the significant role of efflux in the increased tolerance of *E. coli* to kanamycin in the presence of glyphosate (83). Additionally, in another study, the deletion of two genes associated with efflux, *acrA*, and *tolC*, decreased the resistance of *E. coli* when exposed to Roundup compared to the wild type, specifically about ciprofloxacin (77).

It is widespread for microorganisms to inactivate transporter genes when exposed to toxic substances (85). Glyphosate and its structural analogs can enter the cell via amino acid transporters (81). Indeed, the study conducted by Wicke *et al.* were revealed that *B. subtilis* develops resistance to glyphosate quickly by acquiring mutations in the *gltT* and *gltP* genes, which encode GltT and GltP, two membrane transporters that belong to the dicarboxylate/amino

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356 acid: cation (Na+ or H+) symporter (DAACS) family (86). Another study also found that *S.*
357 *cerevisiae* strains became more resistant to glyphosate when the *dip5* gene, which encodes a
358 membrane transporter in the amino acid-polyamine-organocation (APC) superfamily, was altered
359 (79,87).

360 **6. Glyphosate-MIC determination: from in silico to in vitro resistance**

361 Determining the minimal inhibitory concentration (MIC) of glyphosate involves assessing the
362 lowest concentration of the herbicide that effectively inhibits the growth of a specific organism.
363 In literature, glyphosate MICs have been determined for bacteria, fungi, and even plants (83).
364 Comparing data on glyphosate MIC in the literature is challenging due to an absence of a
365 consensus procedure. Additionally, it is important to note that there are different definitions of
366 tolerance: one based on bioinformatic analysis, which may or may not include the study of
367 EPSPS enzyme activity, and another based on MIC determination. This distinction adds
368 complexity when comparing and interpreting the data from different studies

369 **6.1 Technical considerations**

370 Researchers have developed various approaches to evaluate the inhibitory effects of glyphosate
371 on different organisms as Gram negative bacteria (GNB), Gram positive bacteria (GPB) and
372 fungi (Table 1). The most commonly employed methods used to determine glyphosate MIC are
373 broth microdilution (BMD) (88–92), broth dilution (BD) sometimes followed by agar plating
374 (92), and agar dilution (AD) (92). These techniques involve supplementing the medium,
375 typically a minimal medium (triphenylphosphate glucose (TPG), MOPS, Muller Hinton broth
376 (MHB)) occasionally enriched with nutritive substances such as sorbitol, glucose, and amino
377 acids (93), with varying concentrations of glyphosate or glyphosate-based formulation (GBF).
378 However, different culture media adapted for the growth of obligate and facultative anaerobic

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379 organisms are also used, such as reinforced clostridial medium (RCM), anaerobic basal broth
380 (ABB), deMan Rogosa and Sharp broth (MRS), and brain heart infusion broth (BHI). Studies
381 have reported MICs that are tested by different culture media for the same strain in order to
382 compare the different factors influencing the determination of MICs like the composition and the
383 pH of the culture media. For example, Nielson et al. reported MICs for a collection of reference
384 strains from different genera (*Akkermensia*, *Bacteroides*, *Bifidobacterium*, *Enterococcus* etc…)
385 tested in anaerobic conditions using both BHI and RCM medias (91). In addition, culture
386 conditions such as temperature (25°C to 37°C), incubation time, and atmospheric conditions
387 (aerobic, anaerobic, or microaerophilic) were been optimized for each microorganism. Anaerobic
388 conditions were used by Nielsen et al. in order to test the glyphosate MICs for commensal
389 bacteria of the animal gut (91). The commonly used temperature is 37°C, and the incubation time
390 ranges from 18 to 72 hours except for some bacterial genera with slow growth rate such as
391 *Ochrobactrum*, *Rhizobium*, *Pedobacter* and *Phyllobacterium*, where the strains were tested using
392 AD method with six days of incubation (65). In addition to variations in the determination
393 method and culture medium employed, the choice of glyphosate formulation is a major factor.
394 Besides pure glyphosate, various formulations were tested, differing in the type of glyphosate
395 salt used, including isopropylamine (IPA), dimethylamine (DMA), and potassium (P) salts
396 (Table 2). These different salts result in formulations with a less acidic pH, ranging between 4.4
397 and 5.1, compared to pure glyphosate, which has a pH of around 2.5 (Sigma Aldrich, USA).
398 Some of GBFs used contains adjuvants that have antimicrobial effect on bacteria. This is the case
399 of Jablo® and Roundup Ultra Max® that contains polyethoxylated tallowamine (POEA), a toxic
400 surfactant that considerably affects MIC values (94). In general, the composition of GBFs is
401 critical and affect the interpretation of the MICs especially that there is GBFs formulations were

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402 the composition is a secret recipe like Radikal Green® (containg IPA and an unknown quaternary
403 ammonium) (unpublished study). Currently, more than 750 products containing glyphosate can
404 be found in different salt types (isopropylamine (IPA) salt, dimethylamine (DMA) salt and
405 potassium (P) salt).

406 **6.2 MIC range in literature**

407 Glyphosate-MICs and GBF-MICs found in the bibliography varies widely, from 0.07 to 160 g/L
408 (Table 1). The lowest MICs found correspond in most cases to strains tested with pure
409 glyphosate (88), Jablo® (95) and Roundup Ultra Max (92). These low MICs (not exceeding 10
410 g/L) are mainly due to the to the high activity of the pure glyphosate, as well as the toxicity of
411 Jablo® and Roundup Ultra Max, that contains POEA. The MIC values for glyphosate vary
412 significantly among bacterial species, formulations, and culture conditions. Among the GNB
413 tested for glyphosate and GBF MIC, *Escherichia coli*, *Salmonella spp.* and *Pseudomonas*
414 *aeruginosa* were the most frequently studied, with MICs ranging between 0. 845-80 g/L, 5-160
415 g/L, 0.8-26.16 g/L, respectively. The variation in MICs (2.93-26.16 g/L) for *Pseudomonas*
416 *aeruginosa* strains in Háhn *et al*. suggests strain-specific and formulation-specific differences in
417 susceptibility to glyphosate and GBF. For instance, using the same culture media (Luria-Bertani
418 broth, LBB) and method (BMD), the highest MIC for *P. aeruginosa* ATCC 15442 was 23 g/L
419 with Fozat 480, while for *P. aeruginosa* P66, the highest MIC was 13.08 g/L with Roundup®
420 Mega formulation. *Akkermansia muciniphila* shows a MIC of 20 g/L for Glyfonova® 450 Plus,
421 which is consistent across different media (BHI and RCM). However, for the same formulation,
422 strains from the *Bacteroides* genera exhibits MICs that are two to eight times higher in RCM
423 medium compared to BHI medium. Conversely, for *E. coli* (ATCC25922 and DSM 18039
424 strains), the MICs values are lower in RCM medium than in BHI medium. *Bacteroides vulgatus*

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shows a board range of MIC values (0.02-20 g/L) depending on the GBF and the medium. The lowest MIC was observed with pure glyphosate and BHI medium (0.02 g/L). In addition, strains of *Ochrobactrum* and *Rhizobium* genera have MICs that not exceed 6 g/L. Comparing the MICs of these strains with other genera was impossible as only pure glyphosate was tested. Roundup® LB Plus MICs of *Salmonella* spp. were the same of those of *E. coli* using the same media and the same formulation. The highest MIC found, which is 160 g/L, was found in the literature for *Salmonella* Enteritidis and *S.* Typhimurium strains tested for Roundup® LB Plus after extinction and stability passages.

On the other hand, among Gram-positive strains, *Bifidobacterium spp.* and *Lactobacillus spp.* were the most studied with MICs ranging between 0.07-20 g/L and 0.312-40 g/L respectively. For bacteria of the *Bifidobacterium* and *Clostridium* genera, the MICs for Glyfonova® 450 Plus were two times higher when using RCM media compared to BHI, similar to the pattern observed in Gram-negative *Bacteroides*. In contrast, for *Enterococcus faecalis* strains MIC in BHI was higher than MIC in RCM, there was no change in the MIC between the two media for all *Lactobacillus* strains tested. *Bacillus* and *Staphylococcus* strains were more sensitive to Roundup UltraMax® than *Clostridium* strains, with MICs for *Bacillus* being four to almost 33 times lower than those for *Clostridium*.

Finally, there is insufficient data on glyphosate or GBF MICs for fungi. In total four fungi genera were tested (90,96–98). The same MIC of pure glyphosate (> 0.256 g/L) was found by Carneiro et al. for all *Cryptococcus gattii* strains tested in his study under the same culture conditions. Among all fungi strains tested, *Saccharomyces cerevisiae* SHY2/pHR2 have the highest MIC (6 g/L) (27), but unfortunately Kunze *et al.* did not provide information about the GBF and culture

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447 media used in his study, making it difficult to assess differences in fungi susceptibility to
448 glyphosate compared to bacteria which appear to be more tolerant to both glyphosate and GBF.

449 These results highlight the significant impact of media, glyphosate formulations, and bacterial
450 species on glyphosate susceptibility, emphasizing the need for customized susceptibility testing
451 under various conditions.

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7. **Conclusion and perspectives**

In summary, the EPSPS enzyme plays a crucial role in the shikimate pathway, making it a primary target for glyphosate. Mutations in the EPSPS gene and related genes, which are indirectly linked to the shikimate pathway, enhance glyphosate resistance by either increasing enzyme efficiency or decreasing glyphosate binding affinity. Future research should explore unclassified EPSPS sequences to understand their structural and functional features, that can uncover novel resistance mechanisms. Additionally, analyzing the evolutionary relationships between EPSPS classes across diverse bacterial genera may provide insights into the spread and evolution of tolerance traits. The more complex, multi-domain structure of fungal EPSPS, which shares one domain with bacterial EPSPS, represents an underexplored area that could reveal new mechanisms of glyphosate tolerance or sensitivity in eukaryotes. Further studies are needed on the effects of glyphosate on fungi, especially those involved in agriculture or ecosystem services. Several bacterial species have glyphosate degradation pathways, allowing them to use glyphosate as a phosphorus or nitrogen source, with significant implications for bioremediation and the development of glyphosate-tolerant crops through genetic modification. Investigating the prevalence and transfer mechanisms of the phn operon across bacterial species, particularly in soil and aquatic microbiomes, could reveal how environmental factors, such as glyphosate use, influence the spread of the operon and drive the evolution of microbial populations capable of utilizing glyphosate as a phosphorus source. Glyphosate tolerance in microorganisms is often due to changes in cell permeability, with key mechanisms including increased efflux and reduced influx, a mechanism also well known in antibiotic resistance. These findings highlight that microorganisms rely on these strategies in addition of direct changes to glyphosate's target enzymes. Understanding these resistance mechanisms is essential for managing glyphosate

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resistance in agriculture and the environment. The variability in glyphosate MICs across different organisms due to factors, like culture media, glyphosate formulations, and microorganism strains, complicates comparison across studies. The lack of standardized MIC determination protocols exacerbates this issue, with differences in pH, adjuvants, and formulation compositions influencing MIC values. The complexity of resistance mechanisms and strain-specific responses underscore the need for standardized testing procedures and a better understanding of factors affecting MIC outcomes, which is vital for assessing the environmental and health impacts of glyphosate-based herbicides.

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928 **Tables and figures:**

929 **Tables:**

930 Table 1: Glyphosate Minimum Inhibitory Concentrations (MICs) reported in the bibliography
931 and test conditions..... 49
932 Table 2: Composition of glyphosate-based formulations cited in Table 1 55
933 Table 3: Mutations occurring in bacterial class I and class II EPSPS protein sequences..... 59
934 Table 4: Bacterial strains that are able to degrade glyphosate with their glyphosate catabolism
935 pathway. 60

936 **Figures:**

937 Figure 1: The single-domain AroA protein of Escherichia coli K-12 (P0A6D3) compared to the
938 pentafuctional AroM protein containing six domains in Saccharomyces cerevisiae strain ATCC
939 204508 (P08566). The protein functional classification was conducted using InterProScan.
940 DHQS: 3-dehydroquinate synthase; SKI: shikimate kinase; DHQase-I: 3-dehydroquinate
941 dehydratase type I; S-DH-N: shikimate dehydrogenase substrate binding, N-terminal; NAD-
942 bind-S-DH: nicotinamide adenine dinucleotide binding domain of shikimate dehydrogenase. .. 56
943 Figure 2: (A) Phylogenetic classification of a total of 548 EPSPS protein sequences from Uniprot
944 reviewed protein database using Fast Tree, reference sequences and plant sequence show two
945 main distinct EPSPS class, class I (blue) and class II (orange). Reference sequences were
946 highlighted in yellow and starred. No class III EPSPS (brown) sequence is found in Uniprot
947 reviewed protein database, and only one sequence (Brevundimonas vesicularis, CAR82018) of
948 this class is shown in the analysis due to rarety of this sequence. 57
949 Figure 3: Mechanisms of bacterial tolerance to glyphosate. 1: AroA mutations modifying the
950 activity of the EPSPS enzyme; 2: Natural amplification of the aroA gene in the bacterial genome;

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3: Mutation of ppsR, encoding for PPSR, the regulatory protein of the synthesis of PEPS, indirectly affecting PEP; 4: Bacterial degradation of glyphosate using multiple pathways to utilize glyphosate as a nutrient source; 5, 6: Mutations of gltT and gltP genes, encoding for GltT and GltP, transmembrane proteins that transport glyphosate to the cytoplasm; 7: Efflux pumps. GLY: glyphosate; DAHP: 3-deoxy-D-arabino-heptulosonate-7-phosphate; PEPS: phosphoenolpyruvate synthase; PEP: phosphoenolpyruvate; EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase; EPSP: 5-enolpyruvylshikimate-3-phosphate; GOX: glyphosate oxidoreductase; AMPA: aminomethylphosphonic acid; GNAT: glyphosate N-acetyltransferase.

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961 Table 1: Glyphosate Minimum Inhibitory Concentrations (MICs) reported in the bibliography and test conditions.

Gram-negative bacteria	MIC-Gly (g/L)	Glyphosate formulation	Culture medium	MIC determination method	Culture condition	Highest concentration tested	Reference
Akkermansia muciniphila	20	Glyfonova® 450 PLUS	BHI	BMD	37°C, 24-72 h, anaerobic	80 g/L found	(91)
A. muciniphila	20	Glyfonova® 450 PLUS	RCM	BMD	37°C, 24-72 h, anaerobic	80 g/L found	(91)
Bacteroides spp. (B. fragilis, B. ovatus, B. uniformis)	5 [5-10]	Glyfonova® 450 PLUS	BHI	BMD	37°C, 24-72 h, anaerobic	80 g/L found	(91)
Bacteroides spp. (B. fragilis, B. ovatus, B. uniformis)	20 [10-40]	Glyfonova® 450 PLUS	RCM	BMD	37°C, 24-72 h, anaerobic	80 g/L found	(91)
Phocaeicola vulgatus	0.6	Roundup UltraMax®	RCM	BD then Agar plating	37°C, 48 h, anaerobic	5.0 g/L	(92)
P. vulgatus	0.02	Pure Gly[a]	BHI	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
P. vulgatus	0.82	Jablo™ Glyfosat	BHI	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
P. vulgatus (n=2)	[5-20]	Glyfonova® 450 PLUS	BHI	BMD	37°C, 24-72 h, anaerobic	80 g/L found	(91)
Burkholderia pseudomallei	3 % (w/v)	Gylphosate-48	MHB	BMD	37°C, 18-24 h	24% (w/v)	(99)
Campylobacter coli	0.15	Roundup UltraMax®	Caso-modifies broth	BD then Agar plating	37°C, 48 h, microaerophilic	5.0 g/L	(92)
Campylobacter jejuni	0.15	Roundup UltraMax®	Caso-modifies broth	BD then Agar plating	37°C, 48 h, microaerophilic	5.0 g/L	(92)
Escherichia coli (n=238)	10 [<1.25-40]	40% Pure Gly[b]	MH I	BMD	37°C, 16-20 h, aerobic	80 g/L	(88)
E. coli (n=238)	20 [10-40]	40% Pure Gly[b]	MH II	BMD	37°C, 16-20 h, aerobic	160 g/L	(88)
E. coli	80	Roundup® LB Plus + NaOH	MHB	BMD	37°C, 16-20 h, aerobic	160 g/L	(100)
E. coli	0.845	Pure Gly[c]	TPG/MOPS	BMD	37°C, 16-18 h	1690 mg/L	(16)
E. coli	1.2	Roundup UltraMax®	RCM	BD then Agar plating	37°C, 24 h, aerobic	5.0 g/L	(92)
E. coli	40	Roundup® LB Plus	MHB	BMD	37°C, 16-20 h, aerobic	160 g/L	(100)
E. coli (n=238)	40 [20-80]	Roundup® LB Plus	MH I	BMD	37°C, 16-20 h, aerobic	80 g/L	(88)

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E. coli (n=238)	40 [20-80]	Roundup® LB Plus	MH II	BMD	37°C, 16-20 h, aerobic	160 g/L	(88)
E. coli (n=2)	80	Glyfonova® 450 PLUS	BHI	BMD	37°C, 24-72 h, anaerobic	80 g/L	(91)
E. coli (n=2)	20	Glyfonova® 450 PLUS	RCM	BMD	37°C, 24-72 h, anaerobic	80 g/L	(91)
E. coli	>10	Pure Gly[a]	BHI	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
E. coli	>10	Jablo™ Glyfosat	BHI	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
E. coli	7.4	Roundup® weed killer	LB	AD	37°C, > 4 d	ND	(83)
E. coli	0.3	Pure Gly[d]	M9 + sorbitol	BMD	37°C, 24 h	2.5 g/L	(93)
E. coli	0.6	Pure Gly[d]	M9 + glucose	BMD	37°C, 24 h	2.5 g/L	(93)
E. coli	> 2.5	Pure Gly[d]	M9 + sorbitol + (20 µg/mL) aaa	BMD	37°C, 24 h	2.5 g/L	(93)
E. coli	1.2	Roundup UltraMax®	RCM	BD then Agar plating	37°C, 24 h, aerobic	5.0 g/L	(92)
Mitsuokella jalaludinii	10	Pure Gly[a]	BHI	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
M. jalaludinii	2.86	Jablo™ Glyfosat	BHI	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
Ochrobactrum anthropi	5.882	Pure Gly[e]	MSB	AD	25°C, 6 d	10000 mg Gly/Kg	(65)
Ochrobactrum haematophilum	5.882	Pure Gly[e]	MSB	AD	25°C, 6 d	10000 mg Gly/Kg	(65)
Pedobacter nutrimenti	0.5882	Pure Gly[e]	MSB	AD	25°C, 6 d	10000 mg Gly/Kg	(65)
Phyllobacterium myrsinacearum	5.882	Pure Gly[e]	MSB	AD	25°C, 6 d	10000 mg Gly/Kg	(65)
Pseudomonas aeruginosa	> 0.8	Pure Gly[f]	LBB	BMD	28°C, 24 h	0.8 g/L	(101)
P. aeruginosa	6.1 [6.1-24.4]	Dominator Extra 608 SL	LBB	BMD	28°C, 24 h	4% (v/v)	(101)
P. aeruginosa	5.75 [2.875-23]	Fozat 480	LBB	BMD	28°C, 24 h	4% (v/v)	(101)
P. aeruginosa	2.93 [1.465-11.72]	Gladiator 480 SL	LBB	BMD	28°C, 24 h	4% (v/v)	(101)
P. aeruginosa	13.08 [1.635-26.16]	Roundup® Mega	LBB	BMD	28°C, 24 h	4% (v/v)	(101)

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Rhizobium spp. (*R. freirei, R. lusitanum, R. mayense, R. miluonense, R. vallis*)	4.412 [0.294-5.882]	Pure Gly[e]	MSB	AD	25°C, 6 d	10000 mg Gly/Kg	(65)
Riemerella anatipestifer	0.15	Roundup UltraMax®	RCM	BD then Agar plating	37°C, 24 h, aerobic	5.0 g/L	(92)
Salmonella enterica (n=225)	40 [10-80]	40% Pure Gly[b]	MHB	BMD	37°C, 24 h	80 g/L	(89)
Salmonella enterica (n=225)	40 [20-80]	Roundup® LB Plus	MHB	BMD	37°C, 24 h	80 g/L	(89)
Salmonella enterica (n=2)	>10	Pure Gly[a]	BHI	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
Salmonella enterica (n=2)	>10	Jablo™ Glyfosat	BHI	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
Salmonella enterica (n=11)	40 [40-80]	Roundup® LB Plus	MHI	BMD	37°C, 24 h	160 g/L	(102)
Salmonella enterica ** (n=3)	160	Roundup® LB Plus	MHI	BMD	37°C, 24 h	160 g/L	(102)
Salmonella enterica	80	Roundup® LB Plus + NaOH	MHB	BMD	37°C, 16-20 h, aerobic	160 g/L	(100)
Salmonella enterica (n=2)	5	Roundup UltraMax®	RCM	BD then Agar plating	37°C, 24 h, aerobic	5.0 g/L	(92)
Salmonella enterica	6.19	Roundup® weed killer	LB	AD	37°C, > 4 d	ND	(83)
Gram-positive bacteria	**MIC-Gly (g/L)**	**Glyphosate formulation**	**Culture medium**	**MIC determination method**	**Culture condition**	**Highest concentration tested**	**Reference**
Bacillus badius	0.3	Roundup UltraMax®	RCM	Serial dilutions	37°C, ND	5.0 g/L	(92)
B. badius	0.15	Roundup UltraMax®	RCM	BD then Agar plating	37°C, 24 h, aerobic	5.0 g/L	(92)
Bacillus cereus	0.3	Roundup UltraMax®	RCM	BD then Agar plating	37°C, 24 h, aerobic	5.0 g/L	(92)
Bifidobacterium spp. (adolensis, B. animalis, B. bifidum, B. breve, B. longum)	10	Glyfonova® 450 PLUS	BHI	BMD	37°C, 24-72 h, anaerobic	80 g/L found	(91)
Bifidobacterium spp. (adolensis, B. animalis, B. bifidum, B. breve, B. longum)	20	Glyfonova® 450 PLUS	RCM	BMD	37°C, 24-72 h, anaerobic	80 g/L found	(91)
B. adolescentis	0.15	Roundup UltraMax®	RCM	Serial dilutions	37°C, ND	5 g/L	(103)
B. adolescentis	0.075	Roundup UltraMax®	MRS	BD then Agar plating	37°C, 48 h, anaerobic	5 g/L	(92)
B. adolescentis	0.02	Pure Gly[a]	BHI	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)

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B. adolescentis	0.07	Jablo™ Glyfosat	BHI	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
Clostridium botulinum (n=2)	1.2	Roundup UltraMax®	RCM	BD then Agar plating	37°C, 48 h, anaerobic	5.0 g/L	(92)
Clostridium spp. (C. leptum, C. nexile, C. perfringens)	10	Glyfonova® 450 PLUS	BHI	BMD	37°C, 24-72 h, anaerobic	80 g/L found	(91)
Clostridium spp. (C. leptum, C. nexile, C. perfringens)	20	Glyfonova® 450 PLUS	RCM	BMD	37°C, 24-72 h, anaerobic	80 g/L found	(91)
C. perfringens	5	Roundup UltraMax®	RCM	BD then Agar plating	37°C, 24 h, aerobic	5.0 g/L	(92)
C. perfringens	0.23	Pure Gly[a]	ABB	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
C. perfringens	0.23	Jablo™ Glyfosat	ABB	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
Enterococcus faecalis	0.3	Roundup UltraMax®	RCM	Serial dilutions	37°C, ND	5 g/L	(103)
E. faecalis	0.15	Roundup UltraMax®	RCM	BD then Agar plating	37°C, 24 h, aerobic	5.0 g/L	(92)
E. faecalis (n=2)	80	Glyfonova® 450 PLUS	BHI	BMD	37°C, 24-72 h, anaerobic	80 g/L found	(91)
E. faecalis (n=2)	40	Glyfonova® 450 PLUS	RCM	BMD	37°C, 24-72 h, anaerobic	80 g/L found	(91)
Enterococcus faecium	0.15	Roundup UltraMax®	RCM	BD then Agar plating	37°C, 24 h, aerobic	5.0 g/L	(92)
E. faecium	>10	Pure Gly[a]	MRS	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
E. faecium	10	Jablo™ Glyfosat	MRS	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
Lactobacillus spp. (L. buchneri, L. casei)	0.6	Roundup UltraMax®	MRS	BD then Agar plating	37°C, 48 h, anaerobic	5.0 g/L	(92)
Lactobacillus delbrueckii	1	Roundup® R450	ND	BMD	ND	ND	(96)
Lactobacillus harbinensis	0.6	Roundup UltraMax®	MRS	BD then Agar plating	37°C, 48 h, anaerobic	5.0 g/L	(92)
Lactobacillus spp. (L. johnsonii, L. planetarum, L. reuteri, L. rhamnosus)	40 [20-40]	Glyfonova® 450 PLUS	BHI	BMD	37°C, 24-72 h, anaerobic	80 g/L found	(91)
Lactobacillus spp. (L. johnsonii, L. planetarum, L. reuteri, L. rhamnosus)	40 [20-40]	Glyfonova® 450 PLUS	RCM	BMD	37°C, 24-72 h, anaerobic	80 g/L found	(91)
Lactobacillus spp. (L. reuteri,	2.86 [0.82-2.86]	Pure Gly[a]	MRS	BMD	37°C, 18-24 h,	10 g/L	(95)

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L. salivarius, L. sobrius)					aerobic		
Lactobacillus spp. (L. reuteri, L. salivarius, L. sobrius)	2.86 [0.82->10]	Jablo™ Glyfosat	MRS	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
Lactococcus cremoris	0.312	Roundup® R450	ND	BMD	ND	ND	(96)
Salinicoccus spp SH5	2250 ppm	ND	MHB	BD	30°C, 48 h	ND	(104)
Staphylococcus spp. (S. aureus, S. haemolyticus, S. lentus)	0.3	Roundup UltraMax®	RCM	BD then Agar plating	37°C, 24 h, aerobic	5 g/L	(92)
S. aureus	0.07	Pure Gly[a]	BHI	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
S. aureus	0.82	Jablo™ Glyfosat	BHI	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
Streptococcus alactolyticus	0.07	Pure Gly[a]	MRS	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
S. alactolyticus	>10	Jablo™ Glyfosat	MRS	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
Streptococcus hyointestinalis	0.07	Pure Gly[a]	MRS	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
S. hyointestinalis	0.07	Jablo™ Glyfosat	MRS	BMD	37°C, 18-24 h, aerobic	10 g/L	(95)
Fungi	**MIC-Gly (g/L)**	**Glyphosate formulation**	**Culture medium**	**MIC determination method**	**Culture condition**	**Highest concentration tested**	**Reference**
Cryptococcus gattii (n=13)	> 0.256	Pure Gly[g]	RPMI	BMD and spot-plating on SGA	35°C, 72 h	256 mg/L	(90)
Geotrichum candidum	1	MAMBA TM DMA 480 SL®	PDA	BMacroD then Agar plating	27°C, 5 d	2 g/L	(98)
G. candidum (n=2)	0.362 [0.1-0.625]	Roundup® R400	ND	BMD	ND	ND	(96)
Penicillium digitatum	0.24	MAMBA TM DMA 480 SL®	PDA	BMacroD then Agar plating	27°C, 5 d	2 g/L	(98)
Penicillium italicum	0.24	MAMBA TM DMA 480 SL®	PDA	BMacroD then Agar plating	27°C, 5 d	2 g/L	(98)
Saccharomyces cerevisiae (n=2)	4.5 [3-6]	ND	ND	AD at pH 4.0	ND	ND	(97)
S. cerevisiae	2	ND	ND	AD pH 6.8	ND	ND	(97)

962 ***: After extinction and after stability passage; aaa: aromatic amino acids; ABB: Anaerobic Basal Broth (Oxoid CM0957); AD: agar dilution; BHI: Brain Heart

963 Infusion broth (VWR 84626); BD: broth dilution; BMD: broth microdilution; BMacroD: broth microdilution; CATC: Citrat- AzidTween- Carbonat Agar; Gly:

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964 Glyphosate; LBB: Luria-Bertani Broth; MHB: Mueller Hinton Broth; MOPS: minimal medium; MRS: deMan Rogosa and Sharp broth (Merck 1.10661); MSB-
965 Gly: Basal salt-glyphosate medium broth; ND: PDA: Potato dextrose agar; RCM : Reinforced Clostridial Medium; RPMI: Roswell Park Memorial Institute;
966 SGA: Sabouraud glucose agar; TPG: minimal medium. The source of the seven pure glyphosate formulations mentioned in this table is as follow: a Sigma-
967 Aldrich®, CAS number 38641-94-0; b Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany; c Monsanto, Brazil; d Sigma-Aldrich®, St Louis, MO, USA; e
968 Sigma-Aldrich®, Buenos Aires, Argentina; f Merck Ltd., Germany; g Sigma-Aldrich®, Sao Paulo, Brazil.

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970 Table 2: Composition of glyphosate-based formulations cited in Table 1

Glyphosate formulation	Company/Country	Gly acid (g/L)	Gly salt (g/L)	Type of Gly salt	Density (g/mL)	pH
Dominator Extra 608 SL	NA	480	608	DMA	1.22	4.8
Fozat 480	NA	360	480	IPA	1.16 -1.17	4.5 - 5.5
Gladiator 480 SL	NA	360	486	IPA	1.152 - 1.192	4.4 - 4.9
Glyfonova® 450 PLUS	FMC Corporation	450	607	IPA	1.201	5.1
Gylphosate-48	VIV Interchem Ltd., Thailand	NA	480	IPA	NA	NA
Jablo™ Glyfosat	Monsanto Crop Sciences, Denmark	360	480	IPA	NA	NA
MAMBA TM DMA 480 SL®	DowagroScience	480	608	DMA	1.21	4.6
Roundup weed killer	Monsanto, Australia	NA	360	IPA	1.00	6.6 - 6.9
Roundup LB Plus	German registration number 024142-00	NA	486	IPA	1.166	4.8
Roundup Mega	MONSANTO, Europe	450	551	P	1.308	4.8
Roundup Ultra Max®	Monsanto, USA	NA	570	P	1.379	4.3 - 4.8
Roundup® R400	Monsanto, Anvers, Belgium	400	NA	NA	NA	NA
Roundup® R450	Monsanto, Anvers, Belgium	450	NA	NA	NA	NA

971 Gly: Glyphosate; IPA: Isopropylamine; DMA: Dimethylamine; P: Potassium; NA: Not Available

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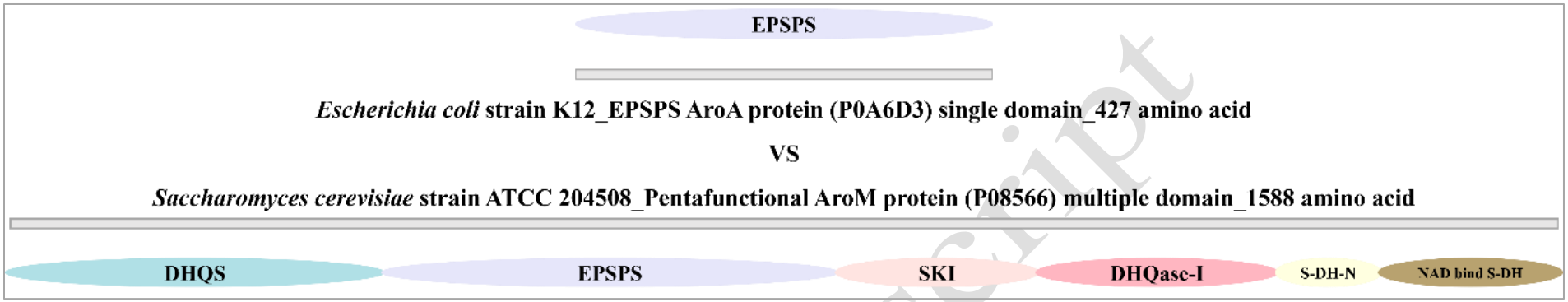

973

974 Figure 1: The single-domain AroA protein of *Escherichia coli* K-12 (P0A6D3) compared to the pentafuctional AroM protein
975 containing six domains in *Saccharomyces cerevisiae* strain ATCC 204508 (P08566). The protein functional classification was
976 conducted using InterProScan. DHQS: 3-dehydroquinate synthase; SKI: shikimate kinase; DHQase-I: 3-dehydroquinate dehydratase
977 type I; S-DH-N: shikimate dehydrogenase substrate binding, N-terminal; NAD-bind-S-DH: nicotinamide adenine dinucleotide binding
978 domain of shikimate dehydrogenase.

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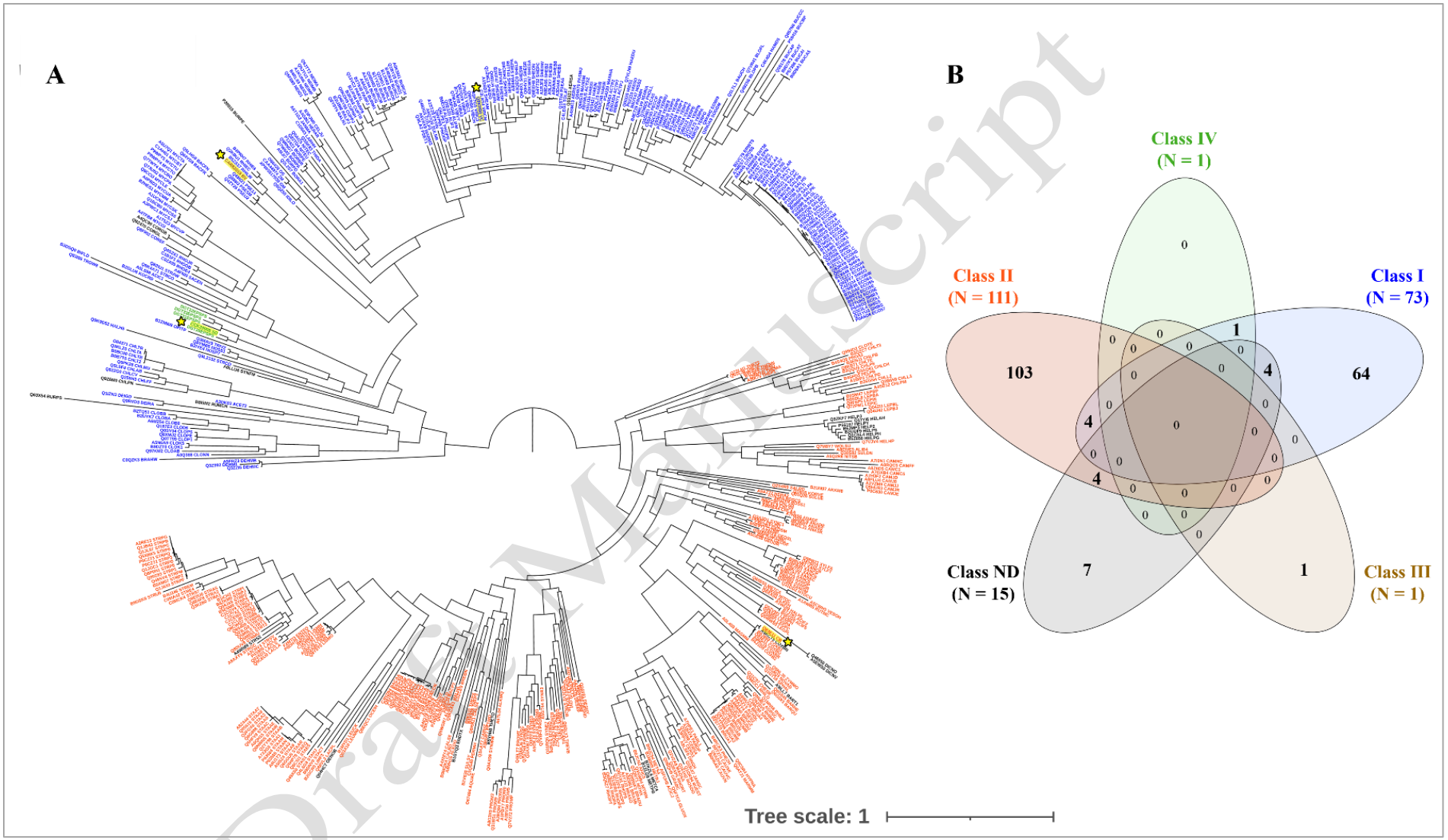

980

981 Figure 2: (A) Phylogenetic classification of a total of 548 EPSPS protein sequences including 541 reviewed proteins from Uniprot
982 (https://www.uniprot.org/), four reference sequences from the ppuigbo EPSPSClass database (https://ppuigbo.me/programs/EPSPSClass/) and

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three plant sequences using Fast Tree. Two distinct EPSPS classes, class I (blue) and class II (orange) are observed. Reference sequences are highlighted in yellow and starred. No class III EPSPS (brown) sequence is found in Uniprot reviewed protein database, and only one sequence (*Brevundimonas vesicularis*, CAR82018) of this class is shown in the analysis due to rarity of this sequence.

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986 Table 3: Mutations occurring in bacterial class I and class II EPSPS protein sequences.

	Mutation	R/S	Origin strain	Reference
Class I	P101S	R	*Salmonella typhimurium*	(40)
	G96A	R	*Klebsiella pneumoniae*	(41,43,105)
	T42M	R	-	(106)
	A183T	R	-	(105)
	P101G	S	-	(42)
	P101A	S	-	(42)
	P101L	S	-	(42)
	T97I	S	-	(44)
	T97I/ P101S	R	-	(44)
Class II	-	R	*Agrobacterium sp. CP4*	
	S130A	R	*Pseudomonas stutzeri A1501*	(37)
	N267S/ P318R/ M425T	R	*Ochrobactrum anthropi*	(39)
	T355S	R	*Halothermothrix orenii H168*	(32)
	G100A	R	*Alcanivorax sp. L27*	(33)
	G96A/T98I/P102S	R	*Pantoea sp.*	(46)
	P101L	R	*Ochrobactrum haematophilum P6BS-III*	(34)
	R21C/N265S/A329T	S	*Pseudomonas nitroreducens FY43M-4*	(107)
	L35F/A242V	S	*P. nitroreducens FY47M-9*	(107)
	P71L	S	*P. nitroreducens FY43M-8*	(107)
	T258A	S	*Pseudomonas nitroreducens FY47M-2*	(107)
	L184F/G292C	R^*	*Pseudomonas nitroreducens FY47M-6*	(107)
	G292S	R	*Pseudomonas nitroreducens FY47M-7*	(107)

987 S: susceptible; R: resistant; R^*: reduced resistance

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989 Table 4: Bacterial strains that are able to degrade glyphosate with their glyphosate catabolism pathway.

Gram negative bacteria	**Source**	**Pathway**	**Source**	**Reference**
Achromobacter sp. MPK 7	Glyphosate-contaminated soil	C-P lyase	Phosphorus	(108)
Achromobacter sp. MPS 12	Alkylphosphonates-contaminated soil	C-P lyase	Phosphorus	(108)
Achromobacter sp. MPS 12A	Alkylphosphonates-contaminated soil	C-P lyase	ND	(109)
Achromobacter denitrificans SOS5	Different environmental samples from an agriculture area treated with glyphosate	C-P lyase	Not shown	(110)
Achromobacter insolitus SOR2	Different environmental samples from an agriculture area treated with glyphosate	C-P lyase	Not shown	(110)
Achromobacter xylosoxidans SOS3	Different environmental samples from an agriculture area treated with glyphosate	C-P lyase	Not shown	(110)
Agrobacterium radiobacter	Sludge from waste treatment plant	C-P lyase	Phosphorus	(111)
Agrobacterium tumefaciens CNI28	River biofilm with strong capacity to dissipate glyphosate	C-P lyase	Phosphorus	(112)
Pseudomonas aeruginosa-ATCC 9027	ND	C-P lyase	Phosphorus	(113)
Pseudomonas sp. PG2982	ND	C-P lyase	Phosphorus	(113)
Pseudomonas sp. GLC11	Mutant of *Pseudomonas sp*. PAO1 on selective medium	C-P lyase	Phosphorus	(114)
Pseudomonas sp. 4ASW	Glyphosate-contaminated soil*	C-P lyase	Phosphorus	(115)
Ochrobactrum intermedium Sq20	Glyphosate-contaminated soil	C-P lyase	Carbon	(47)
Ochrobactrum pituitosum CNI52	River biofilm with strong capacity to dissipate glyphosate	C-P lyase	Phosphorus	(112)
Ochrobactrum haematophilum SR	Different environmental samples from an agriculture area treated with glyphosate	C-P lyase	Not shown	(110)
Alcaligenes sp. GL	Non-axenic cultures of Anacystis nidulans	C-P lyase	Phosphorus	(116)
Rhizobiaeae meliloti 1021	Spontaneous mutation of a wild-type strain	C-P lyase	Phosphorus	(117)
Enterobacter cloacae K7	Rhizoplane of various plants in Russia	C-P lyase	Phosphorus	(118)
Acidovorax sp. CNI26	River biofilm with strong capacity to dissipate glyphosate	C-P lyase	Phosphorus	(112)
Novosphingobium sp. CNI35	River biofilm with strong capacity to dissipate glyphosate	C-P lyase	Phosphorus	(112)
Flavobacterium sp. GD1	Industrial activated sludge	GOX	Phosphorus	(119)
Ochrobactrum sp. GDOS	Soil treated with glyphosate in Iran	GOX	Phosphorus	(120)
Ochrobactrum anthropi GPK 3	Glyphosate-contaminated soil	GOX	Carbon and phosphorus	(108)
Enterobacter sp. Bisph2	Sandy soil in Algeria	GOX	Phosphorus	(121)
Achromobacter sp. LW9	Activated sludge from glyphosate process waste stream	GOX	Carbon	(122)
Pseudomonas pseudomallei 22	Soil	GOX	Phosphorus	(69)
Pseudomonas spp. strains GA07 and GC04	Glyphosate-contaminated soil in China	GOX	Carbon	(10)
Chryseobacterium sp. Y16C	Activated sludge in a pesticide factory	GOX	ND	(123)

Ochrobacterium anthropic GPK3	Glyphosate-contaminated soil	GOX and C-P lyase	Carbon and phosphorus	(109)
Pseudomonas sp. LBr	Glyphosate process waste stream	GOX and C-P lyase	Phosphorus	(124)
Pseudomonas spp. strains GA09	Glyphosate-contaminated soil in China	GOX and C-P lyase	Carbon	(10)
Comamonas odontotermitis P2	Glyphosate-contaminated soil in Australia	GOX and C-P lyase	Carbon and phosphorus	(125)
Rhizobium leguminosarum	Soils	GOX and C-P lyase	Carbon and phosphorus	(2)
Achromobacter sp. Kg 16	Glyphosate-contaminated soil	GOX and GAT	Phosphorus	(108)
Achromobacter sp. Kg 16	Glyphosate-contaminated soil	GAT	Phosphorus	(126)
Agrobacterium tumefaciens CHLDO	Different environmental samples from an agriculture area treated with glyphosate	*thiO* and C-P lyase	Not shown	(110)
Bradyrhizobium jicamae	Soil with diferent glyphosate exposure in Argentina	*thiO*	Phosphorus	(127)
Bradyrhizobium elkanni	Soil with diferent glyphosate exposure in Argentina	*thiO*	Phosphorus	(127)
Bradyrhizobium japonicum	Soil with diferent glyphosate exposure in Argentina	*thiO*	Phosphorus	(128)
Acetobacter sp.	Glyphosate-contaminated soil from rice field	Not shown	Carbon and/or phosphorus	(128)
Pseudomonas fluorescens	Glyphosate-contaminated soil from rice field	Not shown	Carbon and/or phosphorus	(128)
Burkholderia vietnamiensis AO5–12 and Burkholderia sp. AO5–13	Glyphosate contaminated agriculture land in Malaysia	Not shown	Phosphorus	(129)
Proteus mirabilis strain UMTFA2	Agriculture soil in Johor	Not shown	Not shown	(130)

Gram positive bacteria	**Origin**	**Pathway**	**Source**	**Reference**
Streptomycete sp. StC	Activated sludge from a municipal sewage treatment plant	C-P lyase	Phosphorus and/or nitrogen	(131)
Arthrobacter sp. GLP-1	Mixture culture with Klebsiella pneumoniae	C-P lyase	Phosphorus	(132)
Bacillus aryabhattai FACU	Glyphosate treated soil in Egypt	GOX	ND	(133)
Geobacillus caldoxylosilyticus T20	Central heating system water	GOX	Phosphorus	(66)
Arthrobacter atrocyaneus ATCC 13752	Collection of microorganisms and cell cultures, Germany	GOX	Phosphorus	(134)
Bacillus subtilis	Soils	GOX and C-P lyase	Carbon and phosphorus	(2)

Bacillus cereus CB4	Glyphosate-contaminated soil	GOX and C-P lyase	Carbon	(135)
Streptomyces sp.	Soils	GOX and C-P lyase	Carbon and phosphorus	(2)
Bacillus subtilis Bs-15	Rhizospheric soil of a pepper plant	Not shown	Carbon and phosphorus	(136)
Bacillus cereus 6 P	Glyphosate-exposed orange plantation site	Not shown	Phosphorus	(137)
Bacillus tropicus strain UMTFA	Agriculture soil in Johor	Not shown	Not shown	(130)
Salinicoccus spp.	Environemental soil samples from Lake in Iran	Not shown	Not shown	(104)

990 GOX: Glyphosate oxidoreductase; GAT: Glyphosate N-acetyltransferase

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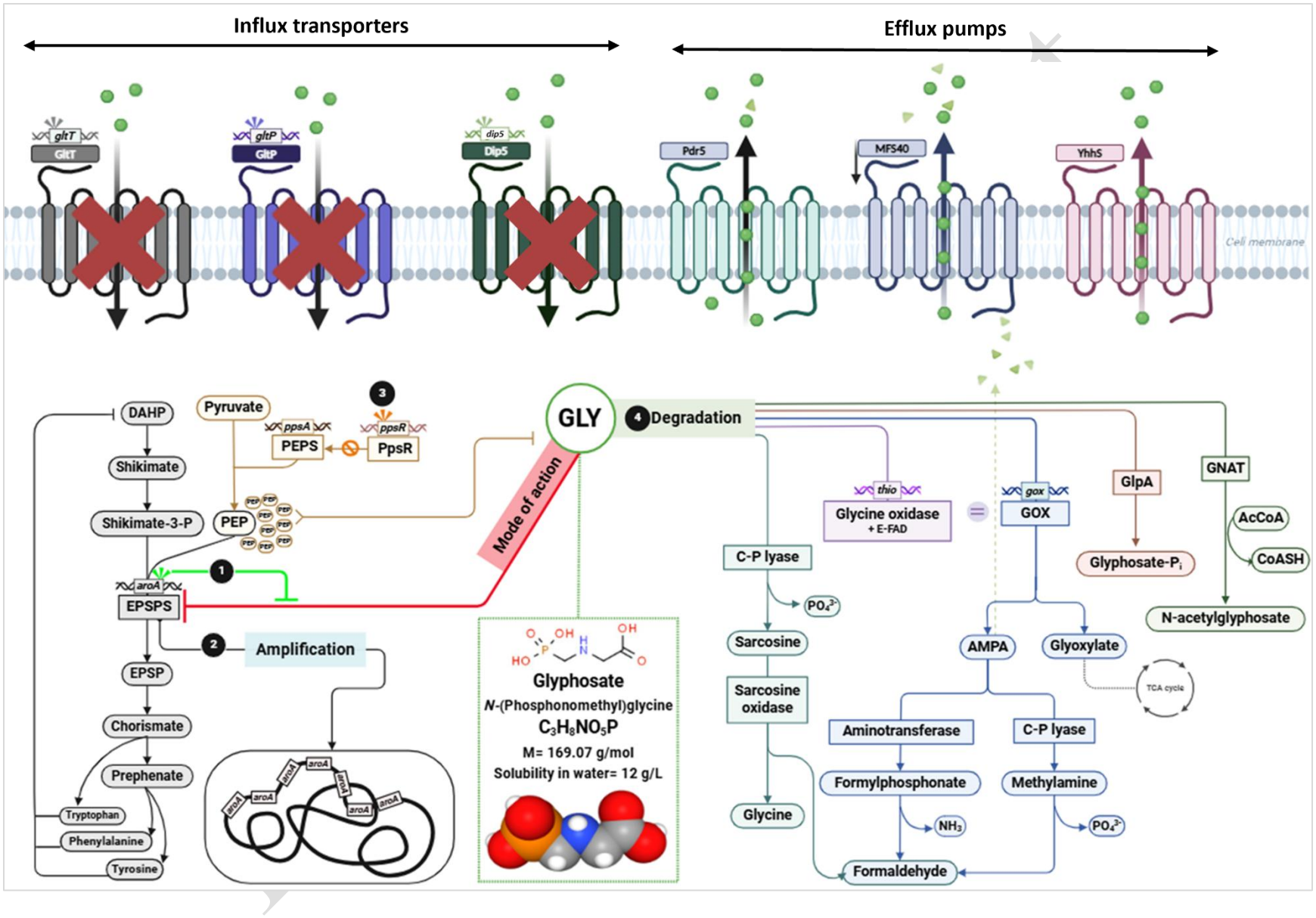
Influx transporters
Efflux pumps
gltT
GltT
gltP
GltP
dip5
Dip5
PdrS
MFS40
YhhS
Cell membrane
DAHP
Shikimate
Shikimate-3-P
EPSPS
EPSP
Chorismate
Prephenate
Tryptophan
Phenylalanine
Tyrosine
Pyruvate
ppsA
PEPS
ppsR
PpsR
PEP
aroA
1
2
Amplification
3
GLY
4 Degradation
Mode of action
Glyphosate
N-(Phosphonomethyl)glycine
C3H8NO5P
M= 169.07 g/mol
Solubility in water= 12 g/L
thio
Glycine oxidase
+ E-FAD
gox
GOX
GlpA
Glyphosate-Pi
GNAT
AcCoA
CoASH
N-acetylglyphosate
C-P lyase
PO4 3-
Sarcosine
Sarcosine oxidase
Glycine
AMPA
Glyoxylate
TCA cycle
Aminotransferase
C-P lyase
Formylphosphonate
Methylamine
NH3
PO4 3-
Formaldehyde

991

Figure 3: Synthesis of bacterial tolerance mechanisms to glyphosate. 1: AroA mutations modify the activity of the EPSPS enzyme; 2: *aroA* gene amplification in the bacterial genome; 3: Upregulation of PEP synthesis due to *ppsR* mutation; 4: Bacterial degradation of glyphosate using multiple pathways to utilize glyphosate as a nutrient source; 5, 6: Mutations of *gltT* and *gltP* genes, encoding the transmembrane transporters GltT and GltP, inhibit the transport of glyphosate into the cytoplasm; 7: Efflux mechanism. GLY: glyphosate; DAHP: 3-deoxy-D-arabino-heptulosonate-7-phosphate; PEPS: phosphoenolpyruvate synthase; PEP: phosphoenolpyruvate; EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase; EPSP: 5-enolpyruvylshikimate-3-phosphate; GOX: glyphosate oxidoreductase; AMPA: aminomethylphosphonic acid; GNAT: glyphosate N-acetyltransferase.

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Supplementary table: Classification of EPSPS sequences using EPSPS Class ppuigbo.

Species	Strain	Accession	Code	ClassIalpha	ClassIbeta	ClassII	ClassIII	ClassIV
Acetivibrio thermocellus	ATCC 27405_DSM 1237_JCM 9322_NBRC 103400_NCIMB 10682_NRRL B_4536_VPI 7372	A3DK03	ACET2	0.92	1	0.579	0.373 (0/18)	0.433
Acidothermus cellulolyticus	ATCC 43068_DSM 8971_11B	A0LS98	ACIC1	1	0.889	0.526	0.384 (0/18)	0.467
Actinobacillus pleuropneumoniae serotype 5b	L20	A3N063	ACTP2	1	1	0.632	0.465 (0/18)	0.267
Actinobacillus pleuropneumoniae serotype 7	AP76	B3GXD4	ACTP7	1	1	0.632	0.465 (0/18)	0.267
Actinobacillus pleuropneumoniae serotype 3	JL03	B0BNY1	ACTPJ	1	1	0.632	0.465 (0/18)	0.267
Actinobacillus succinogenes	ATCC 55618_DSM 22257_CCUG 43843_130Z	A6VMU5	ACTSZ	1	1	0.605	0.459 (0/18)	0.3
Aeromonas salmonicida	A449	A4SM13	AERS4	1	1	0.579	0.465 (0/18)	0.233
Aliivibrio fischeri	MJ11	B5FG60	ALIFM	1	1	0.579	0.454 (0/18)	0.233
Aliivibrio fischeri	ATCC 700601_ES114	Q5E3Z0	ALIF1	1	1	0.579	0.449 (0/18)	0.233
Aliivibrio salmonicida	LFI1238	B6EIX8	ALISL	1	1	0.579	0.449 (0/18)	0.233
Bacteroides fragilis	ATCC 25285_DSM 2151_CCUG 4856_JCM 11019_NCTC 9343_Onslow	Q5LHG9	BACFN	1	1	0.579	0.384 (0/18)	0.267
Bacteroides fragilis	YCH46	Q64YD8	BACFR	1	1	0.579	0.389 (0/18)	0.267
Baumannia cicadellinicola subsp. Homalodisca coagulata	ND	Q1LTL1	BAUCH	0.96	1	0.553	0.378 (0/18)	0.2
Bifidobacterium longum	DJO10A	B3DSQ0	BIFLD	1	0.889	0.553	0.411 (0/18)	0.333
Blochmannia floridanus	ND	Q7VR41	BLOFL	1	1	0.579	0.454 (0/18)	0.267
Blochmannia pennsylvanicus	BPEN	Q492S6	BLOPB	1	1	0.553	0.438 (0/18)	0.233
Bordetella avium	197N	Q2L2S7	BORA1	1	1	0.553	0.465 (0/18)	0.233
Bordetella bronchiseptica	NCTC 13252	Q9RND7	BORBR	1	1	0.553	0.459 (0/18)	0.233
Bordetella parapertussis	12822_ATCC BAA-587_NCTC 13253	Q7W602	BORPA	1	1	0.553	0.459 (0/18)	0.233
Bordetella pertussis	Tohama_I_ATCC BAA-589_NCTC 13251	P12421	BORPE	1	1	0.553	0.454 (0/18)	0.233

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Bordetella petrii	ATCC BAA-461_DSM 12804_CCUG 43448	A9IJI7	BORPD	1	1	0.553	0.481 (0/18)	0.233
Brachyspira hyodysenteriae	ATCC 49526_WA1	C0QZK3	BRAHW	0.88	1	0.526	0.362 (0/18)	0.3
Buchnera aphidicola subsp. Acyrthosiphon pisum	Tuc7	B8D7K3	BUCAT	1	1	0.605	0.432 (0/18)	0.167
Buchnera aphidicola subsp. Acyrthosiphon pisum	5A	B8D9A1	BUCA5	1	1	0.605	0.438 (0/18)	0.167
Buchnera aphidicola subsp. Acyrthosiphon pisum	APS	P57396	BUCAI	1	1	0.605	0.438 (0/18)	0.167
Buchnera aphidicola subsp. Baizongia pistaciae	Bp	P59416	BUCBP	1	1	0.605	0.395 (0/18)	0.267
Buchnera aphidicola subsp. Cinara cedri	Cc	Q057N6	BUCCC	1	1	0.553	0.416 (0/18)	0.2
Buchnera aphidicola subsp. Schizaphis graminum	Sg	Q59178	BUCAP	1	1	0.579	0.427 (0/18)	0.2
Burkholderia ambifaria	MC40_6	B1YV33	BURA4	1	1	0.553	0.497 (0/18)	0.233
Burkholderia ambifaria	ATCC BAA-244_AMMD	Q0BH93	BURCM	1	1	0.553	0.492 (0/18)	0.233
Burkholderia cenocepacia	HI2424	A0K5M1	BURCH	1	1	0.553	0.492 (0/18)	0.233
Burkholderia cenocepacia	ATCC BAA-245_DSM 16553_LMG 16656_NCTC 13227_J2315_CF5610	B4EB42	BURCJ	1	1	0.553	0.497 (0/18)	0.233
Burkholderia lata	ATCC 17760_DSM 23089_LMG 22485_NCIMB 9086_R18194_383	Q39IG1	BURL3	1	1	0.553	0.492 (0/18)	0.233
Burkholderia multivorans	ATCC 17616_249	A9ADV6	BURM1	1	1	0.553	0.503 (0/18)	0.233
Burkholderia orbicola	MC0_3	B1JXR9	BURO0	1	1	0.553	0.492 (0/18)	0.233
Burkholderia orbicola	AU_1054	Q1BY28	BURO1	1	1	0.553	0.492 (0/18)	0.233
Burkholderia vietnamiensis	G4_LMG 22486	A4JCH4	BURVG	1	1	0.553	0.492 (0/18)	0.233
Cellvibrio japonicus	Ueda107	B3PJM0	CELJU	1	1	0.526	0.497 (0/18)	0.267
Chlamydia abortus	DSM 27085_S26_3	Q5L5F4	CHLAB	0.92	1	0.5	0.405 (0/18)	0.4
Chlamydia caviae	ATCC VR-813_DSM 19441_03DC25_GPIC	Q822G0	CHLCV	0.92	1	0.526	0.405 (0/18)	0.4
Chlamydia felis	Fe_C_56	Q255H3	CHLFF	0.88	1	0.526	0.389 (0/18)	0.4
Chlamydia muridarum	MoPn_Nigg	Q9PK28	CHLMU	0.92	1	0.632	0.411 (0/18)	0.233
Chlamydia trachomatis	D_UW_3_Cx	O84371	CHLTR	0.92	1	0.605	0.416 (0/18)	0.267
Chlamydia trachomatis serovar A	ATCC VR-571B_DSM 19440_HAR_13	Q3KLZ0	CHLTA	0.92	1	0.605	0.416 (0/18)	0.267
Chlamydia trachomatis serovar L2	434_Bu_ATCC VR-902B	B0B7T5	CHLT2	0.92	1	0.605	0.422 (0/18)	0.267

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Chlamydia trachomatis serovar L2b	UCH_1_proctitis	B0BC00	CHLTB	0.92	1	0.605	0.422 (0/18)	0.267
Citrobacter koseri	ATCC BAA-895_CDC_4225_83_SGSC4696	A8AIH5	CITK8	1	1	0.605	0.470 (0/18)	0.233
Clostridioides difficile	630	Q187E3	CLOD6	0.92	1	0.553	0.384 (0/18)	0.267
Clostridium acetobutylicum	ATCC 824_DSM 792_JCM 1419_LMG 5710_VKM_B_1787	Q97KM2	CLOAB	0.96	1	0.579	0.384 (0/18)	0.3
Clostridium beijerinckii	ATCC 51743_NCIMB 8052	A6M254	CLOB8	0.92	1	0.526	0.389 (0/18)	0.333
Clostridium botulinum	Eklund_17B_Type_B	B2TQ51	CLOBB	0.92	1	0.579	0.373 (0/18)	0.233
Clostridium botulinum	Alaska_E43_Type_E3	B2UYK7	CLOBA	0.92	1	0.579	0.384 (0/18)	0.233
Clostridium kluyveri	ATCC 8527_DSM 555_NCIMB 10680	A5N6A9	CLOK5	0.96	1	0.605	0.357 (0/18)	0.433
Clostridium kluyveri	NBRC 12016	B9DZT0	CLOK1	0.96	1	0.605	0.357 (0/18)	0.433
Clostridium novyi	NT	A0Q388	CLONN	0.92	1	0.553	0.368 (0/18)	0.3
Clostridium perfringens	SM101_Type_A	Q0SV34	CLOPS	0.92	1	0.526	0.405 (0/18)	0.3
Clostridium perfringens	ATCC 13124_DSM 756_JCM 1290_NCIMB 6125_NCTC 8237_Type_A	Q0TT99	CLOP1	0.92	1	0.526	0.416 (0/18)	0.3
Clostridium perfringens	13_Type_A	Q8XMJ2	CLOPE	0.92	1	0.526	0.416 (0/18)	0.3
Colwellia psychrerythraea	34H_ATCC BAA-681	Q482G5	COLP3	1	1	0.553	0.449 (0/18)	0.3
Corynebacterium efficiens	DSM 44549_YS_314_AJ_12310_JCM 11189_NBRC 100395	Q8FRI2	COREF	1	0.889	0.553	0.378 (0/18)	0.433
Cronobacter sakazakii	ATCC BAA-894	A7MES7	CROS8	1	1	0.605	0.459 (0/18)	0.233
Cupriavidus necator	ATCC 17699_DSM 428_KCTC 22496_NCIMB 10442_H16_Stanier_337	Q0KDH9	CUPNH	1	1	0.526	0.476 (0/18)	0.233
Cupriavidus pinatubonensis	JMP_134_LMG 1197	Q46Y50	CUPPJ	1	1	0.579	0.481 (0/18)	0.233
Cupriavidus taiwanensis	DSM 17343_BCRC 17206_CCUG 44338_CIP 107171_LMG 19424_R1	B3R368	CUPTR	1	1	0.526	0.470 (0/18)	0.233
Dehalococcoides mccartyi	ATCC BAA-2100_JCM 16839_KCTC 5957_BAV1	A5FRZ3	DEHMB	0.88	1	0.526	0.400 (0/18)	0.367
Dehalococcoides mccartyi	ATCC BAA-2266_KCTC 15142_195	Q3Z992	DEHM1	0.88	1	0.526	0.373 (0/18)	0.367
Dehalococcoides mccartyi	CBDB1	Q3ZZI5	DEHMC	0.88	1	0.526	0.400 (0/18)	0.367
Deinococcus geothermalis	DSM 11300_AG_3a	Q1IZN3	DEIGD	0.88	1	0.605	0.362 (0/18)	0.4
Deinococcus radiodurans	ATCC 13939	Q9RVD3	DEIRA	0.88	1	0.605	0.362 (0/18)	0.367
Edwardsiella ictaluri	93_146	Q9X4H2	EDWI9	1	1	0.579	0.432 (0/18)	0.233

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Enterobacter sp.	638	A4W8S8	ENT38	1	1	0.605	0.476 (0/18)	0.233
Erwinia tasmaniensis	DSM 17950_CFBP_7177_CIP 109463_NCPPB_4357_Et1_99	B2VC79	ERWT9	1	1	0.605	0.476 (0/18)	0.233
Escherichia coli	K12	P0A6D3	ECOLI	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli	ATCC 8739_DSM 1576_NBRC 3972_NCIMB 8545_WDCM 00012_Crooks	B1IW23	ECOLC	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli	SMS_3_5_SECEC	B1LJV6	ECOSM	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli	K12_DH10B	B1X848	ECODH	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli	SE11	B6I8Y0	ECOSE	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli	55989_EAEC	B7LDA0	ECO55	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli	K12_MC4100_BW2952	C4ZQ34	ECOBW	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli	ND	P0A6D4	ECO57	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli	UTI89_UPEC	Q1RDV0	ECOUT	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli O1:K1	ND	A1A9I5	ECOK1	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli O127:H6	E2348_69_EPEC	B7UMZ4	ECO27	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli O139:H28	E24377A_ETEC	A7ZJZ7	ECO24	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli O157:H7	EC4115_EHEC	B5YT42	ECO5E	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli O17:K52:H18	UMN026_ExPEC	B7NAQ7	ECOLU	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli O45:K1	S88_ExPEC	B7MHL7	ECO45	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli O6:H1	CFT073_ATCC 700928_UPEC	Q8FJB6	ECOL6	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli O6:K15:H31	536_UPEC	Q0TJE5	ECOL5	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli O7:K1	IAI39_ExPEC	B7NM67	ECO7I	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli O8	IAI1	B7M836	ECO8A	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli O81	ED1a	B7MS23	ECO81	1	1	0.605	0.443 (0/18)	0.233
Escherichia coli O9:H4	HS	A7ZYL1	ECOHS	1	1	0.605	0.443 (0/18)	0.233
Haemophilus ducreyi	35000HP_ATCC 700724	Q7VLN9	HAEDU	1	1	0.632	0.443 (0/18)	0.233
Haemophilus influenzae	PittEE	A5UCH8	HAEIE	1	1	0.632	0.459 (0/18)	0.267
Haemophilus influenzae	PittGG	A5UJ35	HAEIG	1	1	0.632	0.454 (0/18)	0.267
Haemophilus influenzae	ATCC 51907_DSM 11121_KW20_Rd	Q03421	HAEIN	1	1	0.632	0.454 (0/18)	0.267
Haemophilus influenzae	86_028NP	Q4QL19	HAEI8	1	1	0.632	0.459 (0/18)	0.267
Halalkalibacterium halodurans	ATCC BAA-125_DSM 18197	Q9K9D52	HALH5	1	0.944	0.526	0.497 (0/18)	0.467

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Hamiltonella defensa subsp. Acyrthosiphon pisum	5AT	C4K4D4	HAMD5	1	1	0.553	0.459 (0/18)	0.267
Herminiimonas arsenicoxydans	ND	A4G861	HERAR	1	1	0.605	0.486 (0/18)	0.233
Histophilus somni	2336	B0UT47	HISS2	1	1	0.605	0.443 (0/18)	0.167
Histophilus somni	ND	P52310	HISSO	1	1	0.605	0.443 (0/18)	0.167
Histophilus somni	129Pt	Q0I320	HISS1	1	1	0.605	0.443 (0/18)	0.167
Idiomarina loihiensis	ATCC BAA-735_DSM 15497_L2_TR	Q5QZ50	IDILO	1	1	0.579	0.416 (0/18)	0.333
Janthinobacterium sp.	Marseille	A6T1G3	JANMA	1	1	0.579	0.476 (0/18)	0.233
Klebsiella pneumoniae	342	B5XY87	KLEP3	1	1	0.579	0.465 (0/18)	0.233
Klebsiella pneumoniae	ND	P24497	KLEPN	1	1	0.605	0.470 (0/18)	0.233
Klebsiella pneumoniae subsp. pneumoniae	ATCC 700721_MGH_78578	A6T701	KLEP7	1	1	0.605	0.470 (0/18)	0.233
Kocuria rhizophila	ATCC 9341_DSM 348_NBRC 103217_DC2201	B2GLU6	KOCRD	1	0.889	0.526	0.400 (0/18)	0.4
Laribacter hongkongensis	HLHK9	C1D543	LARHH	1	1	0.605	0.514 (0/18)	0.267
Mannheimia haemolytica	ND	P54220	MANHA	1	1	0.605	0.465 (0/18)	0.267
Mannheimia succiniciproducens	MBEL55E	Q65S78	MANSM	1	1	0.605	0.459 (0/18)	0.3
Marinomonas sp.	MWYL1	A6VXX9	MARMS	1	1	0.579	0.465 (0/18)	0.233
Mycobacterium bovis	BCG_Tokyo_172_ATCC 35737_TMC 1019	C1AH01	MYCBT	1	0.889	0.5	0.405 (0/18)	0.467
Mycobacterium bovis	ATCC BAA-935_AF2122_97	Q7TWY4	MYCBO	1	0.889	0.5	0.405 (0/18)	0.467
Mycobacterium leprae	TN	Q9CCI3	MYCLE	1	0.889	0.5	0.389 (0/18)	0.467
Mycobacterium marinum	ATCC BAA-535_M	B2HES1	MYCMM	1	0.889	0.5	0.373 (0/18)	0.433
Mycobacterium sp.	KMS	A1UCN4	MYCSK	1	0.889	0.526	0.427 (0/18)	0.433
Mycobacterium sp.	JLS	A3PWC1	MYCSJ	1	0.889	0.526	0.422 (0/18)	0.433
Mycobacterium sp.	MCS	Q1BCB0	MYCSS	1	0.889	0.526	0.427 (0/18)	0.433
Mycobacterium tuberculosis	ATCC 25618_H37Rv	P9WPY5	MYCTU	1	0.889	0.5	0.405 (0/18)	0.467
Mycobacterium tuberculosis	ATCC 25177_H37Ra	A5U7Q1	MYCTA	1	0.889	0.5	0.405 (0/18)	0.467
Mycobacterium tuberculosis	CDC_1551_Oshkosh	P9WPY4	MYCTO	1	0.889	0.5	0.405 (0/18)	0.467
Mycobacterium ulcerans	Agy99	A0PRD3	MYCUA	1	0.889	0.5	0.373 (0/18)	0.433
Mycolicibacterium gilvum	PYR_GCK	A4TFB8	MYCGI	1	0.889	0.526	0.422 (0/18)	0.4

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Mycolicibacterium paratuberculosis	ATCC BAA-968_K_10	Q73UN2	MYCPA	1	0.889	0.5	0.405 (0/18)	0.433
Mycolicibacterium vanbaalenii	DSM 7251_JCM 13017_BCRC 16820_KCTC 9966_NRRL B_24157_PYR_1	A1T5Z3	MYCVP	1	0.889	0.526	0.432 (0/18)	0.4
Neisseria gonorrhoeae	NCCP11945	B4RL93	NEIG2	1	1	0.605	0.427 (0/18)	0.267
Neisseria gonorrhoeae	ATCC 700825_FA_1090	Q5F889	NEIG1	1	1	0.605	0.422 (0/18)	0.267
Neisseria meningitidis serogroup A serotype 4A	DSM 15465_Z2491	Q9JTT3	NEIMA	1	1	0.579	0.427 (0/18)	0.267
Neisseria meningitidis serogroup B	MC58	Q9JYU1	NEIMB	1	1	0.579	0.427 (0/18)	0.267
Neisseria meningitidis serogroup C serotype 2a	ATCC 700532_DSM 15464_FAM18	A1KUN6	NEIMF	1	1	0.579	0.427 (0/18)	0.267
Nitrosomonas europaea	ATCC 19718_CIP 103999_KCTC 2705_NBRC 14298	Q82TD4	NITEU	1	1	0.553	0.465 (0/18)	0.233
Nitrosomonas eutropha	DSM 101675_C91_Nm57	Q0AIZ5	NITEC	1	1	0.526	0.443 (0/18)	0.2
Nostoc punctiforme	ATCC 29133_PCC_73102	B2IYE4	NOsp.7	1	1	0.5	0.443 (0/18)	0.567
Nostoc sp.	PCC_7120	Q8YMB5	NOSS1	1	1	0.526	0.427 (0/18)	0.533
Opitutus terrae	DSM 11246_JCM 15787_PB90_1	B1ZMW8	OPITP	1	0.944	0.526	0.405 (0/18)	0.5
Paraburkholderia phymatum	DSM 17167_CIP 108236_LMG 21445_STM815	B2JF04	PARP8	1	1	0.526	0.465 (0/18)	0.233
Paraburkholderia phytofirmans	DSM 17436_LMG 22146_PsJN	B2T630	PARPJ	1	1	0.579	0.476 (0/18)	0.233
Paraburkholderia xenovorans	LB400	Q13VC2	PARXL	1	1	0.579	0.476 (0/18)	0.233
Pasteurella multocida	Pm70	Q04570	PASMU	1	1	0.605	0.454 (0/18)	0.233
Pectobacterium atrosepticum	SCRI_1043_ATCC BAA-672	Q6D401	PECAS	1	1	0.579	0.454 (0/18)	0.233
Pectobacterium carotovorum subsp. Carotovorum	PC1	C6DF65	PECCP	1	1	0.579	0.459 (0/18)	0.233
Photobacterium damsela subsp. piscicida	ND	Q8VP65	PHODP	1	1	0.605	0.459 (0/18)	0.233
Photobacterium profundum	SS9	Q6LPE1	PHOPR	1	1	0.579	0.459 (0/18)	0.233
Photorhabdus laumondii subsp. laumondii	DSM 15139_CIP 105565_TT01	Q7N6D5	PHOLL	1	1	0.605	0.449 (0/18)	0.233
Proteus mirabilis	HI4320	B4ET25	PROMH	1	1	0.605	0.459 (0/18)	0.233
Pseudoalteromonas atlantica	T6c_ATCC BAA-1087	Q15T04	PSEA6	1	1	0.579	0.438 (0/18)	0.267

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Pseudoalteromonas translucida	TAC_125	Q3ILA2	PSET1	1	1	0.579	0.443 (0/18)	0.267
Pseudomonas savastanoi pv phaseolicola	1448A_Race_6	Q48N21	PSE14	1	1	0.605	0.984 (15/18)	0.4
Pseudomonas syringae pv syringae	B728a	Q4ZY26	PSEU2	0.96	1	0.605	0.973 (13/18)	0.4
Pseudomonas syringae pv tomato	ATCC BAA-871_DC3000	Q888I3	PSESM	1	1	0.605	0.984 (15/18)	0.4
Psychromonas ingrahamii	DSM 17664_CCUG 51855_37	A1STZ0	PSYIN	1	1	0.579	0.454 (0/18)	0.233
Ralstonia nicotianae	GMI1000	Q8Y0Y6	RALN1	1	1	0.579	0.492 (0/18)	0.233
Ralstonia pickettii	12J	B2U886	RALPJ	1	1	0.579	0.486 (0/18)	0.233
Rhizobium etli	CIAT_652	B3PRH7	RHIE6	1	1	0.5	0.978 (14/18)	0.433
Rhizobium etli	CFN_42_ATCC 51251	Q2KBU1	RHIEC	1	1	0.5	0.989 (16/18)	0.433
Rhizobium leguminosarum bv trifolii	WSM2304	B5ZS37	RHILW	1	1	0.553	0.962 (13/18)	0.433
Rhodococcus erythropolis	PR4_NBRC 100887	C0ZX05	RHOE4	1	0.889	0.526	0.405 (0/18)	0.467
Rhodococcus jostii	RHA1	Q0S2X1	RHOJR	1	0.889	0.526	0.432 (0/18)	0.467
Rhodococcus opacus	B4	C1B1F1	RHOOB	1	0.889	0.526	0.432 (0/18)	0.433
Saccharopolysp.ora erythraea	ATCC 11635_DSM 40517_JCM 4748_NBRC 13426_NCIMB 8594_NRRL 2338	A4FNI0	SACEN	1	0.889	0.553	0.411 (0/18)	0.5
Salmonella agona	SL483	B5F161	SALA4	1	1	0.579	0.449 (0/18)	0.233
Salmonella arizonae	ATCC BAA-731_CDC346_86_RSK2980	A9MHX5	SALAR	1	1	0.579	0.454 (0/18)	0.233
Salmonella choleraesuis	SC_B67	Q57R23	SALCH	1	1	0.579	0.454 (0/18)	0.233
Salmonella dublin	CT_02021853	B5FQ51	SALDC	1	1	0.579	0.454 (0/18)	0.233
Salmonella enteritidis PT4	P125109	B5QYQ8	SALEP	1	1	0.579	0.454 (0/18)	0.233
Salmonella gallinarum	287_91_NCTC 13346	B5R8J5	SALG2	1	1	0.579	0.454 (0/18)	0.233
Salmonella gallinarum	ND	P22299	SALGL	1	1	0.579	0.454 (0/18)	0.233
Salmonella heidelberg	SL476	B4TD38	SALHS	1	1	0.579	0.454 (0/18)	0.233
Salmonella newport	SL254	B4T142	SALNS	1	1	0.579	0.454 (0/18)	0.233
Salmonella paratyphi A	AKU_12601	B5BBP9	SALPK	1	1	0.579	0.454 (0/18)	0.233
Salmonella paratyphi A	ATCC 9150_SARB42	Q5PGG5	SALPA	1	1	0.579	0.454 (0/18)	0.233
Salmonella paratyphi B	ATCC BAA-1250_sp.B7	A9N7V6	SALPB	1	1	0.579	0.454 (0/18)	0.233
Salmonella paratyphi C	RKS4594	C0PXU4	SALPC	1	1	0.579	0.454 (0/18)	0.233

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Salmonella schwarzengrund	CVM19633	B4TRT9	SALSV	1	1	0.579	0.454 (0/18)	0.233
Salmonella typhi	ND	P19786	SALTI	1	1	0.579	0.454 (0/18)	0.233
Salmonella typhimurium	LT2_SGSC1412_ATCC 700720	P07637	SALTY	1	1	0.579	0.454 (0/18)	0.233
Serratia proteamaculans	568	A8GCH1	SERP5	1	1	0.579	0.465 (0/18)	0.233
Shewanella amazonensis	ATCC BAA-1098_SB2B	A1S6D3	SHEAM	1	1	0.579	0.454 (0/18)	0.233
Shewanella baltica	OS155_ATCC BAA-1091	A3D4A6	SHEB5	1	1	0.579	0.476 (0/18)	0.233
Shewanella baltica	OS185	A6WNN0	SHEB8	1	1	0.579	0.470 (0/18)	0.233
Shewanella baltica	OS195	A9L2X7	SHEB9	1	1	0.553	0.476 (0/18)	0.233
Shewanella baltica	OS223	B8EA95	SHEB2	1	1	0.579	0.481 (0/18)	0.233
Shewanella denitrificans	OS217_ATCC BAA-1090_DSM 15013	Q12NE1	SHEDO	1	1	0.579	0.476 (0/18)	0.233
Shewanella frigidimarina	NCIMB 400	Q081U4	SHEFN	1	1	0.579	0.470 (0/18)	0.2
Shewanella halifaxensis	HAW_EB4	B0TT43	SHEHH	1	1	0.579	0.449 (0/18)	0.233
Shewanella loihica	ATCC BAA-1088_PV_4	A3QEC0	SHELP	1	1	0.579	0.481 (0/18)	0.233
Shewanella oneidensis	MR_1	Q8EEH8	SHEON	1	1	0.579	0.470 (0/18)	0.233
Shewanella pealeana	ATCC 700345_ANG_SQ1	A8H4A4	SHEPA	1	1	0.579	0.454 (0/18)	0.233
Shewanella piezotolerans	WP3_JCM 13877	B8CMH2	SHEPW	1	1	0.579	0.438 (0/18)	0.233
Shewanella putrefaciens	CN_32_ATCC BAA-453	A4Y732	SHEPC	1	1	0.579	0.465 (0/18)	0.233
Shewanella sediminis	HAW_EB3	A8FVN6	SHESH	1	1	0.579	0.476 (0/18)	0.233
Shewanella sp.	ANA_3	A0KWN7	SHESA	1	1	0.579	0.476 (0/18)	0.233
Shewanella sp.	W3_18_1	A1RJF8	SHESW	1	1	0.579	0.465 (0/18)	0.233
Shewanella sp.	MR_4	Q0HIX1	SHESM	1	1	0.579	0.481 (0/18)	0.233
Shewanella sp.	MR_7	Q0HV11	SHESR	1	1	0.579	0.476 (0/18)	0.233
Shewanella woodyi	ATCC 51908_MS32	B1KF47	SHEWM	1	1	0.579	0.459 (0/18)	0.233
Shigella boydii serotype 18	CDC_3083_94_BS512	B2TUH3	SHIB3	1	1	0.605	0.443 (0/18)	0.233
Shigella boydii serotype 4	Sb227	Q31YU3	SHIBS	1	1	0.605	0.443 (0/18)	0.233
Shigella dysenteriae	ND	O87006	SHIDY	1	1	0.605	0.443 (0/18)	0.233
Shigella dysenteriae serotype 1	Sd197	Q32E25	SHIDS	1	1	0.605	0.443 (0/18)	0.233
Shigella flexneri	ND	Q83RY8	SHIFL	1	1	0.605	0.443 (0/18)	0.233
Shigella flexneri serotype 5b	8401	Q0SX08	SHIF8	1	1	0.605	0.443 (0/18)	0.233
Shigella sonnei	Ss046	Q3Z3L4	SHISS	1	1	0.605	0.443 (0/18)	0.233
Shigella sonnei	ND	Q9ZFF7	SHISO	1	1	0.605	0.443 (0/18)	0.233

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Sodalis glossinidius	morsitans	Q2NUA9	SODGM	1	1	0.526	0.432 (0/18)	0.233
Streptomyces avermitilis	ATCC 31267_DSM 46492_JCM 5070_NBRC 14893_NCIMB 12804_NRRL 8165_MA_4680	Q82IU1	STRAW	1	0.889	0.553	0.384 (0/18)	0.467
Streptomyces coelicolor	ATCC BAA-471	Q9K4A71	STRCO	1	0.889	0.553	0.378 (0/18)	0.467
Streptomyces coelicolor	ATCC BAA-471	Q9L2132	STRCO	1	1	0.605	0.470 (0/18)	0.3
Sulfurovum sp.	NBC37_1	A6Q7Q0	SULNB	1	1	0.579	0.454 (0/18)	0.233
Thiobacillus denitrificans	ATCC 25259	Q3SK83	THIDA	1	1	0.579	0.465 (0/18)	0.267
Tolumonas auensis	DSM 9187_TA4	C4LEG3	TOLAT	1	1	0.605	0.481 (0/18)	0.233
Trichormus variabilis	ATCC 29413_PCC_7937	Q3MAV9	TRIV2	1	1	0.526	0.427 (0/18)	0.533
Tropheryma whipplei	TW08_27	Q83I86	TROW8	1	0.889	0.553	0.346 (0/18)	0.433
Vibrio atlanticus	LGP32	B7VM38	VIBA3	1	1	0.605	0.459 (0/18)	0.233
Vibrio campbellii	ATCC BAA-1116	A7N1K3	VIBC1	1	1	0.605	0.459 (0/18)	0.233
Vibrio cholerae serotype O1	ATCC 39315_El Tor Inaba_N16961	Q9KRB0	VIBCH	1	1	0.605	0.481 (0/18)	0.233
Vibrio cholerae serotype O1	ATCC 39541_Classical Ogawa 395_O395	A5F7G5	VIBC3	1	1	0.605	0.481 (0/18)	0.233
Vibrio cholerae serotype O1	M66_2	C3LN54	VIBCM	1	1	0.605	0.481 (0/18)	0.233
Vibrio parahaemolyticus serotype O3:K6	RIMD_2210633	Q87QX9	VIBPA	1	1	0.605	0.465 (0/18)	0.233
Vibrio vulnificus	YJ016	Q7MJ45	VIBVY	1	1	0.605	0.476 (0/18)	0.233
Xenorhabdus nematophila	ATCC 19061_DSM 3370_CCUG 14189_LMG 1036_NCIMB 9965_AN6	Q8RLV9	XENNA	1	1	0.605	0.459 (0/18)	0.233
Yersinia enterocolitica serotype O:8 biotype 1B	NCTC 13174_8081	P19688	YERE8	1	1	0.579	0.459 (0/18)	0.233
Yersinia pestis	Pestoides_F	A4TN18	YERPP	1	1	0.579	0.443 (0/18)	0.233
Yersinia pestis	ND	Q60112	YERPE	1	1	0.579	0.443 (0/18)	0.233
Yersinia pestis bv Antiqua	Angola	A9R7I2	YERPG	1	1	0.579	0.443 (0/18)	0.233
Yersinia pestis bv Antiqua	Antiqua	Q1CA73	YERPA	1	1	0.579	0.443 (0/18)	0.233
Yersinia pestis bv Antiqua	Nepal516	Q1CGG5	YERPN	1	1	0.579	0.443 (0/18)	0.233
Yersinia pseudotuberculosis serotype I	IP32953	Q66CI8	YERPS	1	1	0.579	0.449 (0/18)	0.233
Yersinia pseudotuberculosis serotype IB	PB1_+	B2KA23	YERPB	1	1	0.579	0.449 (0/18)	0.233
Yersinia pseudotuberculosis serotype O:1b	IP_31758	A7FJW9	YERP3	1	1	0.579	0.449 (0/18)	0.233

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Yersinia pseudotuberculosis serotype O:3	YPIII	B1JRD9	YERPY	1	1	0.579	0.449 (0/18)	0.233
Yersinia ruckeri	ND	Q93ED4	YERRU	1	1	0.579	0.459 (0/18)	0.233
Oryza sativa subsp japonica		O80428	ORYSA	0.8	0.778	0.474	0.4 (0/18)	0.3
Zea mays	ND	CAA44974	ZM	1	1	0.579	0.405 (0/18)	0.3
Glycine max	ND	XP_003517039	GM	1	1	0.579	0.438 (0/18)	0.267
Acaryochloris marina	MBIC_11017	B0C218	ACAM1	0.76	0.833	1	0.378 (0/18)	0.333
Achromobacter sp.	LBAA	P0A2Y5	ACHSL	0.76	0.889	1	0.351 (0/18)	0.233
Acidiphilium cryptum	JF_5	A5FUH8	ACICJ	0.76	0.833	1	0.357 (0/18)	0.3
Acidithiobacillus ferrooxidans	ATCC 53993_BNL_5_31	B5EQ20	ACIF5	0.76	0.778	1	0.395 (0/18)	0.2
Acidithiobacillus ferrooxidans	ATCC 23270_DSM 14882_CIP 104768_NCIMB 8455	B7J781	ACIF2	0.76	0.778	1	0.395 (0/18)	0.2
Afipia carboxidovorans	ATCC 49405_DSM 1227_KCTC 32145_OM5	B6JCN2	AFIC5	0.8	0.889	1	0.378 (0/18)	0.333
Agrobacterium sp. CP4	CP4	Q9R4E4	AGRSC	0.72	0.889	1	0.422 (0/18)	0.233
Akkermansia muciniphila	ATCC BAA-835_DSM 22959_JCM 33894_BCRC 81048_CCUG 64013_CIP 107961_Muc	B2UN97	AKKM8	0.8	0.833	1	0.351 (0/18)	0.233
Aliarcobacter butzleri	RM4018	A8EWE5	ALIB4	0.76	0.833	1	0.297 (0/18)	0.167
Alkalihalobacillus clausii	KSM_K16	Q5WGR7	ALKCK	0.8	0.833	1	0.346 (0/18)	0.2
Alkalilimnicola ehrlichii	ATCC BAA-1101_DSM 17681_MLHE_1	Q0AA54	ALKEH	0.8	0.833	1	0.314 (0/18)	0.367
Alkaliphilus metalliredigens	QYMF	A6TL04	ALKMQ	0.8	0.889	1	0.346 (0/18)	0.2
Alkaliphilus oremlandii	OhILAs	A8MH16	ALKOO	0.8	0.889	1	0.308 (0/18)	0.2
Anaeromyxobacter dehalogenans	2CP_1_ATCC BAA-258	B8J8W7	ANAD2	0.8	0.889	1	0.330 (0/18)	0.167
Anaeromyxobacter dehalogenans	2CP_C	Q2IMC8	ANADE	0.8	0.889	1	0.357 (0/18)	0.167
Anaeromyxobacter sp.	Fw109_5	A7H6S6	ANADF	0.8	0.889	1	0.335 (0/18)	0.2
Anaeromyxobacter sp.	K	B4ULJ1	ANASK	0.8	0.889	1	0.330 (0/18)	0.167
Aquifex aeolicus	VF5	O67494	AQUAE	0.8	0.889	1	0.351 (0/18)	0.267
Bacillus anthracis	CDC_684_NRRL 3495	C3LF06	BACAC	0.8	0.889	1	0.324 (0/18)	0.2
Bacillus anthracis	A0248	C3NZR4	BACAA	0.8	0.889	1	0.324 (0/18)	0.2
Bacillus anthracis	ND	Q81P64	BACAN	0.8	0.889	1	0.324 (0/18)	0.2
Bacillus cereus	B4264	B7H636	BACC4	0.8	0.889	1	0.324 (0/18)	0.2

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Bacillus cereus	AH187	B7HVH9	BACC7	0.8	0.889	1	0.330 (0/18)	0.2
Bacillus cereus	G9842	B7ILG9	BACC2	0.8	0.889	1	0.335 (0/18)	0.2
Bacillus cereus	AH820	B7JSV5	BACC0	0.8	0.889	1	0.324 (0/18)	0.2
Bacillus cereus	03BB102	C1EYX7	BACC3	0.8	0.889	1	0.330 (0/18)	0.2
Bacillus cereus	ZK_E33L	Q63A07	BACCZ	0.8	0.889	1	0.324 (0/18)	0.2
Bacillus cereus	ATCC 10987_NRS_248	Q736A7	BACC1	0.8	0.889	1	0.324 (0/18)	0.2
Bacillus cereus	ATCC 14579_DSM 31_CCUG 7414_JCM 2152_NBRC 15305_NCIMB 9373_NCTC 2599_NRRL B_3711	Q81C45	BACCR	0.8	0.889	1	0.324 (0/18)	0.2
Bacillus cytotoxicus	DSM 22905_CIP 110041_391_98_NVH_391_98	A7GN56	BACCN	0.8	0.833	1	0.362 (0/18)	0.2
Bacillus licheniformis	ATCC 14580_DSM 13_JCM 2505_CCUG 7422_NBRC 12200_NCIMB 9375_NCTC 10341_NRRL NRS_1264_Gibson_46	Q65I39	BACLD	0.8	0.833	1	0.335 (0/18)	0.167
Bacillus pumilus	SAFR_032	A8FEJ4	BACP2	0.8	0.889	1	0.314 (0/18)	0.167
Bacillus subtilis	168	P20691	BACSU	0.8	0.833	1	0.308 (0/18)	0.167
Bacillus thuringiensis	Al_Hakam	A0RFD1	BACAH	0.8	0.889	1	0.324 (0/18)	0.2
Bacillus thuringiensis subsp. konkukian	97_27	Q6HHF8	BACHK	0.8	0.889	1	0.324 (0/18)	0.2
Bacillus velezensis	DSM 23117_BGSC_10A6_LMG 26770_FZB42	A7Z612	BACVZ	0.8	0.833	1	0.341 (0/18)	0.167
Bartonella henselae	ATCC 49882_DSM 28221_CCUG 30454_Houston_1	Q6G545	BARHE	0.76	0.833	1	0.389 (0/18)	0.267
Bartonella quintana	Toulouse	Q6G0X3	BARQU	0.76	0.833	1	0.416 (0/18)	0.267
Bradyrhizobium diazoefficiens	JCM 10833_BCRC 13528_IAM_13628_NBRC 14792_USDA_110	Q89WF2	BRADU	0.8	0.889	1	0.351 (0/18)	0.333
Bradyrhizobium sp.	ORS_278	A4YJJ3	BRASO	0.8	0.889	1	0.362 (0/18)	0.333
Bradyrhizobium sp.	BTAi1_ATCC BAA-1182	A5E8B1	BRASB	0.76	0.833	1	0.368 (0/18)	0.333
Brevibacillus brevis	47_JCM 6285_NBRC 100599	C0ZCE9	BREBN	0.8	0.889	1	0.341 (0/18)	0.2
Brucella abortus	S19	B2S7T0	BRUA1	0.76	0.889	1	0.373 (0/18)	0.233
Brucella abortus	2308	Q2YPN6	BRUA2	0.76	0.889	1	0.373 (0/18)	0.233
Brucella abortus biovar 1	9_941	P0C100	BRUAB	0.76	0.889	1	0.373 (0/18)	0.233
Brucella anthropi	ATCC 49188_DSM 6882_CCUG 24695_JCM 21032_LMG	A6WUV8	BRUA4	0.76	0.889	1	0.378 (0/18)	0.233

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	3331_NBRC 15819_NCTC 12168_Alc_37							
Brucella canis	ATCC 23365_NCTC 10854	A9M6N0	BRUC2	0.76	0.889	1	0.378 (0/18)	0.233
Brucella melitensis biotype 1	16M_ATCC 23456_NCTC 10094	Q8YEG1	BRUME	0.76	0.889	1	0.373 (0/18)	0.233
Brucella melitensis biotype 2	ATCC 23457	C0RG90	BRUMB	0.76	0.889	1	0.373 (0/18)	0.233
Brucella suis	ATCC 23445_NCTC 10510	B0CI45	BRUSI	0.76	0.889	1	0.373 (0/18)	0.233
Brucella suis biovar 1	1330	Q8G3C4	BRUSU	0.76	0.889	1	0.378 (0/18)	0.233
Caldanaerobacter subterraneus subsp. tengcongensis	DSM 15242_JCM 11007_NBRC 100824_MB4	Q8RB11	CALS4	0.76	0.833	1	0.330 (0/18)	0.267
Caldicellulosiruptor bescii	ATCC BAA-1888_DSM 6725_KCTC 15123_Z_1320	B9MQK4	CALBD	0.8	0.889	1	0.335 (0/18)	0.167
Caldicellulosiruptor saccharolyticus	ATCC 43494_DSM 8903_Tp8T_6331	A4XMY4	CALS8	0.76	0.833	1	0.351 (0/18)	0.2
Campylobacter concisus	13826	A8Z6D5	CAMC1	0.84	0.944	1	0.324 (0/18)	0.2
Campylobacter curvus	525	A7GXB4	CAMC5	0.8	0.889	1	0.330 (0/18)	0.2
Campylobacter fetus subsp. fetus	82_40	A0RQC5	CAMFF	0.76	0.833	1	0.319 (0/18)	0.167
Campylobacter hominis	ATCC BAA-381_LMG 19568_NCTC 13146_CH001A	A7I0N1	CAMHC	0.76	0.833	1	0.324 (0/18)	0.267
Campylobacter jejuni	RM1221	Q5HUR3	CAMJR	0.76	0.833	1	0.303 (0/18)	0.167
Campylobacter jejuni subsp. doylei	ATCC BAA-1458_RM4099	A7H3F2	CAMJD	0.76	0.833	1	0.319 (0/18)	0.167
Campylobacter jejuni subsp. jejuni serotype O:2	ATCC 700819_NCTC 11168	P0C630	CAMJE	0.76	0.833	1	0.303 (0/18)	0.167
Campylobacter jejuni subsp. jejuni serotype O:23 36	81_176	A1VZM9	CAMJJ	0.76	0.833	1	0.303 (0/18)	0.167
Campylobacter jejuni subsp. jejuni serotype O:6	81116_NCTC 11828	A8FLU4	CAMJ8	0.76	0.833	1	0.308 (0/18)	0.167
Carboxydothermus hydrogenoformans	ATCC BAA-161_DSM 6008_Z_2901	Q3AAT7	CARHZ	0.8	0.889	1	0.357 (0/18)	0.333
Caulobacter sp.	K31	B0T270	CAUSK	0.8	0.889	1	0.346 (0/18)	0.233
Caulobacter vibrioides	NA1000_CB15N	B8H6A8	CAUVN	0.8	0.889	1	0.351 (0/18)	0.233
Caulobacter vibrioides	ATCC 19089_CB15	Q9A2H2	CAUVC	0.8	0.889	1	0.351 (0/18)	0.233
Cereibacter sphaeroides	ATCC 17029	A3PPV7	CERS1	0.76	0.833	1	0.346 (0/18)	0.267
Cereibacter sphaeroides	ATCC 17023_DSM 158	Q3IW89	CERS4	0.76	0.833	1	0.357 (0/18)	0.267

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Chelativorans sp.	BNC1	Q11C97	CHESB	0.8	0.889	1	0.405 (0/18)	0.333
Chlorobaculum parvum	DSM 263_NCIMB 8327	B3QQU6	CHLP8	0.72	0.833	1	0.351 (0/18)	0.2
Chlorobaculum tepidum	ATCC 49652_DSM 12025_NBRC 103806_TLS	Q8KB71	CHLTE	0.72	0.833	1	0.351 (0/18)	0.2
Chlorobium chlorochromatii	CaD3	Q3AQ41	CHLCH	0.72	0.833	1	0.335 (0/18)	0.233
Chlorobium limicola	DSM 245_NBRC 103803_6330	B3EGN4	CHLL2	0.72	0.833	1	0.346 (0/18)	0.2
Chlorobium luteolum	DSM 273_BCRC 81028_2530	Q3B659	CHLL3	0.72	0.833	1	0.314 (0/18)	0.167
Chlorobium phaeobacteroides	DSM 266_SMG_266_2430	A1BIP2	CHLPD	0.72	0.833	1	0.357 (0/18)	0.233
Chlorobium phaeobacteroides	BS1	B3ENJ1	CHLPB	0.72	0.778	1	0.351 (0/18)	0.2
Chlorobium phaeovibrioides	DSM 265_1930	A4SD12	CHLPM	0.72	0.833	1	0.346 (0/18)	0.233
Chloroflexus aggregans	MD_66_DSM 9485	B8GC95	CHLAD	0.8	0.889	1	0.330 (0/18)	0.167
Chloroflexus aurantiacus	ATCC 29366_DSM 635_J_10_fl	A9WBA4	CHLAA	0.8	0.889	1	0.373 (0/18)	0.233
Chloroflexus aurantiacus	ATCC 29364_DSM 637_Y_400_fl	B9LFL8	CHLSY	0.8	0.889	1	0.373 (0/18)	0.233
Chloroherpeton thalassium	ATCC 35110_GB_78	B3QZC7	CHLT3	0.72	0.833	1	0.368 (0/18)	0.2
Citrifermentans bemidjiense	ATCC BAA-1014_DSM 16622_JCM 12645_Bem	B5E9T1	CITBB	0.76	0.778	1	0.341 (0/18)	0.233
Clostridium tetani	Massachusetts_E88	Q894D2	CLOTE	0.8	0.889	1	0.324 (0/18)	0.2
Coxiella burnetii	RSA_493_Nine_Mile_phase_I	Q83E11	COXBU	0.8	0.889	1	0.346 (0/18)	0.233
Coxiella burnetii	RSA_331_Henzerling_II	A9NC18	COXBR	0.8	0.889	1	0.351 (0/18)	0.233
Coxiella burnetii	CbuG_Q212	B6J1D9	COXB2	0.8	0.889	1	0.346 (0/18)	0.233
Coxiella burnetii	CbuK_Q154	B6J892	COXB1	0.8	0.889	1	0.346 (0/18)	0.233
Crocosphaera subtropica	ATCC 51142_BH68	B1WZH9	CROS5	0.76	0.833	1	0.270 (0/18)	0.333
Desulfitobacterium hafniense	DSM 10664_DCB_2	B8G2R0	DESHD	0.8	0.889	1	0.308 (0/18)	0.2
Desulfitobacterium hafniense	Y51	Q24V91	DESHY	0.8	0.889	1	0.308 (0/18)	0.2
Desulforamulus reducens	ATCC BAA-1160_DSM 100696_MI_1	A4J3N2	DESRM	0.76	0.833	1	0.362 (0/18)	0.233
Desulforudis audaxviator	MP104C	B1I3Z0	DESAP	0.8	0.889	1	0.368 (0/18)	0.167
Dinoroseobacter shibae	DSM 16493_NCIMB 14021_DFL_12	A8LSF0	DINSH	0.76	0.833	1	0.357 (0/18)	0.233
Enterococcus faecalis	ATCC 47077_OG1RF	F2MTI3	ENTFO	0.8	0.889	1	0.368 (0/18)	0.167
Enterococcus faecalis	ATCC 700802_V583	P0DH70	ENTFA	0.8	0.889	1	0.362 (0/18)	0.167
Geobacillus kaustophilus	HTA426	Q5KXV5	GEOKA	0.8	0.833	1	0.357 (0/18)	0.267

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Geobacillus thermodenitrificans	NG80_2	A4IQ78	GEOTN	0.8	0.833	1	0.357 (0/18)	0.267
Geobacter metallireducens	ATCC 53774_DSM 7210_GS_15	Q39XB8	GEOMG	0.76	0.778	1	0.319 (0/18)	0.2
Geobacter sp.	M21	C6E2B1	GEOSM	0.76	0.778	1	0.330 (0/18)	0.2
Geobacter sulfurreducens	ATCC 51573_DSM 12127_PCA	Q749Y6	GEOSL	0.8	0.833	1	0.314 (0/18)	0.233
Geotalea daltonii	DSM 22248_JCM 15807_FRC_32	B9LZG2	GEODF	0.76	0.778	1	0.319 (0/18)	0.133
Geotalea uraniireducens	Rf4	A5GE08	GEOUR	0.76	0.778	1	0.319 (0/18)	0.167
Gloeobacter violaceus	ATCC 29082_PCC_7421	Q7NLT3	GLOVI	0.76	0.833	1	0.368 (0/18)	0.267
Gloeothece citriformis	PCC_7424	B7KLE7	GLOC7	0.76	0.833	1	0.346 (0/18)	0.267
Gluconobacter oxydans	ND	Q5FTC0	GLUOX	0.8	0.889	1	0.378 (0/18)	0.233
Granulibacter bethesdensis	ATCC BAA-1260_CGDNIH1	Q0BQH9	GRABC	0.76	0.778	1	0.400 (1/18)	0.333
Halalkalibacterium halodurans	ATCC BAA-125_DSM 18197	Q9KCA61	HALH5	0.8	0.889	1	0.346 (0/18)	0.233
Halorhodospira halophila	DSM 244_SL1	A1WUI9	HALHL	0.8	0.833	1	0.362 (0/18)	0.233
Helicobacter hepaticus	ATCC 51449_3B1	Q7VJV4	HELHP	0.8	0.889	1	0.308 (0/18)	0.167
Herpetosiphon aurantiacus	ATCC 23779_DSM 785_114_95	A9AZ72	HERA2	0.76	0.833	1	0.384 (0/18)	0.233
Hydrogenovibrio crunogenus	DSM 25203_XCL_2	Q31GD2	HYDCU	0.8	0.833	1	0.346 (0/18)	0.133
Hyphomonas neptunium	ATCC 15444	Q0C5R4	HYPNA	0.8	0.889	1	0.432 (0/18)	0.233
Jannaschia sp.	CCS1	Q28UI7	JANSC	0.76	0.833	1	0.362 (0/18)	0.267
Koribacter versatilis	Ellin345	Q1IKI5	KORVE	0.72	0.833	1	0.373 (0/18)	0.167
Lactiplantibacillus plantarum	ATCC BAA-793_NCIMB 8826_WCFS1	Q88VL2	LACPL	0.8	0.833	1	0.351 (0/18)	0.267
Lactococcus lactis subsp. cremoris	MG1363	P43905	LACLM	0.8	0.889	1	0.362 (0/18)	0.2
Lactococcus lactis subsp. cremoris	SK11	Q02XD1	LACLS	0.8	0.889	1	0.341 (0/18)	0.233
Lactococcus lactis subsp. lactis	IL1403	Q9CEU0	LACLA	0.8	0.889	1	0.378 (0/18)	0.233
Legionella pneumophila	Corby	A5IBR1	LEGPC	0.76	0.778	1	0.351 (0/18)	0.233
Legionella pneumophila	Lens	Q5WWS9	LEGPL	0.76	0.778	1	0.346 (0/18)	0.233
Legionella pneumophila	Paris	Q5X5E6	LEGPA	0.76	0.778	1	0.351 (0/18)	0.233
Legionella pneumophila subsp. pneumophila	Philadelphia_1_ATCC 33152_DSM 7513	Q5ZVM1	LEGPH	0.76	0.778	1	0.351 (0/18)	0.233
Leptospira biflexa serovar Patoc	Patoc_1_Ames	B0SDL8	LEPBA	0.8	0.889	1	0.330 (0/18)	0.233

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Leptospira biflexa serovar Patoc	Patoc_1_ATCC 23582_Paris	B0SM47	LEPBP	0.8	0.889	1	0.330 (0/18)	0.233
Leptospira borgpetersenii serovar Hardjo bovis	JB197	Q04U42	LEPBJ	0.76	0.833	1	0.362 (0/18)	0.133
Leptospira borgpetersenii serovar Hardjo bovis	L550	Q04ZI3	LEPBL	0.76	0.833	1	0.362 (0/18)	0.133
Leptospira interrogans serogroup Icterohaemorrhagiae serovar copenhageni	Fiocruz_L1_130	Q72PM1	LEPIC	0.76	0.778	1	0.335 (0/18)	0.133
Leptospira interrogans serogroup Icterohaemorrhagiae serovar Lai	56601	Q8F6P5	LEPIN	0.76	0.778	1	0.330 (0/18)	0.133
Leuconostoc citreum	KM20	B1MYD1	LEUCK	0.76	0.833	1	0.335 (0/18)	0.233
Leuconostoc mesenteroides subsp. mesenteroides	ATCC 8293_DSM 20343_BCRC 11652_CCM_1803_JCM 6124_NCDO_523_NBRC 100496_NCIMB 8023_NCTC 12954_NRRL B_1118_37Y	Q03X10	LEUMM	0.76	0.833	1	0.346 (0/18)	0.233
Limosilactobacillus fermentum	NBRC 3956_LMG 18251	B2GCR0	LIMF3	0.76	0.889	1	0.362 (0/18)	0.233
Listeria innocua serovar 6a	ATCC BAA-680_CLIP_11262	Q92A85	LISIN	0.8	0.833	1	0.362 (0/18)	0.2
Listeria monocytogenes serotype 4a	HCC23	B8DC03	LISMH	0.8	0.833	1	0.357 (0/18)	0.2
Listeria monocytogenes serotype 4b	CLIP80459	C1KWM3	LISMC	0.8	0.833	1	0.362 (0/18)	0.2
Listeria monocytogenes serotype 4b	F2365	Q71Y92	LISMF	0.8	0.833	1	0.362 (0/18)	0.2
Listeria monocytogenes serovar 1 2a	ATCC BAA-679_EGD_e	Q8Y5Y0	LISMO	0.8	0.833	1	0.368 (0/18)	0.2
Listeria welshimeri serovar 6b	ATCC 35897_DSM 20650_CIP 8149_NCTC 11857_SLCC_5334_V8	A0AK35	LISW6	0.8	0.833	1	0.351 (0/18)	0.2
Magnetococcus marinus	ATCC BAA-1437_JCM 17883_MC_1	A0L408	MAGMM	0.8	0.833	1	0.378 (0/18)	0.2
Magnetosp.irillum magneticum	AMB_1_ATCC 700264	Q2VZA8	MAGSA	0.8	0.889	1	0.378 (0/18)	0.4
Maricaulis maris	MCS10	Q0ATJ1	MARMM	0.8	0.889	1	0.357 (0/18)	0.233
Mesorhizobium japonicum	LMG 29417_CECT_9101_MAFF_303099	Q98CC1	RHILO	0.8	0.889	1	0.378 (0/18)	0.2

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Methylobacterium radiotolerans	ATCC 27329_DSM 1819_JCM 2831_NBRC 15690_NCIMB 10815_0_1	B1M0N9	METRJ	0.8	0.889	1	0.378 (0/18)	0.233
Methylobacterium sp.	4_46	B0UC54	METS4	0.8	0.889	1	0.395 (1/18)	0.267
Methylococcus capsulatus	ATCC 33009_NCIMB 11132_Bath	Q608S5	METCA	0.8	0.833	1	0.297 (0/18)	0.2
Nitratiruptor sp.	SB155_2	A6Q2R6	NITSB	0.8	0.889	1	0.314 (0/18)	0.2
Nitrobacter hamburgensis	DSM 10229_NCIMB 13809_X14	Q1QRT1	NITHX	0.8	0.889	1	0.378 (0/18)	0.333
Nitrobacter winogradskyi	ATCC 25391_DSM 10237_CIP 104748_NCIMB 11846_Nb_255	Q3SW64	NITWN	0.76	0.833	1	0.373 (0/18)	0.333
Nitrosococcus oceani	ATCC 19707_BCRC 17464_JCM 30415_NCIMB 11848_C_107	Q3JEN6	NITOC	0.76	0.778	1	0.308 (0/18)	0.233
Novosphingobium aromaticivorans	ATCC 700278_DSM 12444_CCUG 56034_CIP 105152_NBRC 16084_F199	Q2G8Q3	NOVAD	0.72	0.722	1	0.400 (0/18)	0.3
Oceanobacillus iheyensis	DSM 14371_CIP 107618_JCM 11309_KCTC 3954_HTE831	Q8EQC1	OCEIH	0.8	0.833	1	0.330 (0/18)	0.233
Paracoccus denitrificans	Pd_1222	A1B474	PARDP	0.8	0.889	1	0.357 (0/18)	0.267
Parasynechococcus marenigrum	WH8102	Q7U7H8	PARMW	0.76	0.833	1	0.351 (0/18)	0.3
Parvibaculum lavamentivorans	DS_1_DSM 13023_NCIMB 13966	A7HPE1	PARL1	0.8	0.889	1	0.378 (0/18)	0.4
Pelobacter propionicus	DSM 2379_NBRC 103807_OttBd1	A1ANP7	PELPD	0.76	0.778	1	0.319 (0/18)	0.2
Pelodictyon phaeoclathratiforme	DSM 5477_BU_1	B4SFD3	PELPB	0.72	0.833	1	0.362 (0/18)	0.2
Pelotomaculum thermopropionicum	DSM 13744_JCM 10971_SI	A5D1R9	PELTS	0.8	0.889	1	0.368 (0/18)	0.2
Persephonella marina	DSM 14350_EX_H1	C0QS40	PERMH	0.8	0.833	1	0.357 (0/18)	0.167
Petrotoga mobilis	DSM 10674_SJ95	A9BHP7	PETMO	0.8	0.889	1	0.341 (0/18)	0.233
Phenylobacterium zucineum	HLK1	B4RCK3	PHEZH	0.8	0.889	1	0.368 (0/18)	0.3
Prochlorococcus marinus	AS9601	A2BQ94	PROMS	0.8	0.889	1	0.341 (0/18)	0.333
Prochlorococcus marinus	MIT_9515	A2BVS6	PROM5	0.8	0.889	1	0.346 (0/18)	0.267
Prochlorococcus marinus	MIT_9303	A2CAX1	PROM3	0.76	0.833	1	0.368 (0/18)	0.333
Prochlorococcus marinus	MIT_9301	A3PBY7	PROM0	0.8	0.889	1	0.351 (0/18)	0.333
Prochlorococcus marinus	MIT_9215	A8G3X9	PROM2	0.8	0.889	1	0.335 (0/18)	0.367
Prochlorococcus marinus	MIT_9312	Q31BS1	PROM9	0.8	0.889	1	0.341 (0/18)	0.333
Prochlorococcus marinus	NATL2A	Q46LT7	PROMT	0.76	0.833	1	0.351 (0/18)	0.333
Prochlorococcus marinus	MIT_9313	Q7V8F4	PROMM	0.76	0.833	1	0.357 (0/18)	0.333

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Prochlorococcus marinus subsp. pastoris	CCMP1986_NIES_2087_MED4	Q7V272	PROMP	0.8	0.889	1	0.362 (0/18)	0.3
Prosthecochloris aestuarii	DSM 271_SK_413	B4S4Q8	PROA2	0.72	0.778	1	0.341 (0/18)	0.167
Pseudomonas sp.	PG2982	P0A2Y4	PSES2	0.76	0.889	1	0.351 (0/18)	0.233
Rhizobium leguminosarum bv viciae	3841	Q1MN56	RHIL3	0.76	0.889	1	0.411 (0/18)	0.233
Rhizobium meliloti	1021	Q92SV5	RHIME	0.76	0.889	1	0.395 (0/18)	0.2
Rhodopseudomonas palustris	TIE_1	B3Q5Z7	RHOPT	0.8	0.889	1	0.384 (0/18)	0.333
Rhodopseudomonas palustris	BisA53	Q07UI1	RHOP5	0.8	0.889	1	0.373 (1/18)	0.333
Rhodopseudomonas palustris	BisB5	Q13ER0	RHOPS	0.8	0.889	1	0.378 (0/18)	0.3
Rhodopseudomonas palustris	HaA2	Q2J2F6	RHOP2	0.8	0.889	1	0.384 (0/18)	0.333
Rhodopseudomonas palustris	ATCC BAA-98_CGA009	Q6NDP4	RHOPA	0.8	0.889	1	0.384 (0/18)	0.333
Rhodosp.irillum rubrum	ATCC 11170	Q2RXP5	RHORT	0.8	0.889	1	0.351 (0/18)	0.333
Roseiflexus castenholzii	DSM 13941_HLO8	A7NJA5	ROSCS	0.8	0.889	1	0.357 (0/18)	0.233
Roseiflexus sp.	RS_1	A5UZF9	ROSS1	0.8	0.889	1	0.368 (0/18)	0.233
Roseobacter denitrificans	ATCC 33942_OCh_114	Q161H2	ROSDO	0.8	0.889	1	0.351 (0/18)	0.233
Ruegeria sp.	TM1040	Q1GK84	RUEST	0.76	0.833	1	0.324 (0/18)	0.233
Ruthia magnifica subsp. Calyptogena magnifica	ND	A1AWR5	RUTMC	0.8	0.833	1	0.341 (0/18)	0.167
Salinibacter ruber	DSM 13855_M31	Q2S4R9	SALRD	0.76	0.778	1	0.357 (0/18)	0.2
Sinorhizobium fredii	NBRC 101917_NGR234	C3MC80	SINFN	0.76	0.889	1	0.389 (0/18)	0.2
Solibacter usitatus	Ellin6076	Q01Q26	SOLUE	0.72	0.778	1	0.357 (0/18)	0.2
sphingopyxis alaskensis	DSM 13593_LMG 18877_RB2256	Q1GT16	SPHAL	0.72	0.778	1	0.411 (0/18)	0.333
Staphylococcus aureus	JH9	A5ISZ4	STAA9	0.76	0.833	1	0.357 (0/18)	0.267
Staphylococcus aureus	Newman	A6QH15	STAAE	0.76	0.778	1	0.346 (0/18)	0.267
Staphylococcus aureus	JH1	A6U1T3	STAA2	0.76	0.833	1	0.357 (0/18)	0.267
Staphylococcus aureus	Mu3_ATCC 700698	A7X2G5	STAA1	0.76	0.778	1	0.357 (0/18)	0.267
Staphylococcus aureus	USA300_TCH1516	A8Z444	STAAT	0.76	0.778	1	0.346 (0/18)	0.267
Staphylococcus aureus	Mu50_ATCC 700699	P63584	STAAM	0.76	0.778	1	0.357 (0/18)	0.267
Staphylococcus aureus	N315	P63585	STAAN	0.76	0.778	1	0.357 (0/18)	0.267
Staphylococcus aureus	NCTC 8325_PS_47	Q05615	STAA8	0.76	0.778	1	0.346 (0/18)	0.267

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Staphylococcus aureus	USA300	Q2FGX6	STAA3	0.76	0.778	1	0.346 (0/18)	0.267
Staphylococcus aureus	bovine_RF122_ET3_1	Q2YY90	STAAB	0.76	0.778	1	0.357 (0/18)	0.267
Staphylococcus aureus	COL	Q5HFV9	STAAC	0.76	0.778	1	0.346 (0/18)	0.267
Staphylococcus aureus	MSSA476	Q6G998	STAAS	0.76	0.778	1	0.346 (0/18)	0.267
Staphylococcus aureus	MRSA252	Q6GGU5	STAAR	0.76	0.778	1	0.351 (0/18)	0.267
Staphylococcus aureus	MW2	Q8NWN5	STAAW	0.76	0.778	1	0.346 (0/18)	0.267
Staphylococcus carnosus	TM300	B9DNV0	STACT	0.8	0.833	1	0.335 (0/18)	0.233
Staphylococcus epidermidis	ATCC 35984_RP62A	Q5HP79	STAEQ	0.76	0.778	1	0.351 (0/18)	0.267
Staphylococcus epidermidis	ATCC 12228_FDA_PCI_1200	Q8CSI1	STAES	0.76	0.778	1	0.351 (0/18)	0.267
Staphylococcus haemolyticus	JCSC1435	Q4L6G8	STAHJ	0.76	0.778	1	0.330 (0/18)	0.3
Staphylococcus saprophyticus subsp. saprophyticus	ATCC 15305_DSM 20229_NCIMB 8711_NCTC 7292_S_41	Q49XS0	STAS1	0.8	0.833	1	0.357 (0/18)	0.267
Streptococcus agalactiae serotype Ia	ATCC 27591_A909_CDC_SS700	Q3K2B0	STRA1	0.8	0.889	1	0.335 (0/18)	0.2
Streptococcus agalactiae serotype III	NEM316	Q8E6F8	STRA3	0.8	0.889	1	0.324 (0/18)	0.2
Streptococcus agalactiae serotype V	ATCC BAA-611_2603_V_R	Q8E0U0	STRA5	0.8	0.889	1	0.335 (0/18)	0.2
Streptococcus equi subsp. equi	4047	C0MA42	STRE4	0.8	0.889	1	0.351 (0/18)	0.267
Streptococcus equi subsp. zooepidemicus	MGCS10565	B4U246	STREM	0.8	0.889	1	0.351 (0/18)	0.267
Streptococcus equi subsp. zooepidemicus	H70	C0MCK4	STRS7	0.8	0.889	1	0.357 (0/18)	0.267
Streptococcus gordonii	Challis_ATCC 35105_BCRC 15272_CH1_DL1_V288	A8AXY9	STRGC	0.8	0.889	1	0.335 (0/18)	0.133
Streptococcus mutans serotype c	ATCC 700610_UA159	Q8DUV8	STRMU	0.8	0.889	1	0.346 (0/18)	0.133
Streptococcus pneumoniae	Hungary19A_6	B1ICH2	STRPI	0.8	0.889	1	0.351 (0/18)	0.133
Streptococcus pneumoniae	ATCC 700669_Spain_23F_1	B8ZKM0	STRPJ	0.84	0.944	1	0.351 (0/18)	0.133
Streptococcus pneumoniae	70585	C1C7X2	STRP7	0.84	0.944	1	0.351 (0/18)	0.133
Streptococcus pneumoniae	JJA	C1CEW0	STRZJ	0.84	0.944	1	0.351 (0/18)	0.133
Streptococcus pneumoniae	P1031	C1CL80	STRZP	0.84	0.944	1	0.351 (0/18)	0.133
Streptococcus pneumoniae	Taiwan19F_14	C1CQY4	STRZT	0.8	0.889	1	0.346 (0/18)	0.133
Streptococcus pneumoniae	ATCC BAA-255_R6	Q8CWQ7	STRR6	0.84	0.944	1	0.346 (0/18)	0.133

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Streptococcus pneumoniae serotype 19F	G54	B5E5M9	STRP4	0.8	0.889	1	0.357 (0/18)	0.133
Streptococcus pneumoniae serotype 2	D39_NCTC 7466	Q04JX6	STRP2	0.84	0.944	1	0.346 (0/18)	0.133
Streptococcus pneumoniae serotype 4	ATCC BAA-334_TIGR4	Q9S400	STRPN	0.8	0.889	1	0.351 (0/18)	0.133
Streptococcus pyogenes serotype M1	ND	Q99Z83	STRP1	0.8	0.889	1	0.351 (0/18)	0.2
Streptococcus pyogenes serotype M12	MGAS2096	Q1JB43	STRPB	0.8	0.889	1	0.357 (0/18)	0.2
Streptococcus pyogenes serotype M12	MGAS9429	Q1JL87	STRPC	0.8	0.889	1	0.357 (0/18)	0.2
Streptococcus pyogenes serotype M18	MGAS8232	Q8P0H1	STRP8	0.8	0.889	1	0.351 (0/18)	0.2
Streptococcus pyogenes serotype M2	MGAS10270	Q1JGC1	STRPD	0.8	0.889	1	0.357 (0/18)	0.2
Streptococcus pyogenes serotype M28	MGAS6180	Q48SV4	STRPM	0.8	0.889	1	0.357 (0/18)	0.2
Streptococcus pyogenes serotype M3	ATCC BAA-595_MGAS315	P0CZ72	STRP3	0.8	0.889	1	0.357 (0/18)	0.2
Streptococcus pyogenes serotype M3	SSI_1	P0CZ73	STRPQ	0.8	0.889	1	0.357 (0/18)	0.2
Streptococcus pyogenes serotype M4	MGAS10750	Q1J633	STRPF	0.8	0.889	1	0.357 (0/18)	0.2
Streptococcus pyogenes serotype M49	NZ131	B5XM07	STRPZ	0.8	0.889	1	0.357 (0/18)	0.2
Streptococcus pyogenes serotype M5	Manfredo	A2RE13	STRPG	0.8	0.889	1	0.357 (0/18)	0.2
Streptococcus pyogenes serotype M6	ATCC BAA-946_MGAS10394	Q5XBK5	STRP6	0.8	0.889	1	0.357 (0/18)	0.2
Streptococcus sanguinis	SK36	A3CNV3	STRSV	0.8	0.889	1	0.308 (0/18)	0.133
Streptococcus thermophilus	ATCC BAA-491_LMD_9	Q03LG9	STRTD	0.8	0.889	1	0.368 (0/18)	0.133
Streptococcus thermophilus	CNRZ_1066	Q5M0L5	STRT1	0.8	0.889	1	0.368 (0/18)	0.133
Streptococcus thermophilus	ATCC BAA-250_LMG 18311	Q5M551	STRT2	0.8	0.889	1	0.368 (0/18)	0.133
Streptococcus uberis	ATCC BAA-854_0140J	B9DSK8	STRU0	0.76	0.833	1	0.319 (0/18)	0.267
Sulfurihydrogenibium sp.	YO3AOP1	B2V630	SULSY	0.76	0.778	1	0.384 (0/18)	0.2
Sulfurimonas denitrificans	ATCC 33889_DSM 1251	Q30S82	SULDN	0.8	0.889	1	0.341 (0/18)	0.133
Synechococcus elongatus	ATCC 33912_PCC_7942_FACHB_805	Q31RQ5	SYNE7	0.76	0.833	1	0.362 (0/18)	0.3
Synechococcus sp.	WH7803	A5GKJ3	SYNPW	0.76	0.833	1	0.346 (0/18)	0.3

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Synechococcus sp.	RCC307	A5GT44	SYNR3	0.76	0.833	1	0.357 (0/18)	0.333
Synechococcus sp.	CC9311	Q0I9Y0	SYNS3	0.76	0.833	1	0.362 (0/18)	0.3
Synechococcus sp.	JA_2	Q2JLV2	SYNJB	0.76	0.833	1	0.368 (0/18)	0.267
Synechococcus sp.	JA_3_3Ab	Q2JSF1	SYNJA	0.76	0.833	1	0.357 (0/18)	0.233
Synechococcus sp.	CC9605	Q3AKJ6	SYNSC	0.76	0.833	1	0.324 (0/18)	0.3
Synechococcus sp.	CC9902	Q3AXB7	SYNS9	0.76	0.833	1	0.351 (0/18)	0.3
Synechococcus sp.	ATCC 27144_PCC_6301_SAUG_1402_1	Q5N2J9	SYNP6	0.76	0.833	1	0.362 (0/18)	0.3
Synechocystis sp.	PCC_6803_Kazusa	Q59975	SYNY3	0.8	0.889	1	0.378 (0/18)	0.3
Syntrophomonas wolfei subsp. wolfei	DSM 2245B_Goettingen	Q0AX99	SYNWW	0.76	0.833	1	0.373 (0/18)	0.233
Syntrophotalea carbinolica	DSM 2380_NBRC 103641_GraBd1	Q3A3D1	SYNC1	0.76	0.778	1	0.351 (0/18)	0.267
Thermoanaerobacter pseudethanolicus	ATCC 33223_39E	B0K928	THEP3	0.8	0.889	1	0.351 (0/18)	0.233
Thermoanaerobacter sp.	X514	B0K0J1	THEPX	0.8	0.889	1	0.351 (0/18)	0.233
Thermosynechococcus vestitus	NIES_2133_IAM_M_273_BP_1	Q8DLY3	THEVB	0.76	0.833	1	0.346 (0/18)	0.333
Thermotoga maritima	ATCC 43589	Q9WYI0	THEMA	0.8	0.889	1	0.330 (0/18)	0.3
Thermotoga neapolitana	ATCC 49049_DSM 4359_NBRC 107923_NS_E	B9KBV6	THENN	0.8	0.889	1	0.330 (0/18)	0.3
Thermotoga petrophila	ATCC BAA-488_DSM 13995_JCM 10881_RKU_1	A5IK73	THEP1	0.8	0.889	1	0.330 (0/18)	0.3
Thermotoga sp.	RQ2	B1L9E5	THESQ	0.8	0.889	1	0.330 (0/18)	0.3
Thermus thermophilus	ATCC 27634_DSM 579_HB8	Q5SL36	THET8	0.8	0.889	1	0.384 (0/18)	0.333
Thermus thermophilus	ATCC BAA-163_DSM 7039_HB27	Q72LH1	THET2	0.8	0.889	1	0.378 (0/18)	0.333
Vesicomyosocius okutanii subsp. Calyptogena okutanii	HA	A5CWH5	VESOH	0.8	0.889	1	0.335 (0/18)	0.2
Wolinella succinogenes	ATCC 29543_DSM 1740_LMG 7466_NCTC 11488_FDC_602W	Q7M8Y7	WOLSU	0.76	0.833	1	0.330 (0/18)	0.2
Xanthomonas axonopodis pv citri	306	Q8PLY5	XANAC	0.8	0.833	1	0.400 (0/18)	0.3
Xanthomonas campestris pv campestris	B100	B0RUW1	XANCB	0.8	0.833	1	0.405 (0/18)	0.267
Xanthomonas campestris pv campestris	8004	Q4UTD1	XANC8	0.8	0.833	1	0.400 (0/18)	0.267
Xanthomonas campestris pv campestris	ATCC 33913_DSM 3586_NCPPB_528_LMG 568_P_25	Q8PA95	XANCP	0.8	0.833	1	0.400 (0/18)	0.267

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Xanthomonas campestris pv vesicatoria	85_10	Q3BUZ1	XANC5	0.8	0.833	1	0.384 (0/18)	0.3
Xanthomonas oryzae pv oryzae	PXO99A	B2SJJ3	XANOP	0.76	0.778	1	0.389 (0/18)	0.3
Xanthomonas oryzae pv oryzae	MAFF_311018	Q2P356	XANOM	0.76	0.778	1	0.389 (0/18)	0.3
Xanthomonas oryzae pv oryzae	KACC10331_KXO85	Q5H081	XANOR	0.76	0.778	1	0.389 (0/18)	0.3
Xylella fastidiosa	Temecula1_ATCC 700964	Q87BU2	XYLFT	0.8	0.889	1	0.438 (0/18)	0.267
Xylella fastidiosa	9a5c	Q9PB21	XYLFA	0.8	0.833	1	0.416 (0/18)	0.3
Zymomonas mobilis subsp. mobilis	ATCC 31821_ZM4_CP4	Q5NLJ0	ZYMMO	0.68	0.722	1	0.395 (0/18)	0.367
Brevundimonas vesicularis	ND	CAR82018	BV	0.96	0.944	0.605	1 (18 / 18)	0.467
Streptomyces davawensis	JCM 4913	CCK29908	SD	0.96	1	0.5	0.459 (0/18)	1
Streptomyces svecius	DGT-28_ATCC 29083	EDY54343.1	DGT28	0.96	1	0.526	0.465 (0/18)	1
Streptomyces flavogriseus	DGT-31	WP_014153759.1	STRF31	0.96	1	0.5	0.47 (0/18)	1
Streptomyces roseosporus	DGT-32	WP_006127956.1	STRR32	0.96	1	0.526	0.454 (0/18)	1
Streptomyces griseus	DGT-33	WP_012378800.1	STRG33	0.96	1	0.5	0.454 (0/18)	1
Aeromonas salmonicida	ND	Q03321	AERSA	0.92	0.944	0.553	0.449 (0/18)	0.233
Bartonella tribocorum	CIP 105476_IBS 506	A9ILL7	BART1	0.8	0.889	0.974	0.389 (0/18)	0.267
Burkholderia pseudomallei	K96243	Q63X54	BURPS	0.72	0.833	0.474	0.357 (0/18)	0.267
Burkholderia pseudomallei	ND	P39915	BURPE	0.84	0.889	0.684	0.389 (0/18)	0.233
Burkholderia pseudomallei	ND	P39915	BURPE	0.84	0.889	0.684	0.389 (0/18)	0.233
Chlamydia pneumoniae	ND	Q9Z6M0	CHLPN	0.88	0.944	0.579	0.395 (0/18)	0.333
Corynebacterium glutamicum	R	A4QC99	CORGB	0.96	0.889	0.553	0.416 (0/18)	0.5
Corynebacterium glutamicum	ATCC 13032	Q9Z470	CORGL	0.96	0.889	0.553	0.422 (0/18)	0.5
Coxiella burnetii	Dugway_5J108_111	A9KCT5	COXBN	0.8	0.889	0.974	0.346 (0/18)	0.233
Dichelobacter nodosus	VCS1703A	A5EWS0	DICNV	0.8	0.833	0.974	0.335 (0/18)	0.3
Dichelobacter nodosus	ND	Q46550	DICNO	0.8	0.833	0.974	0.335 (0/18)	0.267
Endomicrobium trichonymphae	ND	B1GYQ3	ENDTX	0.76	0.778	0.974	0.400 (0/18)	0.3
Helicobacter acinonychis	Sheeba	Q17YI6	HELAH	0.76	0.833	0.947	0.286 (0/18)	0.233
Helicobacter pylori	Shi470	B2UUF9	HELPS	0.76	0.833	0.947	0.270 (0/18)	0.233
Helicobacter pylori	G27	B5Z850	HELPG	0.76	0.833	0.947	0.292 (0/18)	0.233

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Helicobacter pylori	P12	B6JMP3	HELP2	0.76	0.833	0.947	0.292 (0/18)	0.233
Helicobacter pylori	ATCC 700392_26695	P56197	HELPY	0.76	0.833	0.947	0.292 (0/18)	0.267
Helicobacter pylori	HPAG1	Q1CSL4	HELPH	0.76	0.833	0.947	0.297 (0/18)	0.233
Helicobacter pylori	J99_ATCC 700824	Q9ZKF7	HELPJ	0.76	0.833	0.947	0.286 (0/18)	0.233
Methylorubrum extorquens	CM4_NCIMB 13688	B7KZL6	METC4	0.76	0.833	0.974	0.395 (1/18)	0.267
Methylorubrum populi	ATCC BAA-705_NCIMB 13946_BJ001	B1ZLG6	METPB	0.8	0.889	0.974	0.405 (1/18)	0.267
Oenococcus oeni	ATCC BAA-331_PSU_1	Q04HC7	OENOB	0.72	0.778	0.974	0.368 (0/18)	0.233
Ruminiclostridium cellulolyticum	ATCC 35319_DSM 5812_JCM 6584_H10	B8I5M2	RUMCH	0.88	0.944	0.553	0.357 (0/18)	0.3
Streptococcus suis	98HAH33	A4W069	STRS2	0.76	0.778	0.974	0.373 (0/18)	0.133
Syntrophobacter fumaroxidans	DSM 10017_MPOB	A0LLU6	SYNFM	0.96	0.944	0.632	0.432 (0/18)	0.467
Thermodesulfovibrio yellowstonii	ATCC 51303_DSM 11347_YP87	B5YH68	THEYD	0.8	0.889	0.974	0.324 (0/18)	0.267

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Chapitre II

Mécanismes de tolérance au glyphosate : Approche comparative *in vitro*/ *in silico*

Avant-propos

Le glyphosate agit principalement en inhibant l'enzyme EPSP synthase (EPSPS) codé par le gène *aroA*, présente chez les plantes, les bactéries, et les champignons. Cette enzyme a suscité un grand intérêt, notamment dans le domaine de la bio-ingénierie végétale, où des variétés de plantes ont été transformées avec des EPSPS de classe II ou des EPSPS de classe I modifiées pour les rendre résistantes à l'action du glyphosate (46). Les enzymes utilisées en bio-ingénierie étant issues des bactéries, la recherche s'est initialement concentrée sur l'identification et la caractérisation de ces enzymes pour trouver de nouveaux candidats à utiliser dans la production de plantes transgéniques (24,54). Cependant, l'étude du rôle du glyphosate en tant qu'agent antimicrobien et la capacité de certaines bactéries à développer des stratégies de survie malgré l'exposition à cet herbicide a été largement délaissé. La définition de la tolérance/résistance au glyphosate est complexe. De nombreux articles utilisent le terme résistance en se basant sur l'analyse du gène *aroA*, en observant une diminution de son affinité pour le glyphosate, même si les niveaux de résistance *in vitro* chez les bactéries n'ont pas été évalués.

L'objectif de ce chapitre est d'examiner s'il existe a une concordance entre les niveaux de résistance observés et les données génétiques. Nous soulignons ainsi la nécessité d'harmoniser les définitions de tolérance, de résistance et d'affinité de la cible pour mieux comprendre et comparer la sensibilité des microorganismes au glyphosate.

Dans le premier article de ce chapitre (**Article 2**), nous avons étudié le mécanisme de tolérance à une formulation de glyphosate (HBG) de souches du genre *Klebsiella* isolées à partir d'échantillons fécaux de fermiers de la province de Nan, en Thaïlande, une région connue pour son utilisation intensive de glyphosate en agriculture (55). Parmi les dix souches séquencées par des technologies

de séquençage à haut débit, nous avons découvert que quatre d'entre elles possédaient une copie supplémentaire du gène *aroA* de classe II (*aroA II*), situé sur un transposon et porté par un plasmide, alors que les souches de *Klebsiella* possèdent normalement un gène *aroA* de classe I chromosomique. Nous avons analysé l'environnement génétique de ces souches et avons identifié un autre gène d'intérêt, codant pour une protéine de la famille des Major Facilitator Superfamily (MFS). Nous avons supposé que ce plasmide était d'origine alimentaire et nous avons collecté des échantillons d'aliments dans différents supermarchés de Marseille, pour rechercher le gène *aroA II* ainsi que le gène *CP4 aroA*, classiquement utilisé dans les plantes transgéniques résistantes au glyphosate. Parmi les 159 aliments testés, nous avons trouvé six produits d'origine asiatique positifs pour le gène *aroA II* de notre étude. La culture de ces six échantillons a permis d'isoler une souche de *Klebsiella quasipneumoniae* à partir d'un gingembre "Gian Dai" en provenance de Thaïlande, qui possédait un plasmide porteur du même transposon.

En parallèle, différents biocides utilisés par les fermiers ont été testés pour identifier de potentiels substrats de ce plasmide, et notamment du transporteur MFS-like. Parmi toutes les molécules testées, les souches porteuses du plasmide présentaient des CMI plus élevées au paraquat, un herbicide de la famille des ammoniums quaternaires, par rapport aux autres souches. Ce résultat a été confirmé par une diminution de 32 fois la CMI après curage du plasmide dans la souche d'origine alimentaire.

Enfin, nous avons cherché à caractériser ces deux gènes. Les souches de *K. pneumoniae* présentant déjà une CMI élevée au glyphosate (moyenne de 8 g/L) lorsqu'elles n'ont que le gène *aroA* de classe I, nous n'avons pas observé de diminution de la CMI du glyphosate après curage du plasmide. Nous avons donc évalué l'activité enzymatique d'*aroA II* après expression dans une souche d'*Escherichia coli* BL21(DE3) (en cours).

Dans le deuxième article de ce chapitre (**Article 3**), nous avons voulu démontrer qu'il existait une discordance entre l'appellation « bactérie résistante au glyphosate » basée sur l'analyse *in silico* du gène *aroA* et la détermination de la CMI par une méthode *in vitro*. En effet, nous avons fait face à différentes problématiques en réalisant la revue bibliographique sur cette thématique. L'essentiel de la bibliographie ayant porté sur la recherche en bio-ingénierie, ce terme a été utilisé dès qu'une souche portait une EPSPS avec une faible affinité pour le glyphosate, c'est-à-dire pour des EPSPS de classe II, III ou IV. Or, d'autres mécanismes interviennent dans la résistance d'une bactérie au glyphosate qui ne sont pas pris en compte dans cette définition. D'autres part, les travaux qui ont évalué la CMI des bactéries aux glyphosates ont utilisés différentes méthodes, différents réactifs, différentes formulations de glyphosate…, limitant la comparaison des données. Ainsi, nous avons déterminé cette CMI selon une méthodologie harmonisée sur un panel de 60 bactéries de 57 espèces et avons analysé leur gène *aroA*. Nous proposons de différencier clairement l'utilisation du terme « tolérance/résistance au glyphosate » pour la recherche sur l'impact du glyphosate sur les bactéries et de l'affinité de l'EPSPS utilisée en bio-ingénierie.

Article 2

Identification of a novel transposon carrying new *aroA* gene conferring glyphosate tolerance and a MFS transporter for paraquat efflux in *Klebsiella spp.* strains isolated from a polluted area in Thailand: A new One Health threat ?

Aïcha Hamieh, Hanane Zerrouki, Tram VO, Serge Morand, Sophie Alexandra Baron and Jean-Marc Rolain*

En cours de préparation

Résumé

L'environnement est contaminé par un mélange de produits chimiques qui affectent les organismes vivants, y compris les bactéries. Les herbicides sont massivement utilisés dans le monde entier et persistent dans les sols, mais leurs effets sur les microbiomes restent encore mal compris. La Thaïlande, un acteur majeur dans l'exportation de produits alimentaires et agricoles, dépend fortement des pesticides tels que le glyphosate et le paraquat pour protéger les cultures et augmenter les rendements.

Dans un premier travail (**Article 4**), nous avons isolé des souches du genre *Klebsiella* à partir de prélèvements rectaux de fermiers de la province de Nan, l'une des plus grandes provinces agricoles de Thaïlande. Neuf souches présentant des CMI à 16 g/L d'un herbicide à base de glyphosate (HBG) contenant du sel d'isopropylamine et un composé d'ammonium quaternaire ont été séquencées par des technologies de séquençage à haut débit (Illumina MiSeq et/ou Gridion Nanopore). L'objectif de cette étude était de caractériser les mécanismes moléculaires de la tolérance au glyphosate de ces souches. Toutes les souches séquencées possédaient une enzyme EPSPS de classe I constitutive, qui confère normalement une sensibilité au glyphosate. Parmi elles, quatre portaient une enzyme EPSPS supplémentaire, de classe II. Cette EPSPS de classe II se trouve sur un transposon composite (~15 Kb) présent sur différents plasmides de type IncF. Une recherche par BLAST dans la base de données NCBI et une analyse phylogénétique de cette EPSPS de classe II ont révélé une homologie avec 87 séquences à 100 % d'identité et de couverture, principalement chez des souches du genre *Klebsiella*. Bien que ce gène soit retrouvé majoritairement sur des plasmides de type IncF, il a également été localisé dans le chromosome de deux souches d'*Achromobacter sp.* et d'une souche d'*Enterobacter hormaechei*. De manière

intéressante, cette classe II a également été détectée dans le génome d'une souche de *Pseudomonas sp.*, tolérante au glyphosate, isolée d'un sol pollué en Chine, bien que la localisation de ce gène (plasmidique ou chromosomique) n'ait pas pu être déterminée. Nous avons supposé que ce gène a une origine environnementale, dans lequel la pression de sélection au glyphosate est beaucoup plus forte. Les aliments étant plus fortement contaminés par le glyphosate que les humains, nous avons collecté 159 échantillons alimentaires dans différents supermarchés de Marseille. L'ADN extrait de ces échantillons a été testé par RT-PCR pour détecter le gène *aroA II* (notre étude), ainsi que le gène *aroA* CP4 utilisé par Monsanto pour produire des plantes transgéniques. Six échantillons sont revenus positifs à *l'aroA II*, zéro à *l'aroA* CP4. Après mise en culture de ces échantillons, seule une souche de *Klebsiella quasipneumoniae* (Q7925), isolée d'un échantillon de gingembre « Gian Dai » de Thaïlande est revenue positive pour le gène *aroA II*. Le séquençage de cette souche Q7925 a confirmé la présence du gène *aroA II* en association avec la protéine MFS, au sein du même transposon, sur un plasmide de type IncFIB. Nous avons ensuite curé la souche du plasmide pour identifier les résistances induites par ce plasmide. Plusieurs herbicides (N=6) et antiseptiques (N=3) et antibiotiques (N=27) ont été testé, mais seule la sensibilité du paraquat a diminuée d'un facteur 32 après élimination du plasmide. Au sein du transposon porteur du gène *aroA*, nous avons identifié un transporteur membranaire de la famille « Major Facilitator Superfamily » (MFS), impliqué dans la résistance aux biocides. Nous supposons que ce transporteur pourrait être responsable de la résistance accrue au paraquat. En conclusion, cette étude met en évidence la présence d'un nouveau transposon composite contenant un gène *aroA* de classe II impliqué dans la tolérance au glyphosate et une protéine MFS-like potentiellement impliquée dans la résistance au paraquat. Ces gènes ont été trouvés à la fois sur des plasmides et des chromosomes, suggérant un transfert latéral et horizontal de ces éléments génétiques entre diverses espèces bactériennes,

renforçant ainsi leur capacité à se propager dans divers environnements. En outre, la détection du gène *aroA II* dans des aliments et dans le tube digestif de l'homme suggère un transfert de ces gènes par voie alimentaire. Ce transposon est porté par des plasmides largement répandu dans les souches d'origine humaine, qui peuvent également porter des gènes de résistance. La pression exercée par l'utilisation massive de glyphosate pourrait donc favoriser la co-sélection de gènes de résistance sur ce type de plasmide, renforçant ainsi la dissémination de la résistance aux antibiotiques chez l'homme mais également dans l'environnement.

Title: Identification of a novel transposon carrying new *aroA* gene conferring glyphosate tolerance and a MFS transporter for paraquat efflux in *Klebsiella spp.* strains isolated from a polluted area in Thailand: a new One Health threat?

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Abstract

Background: The environment is contaminated with a "cocktail" of chemicals that impacts living organisms, including bacteria. Herbicides are among these chemicals, and their effects on bacteria are not yet well understood.

Objectives: The aim of this study was to investigate the molecular mechanisms of herbicide tolerance in *Klebsiella spp.* strains isolated from farmers from Nan Province in Thailand, which display high minimum inhibitory concentrations (MIC) values to a glyphosate-based formulation (GBF) (16 g/L).

Material and methods: DNAs were extracted and then sequenced using both Illumina MiSeq and Oxford Nanopore Gridion technologies. EPSPS protein sequences were extracted and classified according to the species degree of tolerance to glyphosate, using existing classifications.

Results: Four of the nine pesticide-tolerant strains (GBF-MIC =16 g/L) harbored a novel composite transposon (~15 Kb) carried by two IncF-type plasmids. This transposon contains an *aroA* gene encoding for a novel class II EPSPS enzyme, the target of glyphosate, as well as a Major Facilitator Superfamily (MFS) transporter associated with efflux of biocides. These two genes were found in NCBI in 87 strains in plasmids or chromosomes of different bacterial species including one *Pseudomonas sp.* isolated from glyphosate polluted soil in China. Six out of 159 food samples analyzed were positive to the class II *aroA* found in our study. An IncFIB plasmid harboring both class II aroA and MFS-like genes was found in a *Klebsiella quasipneumoniae* isolated from a ginger plant originated from Thailand. Curing the plasmid of this strain led to a 32-fold decrease of paraquat MIC.

Conclusions: Overall, our study suggest the vertical and horizontal transfer of a novel composite transposon containing a class II *aroA* gene along with a *MFS-like* gene, conferring resistance to paraquat, a widely used herbicide with mode of action similar to that of antiseptics. This raises the question of a possible selection of antiseptic-resistant bacteria from the environment to clinical settings.

Keywords: Glyphosate-based formulation, paraquat, EPSPS, *aroA*, MFS, Thailand.

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Introduction

The intensive human activities of recent decades have massively contaminated the environment, and recent climate events have encouraged the spread of these contaminants. Soil, waters and living organisms are “polluted” by cocktails of contaminants whose levels and impact on ecosystems are not yet known. Herbicides are agents used in various fields, such as agriculture, aquaculture, fisheries and other domains. They play a crucial role in weed management and food production. However, their use, estimated to 2.6 billion kilograms per year (1), raises concerns about their effects on living organisms and ecosystems, especially that some herbicides persist in the environment for years. The most used herbicides (selective and non-selective) include glyphosate, paraquat, glufosinate-ammonium, atrazine, 2,4-Dichlorophenoxyacetic acid

Thailand, a major player in food and agriculture exports, relies heavily on pesticides to protect crops and boost yields. Pesticides contaminated soil, water sources, and even food itself, posing a threat to human health and the environment (2,3). The top two pesticides import in Thailand are glyphosate and paraquat (1,1-dimethyl-4,4-bipyridyl dichloride, a quaternary ammonium herbicide) (4,5). The use and the harmful effects of these two non-selective herbicides have been documented in Nan province, one of the largest agricultural provinces in Thailand (6).

Glyphosate is known for its effectiveness in controlling a broad range of weeds. It is used in various agricultural, horticultural, and forestry applications and has become one of the most applied herbicides due to its alleged inaction on mammals (7). Indeed, it acts by inhibiting the activity of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an essential enzyme in the shikimate pathway encoded by the *aroA* gene, which is responsible for the biosynthesis of aromatic amino acids in all living beings except mammals, where the pathway is absent (8). (9,10). The EPSPS is classified into five classes (I, II, III, IV and unclassified) based on their

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origin and glyphosate tolerance (10,11). The class I EPSPS enzymes are highly sensitive to glyphosate inhibition due to their tight binding affinity for the herbicide while class II EPSPS enzymes have a natural tolerance or reduced sensitivity to glyphosate. Thus, bacterial class II EPSPS sequences have been employed in the development of genetically modified plants with glyphosate resistance trait like the he CP4-EPSPS or the G2-EPSPS, derived from *Agrobacterium sp.* CP4 (12) and *Pseudomonas fluorescens* G2 strains, respectively (13).

Additionally, paraquat, equally as interesting as glyphosate, leads to the destruction of plant and bacterial cells by generating reactive oxygen species (ROS) through a redox cycle. This process produces superoxide radicals and hydrogen peroxide, causing oxidative damage to cellular components, resulting in the death of plants and bacteria.

In a previous study (submitted), we isolated nine *Klebsiella sp.* strains with a high level of tolerance to a glyphosate-based formulation (GBF) (Radikal green, Fitogal, Valencia, Spain). These strains normally possess a class I EPSPS with high affinity to glyphosate. We used whole-genome-sequencing to identify the molecular support of such resistance. We identified a new *aroA* class II gene that was carried by a plasmid also containing a MFS transporter. Further analysis showed this transporter was able to efflux paraquat, suggesting these strains acquired this plasmid to survive in a highly polluted environment.

Materials and methods

Human strains

Nine *Klebsiella spp.* strains exhibiting a GBF MIC at 16 g/L, equivalent to 12 g/L of glyphosate acid, were recovered from our previous study where we isolated glyphosate tolerant enterobacteria from the rectal swab of healthy humans living in the Nan Province in Thailand. In addition, two glyphosate tolerant isolates, namely *K. pneumoniae* Q6223 and the type strain *K. pneumoniae* ATCC 13883 that showed GBF MICs at 4 et 8 g/L respectively,

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were used as control for our study. This research study was approved by the Mahidol University Ethics Committee of the Faculty of Tropical Medicine under certification number MUTM-2018-035-01.

Food samples collection

In order to check if the *aroA* was present in bacteria from the environment, we collected 159 food samples in supermarkets in France that have been imported from various countries (Supplementary table 1). The collected foods included raw and canned cereals (such as rice, corn and soybean), sauces (primarily soy sauce), vegetables, and fruits. The collection of food samples was carried out in accordance with the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization.

Screening for *aroA* genes by PCR and bacterial isolation

DNA was extracted from food samples using EZ1 DNA Tissue Kit (Qiagen GmbH, Germany) and screened for the new class II *aroA* gene and the *Agrobacterium sp.* CP4 *aroA* using Real-Time PCR. Positive PCR were confirmed by standard PCR followed by sanger sequencing as previously described (14). Specific primers for each system are available in Table 1. Positive food samples were enriched in Tryptic Soy (TS) broth (bioMérieux, Marcy l'Etoile, France) for 24 hours at 37 °C and then inoculated on MacConkey agar (McK) (Becton Dickinson, Le Pont de Claix, France) and Luria-Bertani (LB) agar (Becton Dickinson, Le Pont de Claix, France) supplemented with 16 g/L of GBF (LB16) and incubated at 37 °C for 24–48 hours. Bacteria isolated were subcultured on Trypticase Soy agar (TSA) (bioMérieux) and identified using Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF, Bruker Daltonik, Bremen, Germany). PCRs described above were performed on isolates to confirm the presence of the *aroA* gene.

Chemicals and stock solution

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Herbicides (paraquat (PARA), abamectin (ABA), atrazine (ATR), cypermethrin (CYPR), butachlor (BUTA)) and quaternary ammonium (benzalkonium chloride (BKC), chlorhexidine (CHX), triclosan (TRC)) were purchased from Sigma-Aldrich, (Burlington, Massachusetts, United States (US)). Stock solutions of CHX, BKC, and paraquat were prepared at 0.8 mg/mL, 1 mg/mL, and 10 mg/mL in distilled water, respectively. Stock solutions of abamectin, atrazine, cypermethrin, butachlor, and triclosan were prepared at 10 mg/mL in DMSO (Euromedex, France). The final concentration of DMSO in the well of the 96-plate is 0.2%. For glyphosate, we used a commercial GBF (Radikal, Fitogal, Spain), which contains 360 g/L of glyphosate isopropylamine salt, corresponding to 270 g/L of glyphosate acid and a quaternary ammonium as a coformulant (the specific type and concentration are not disclosed by the manufacturer) (Expiration date of approval: 30/04/2028). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and Phenylalanine-Arginine Beta-Naphthylamide (PAβN) were prepared in a stock solution of 5 mg/mL each, with CCCP is dissolved in DMSO and PaβN in distilled water. Verapamil hydrochloride (Isoptine, Viatris Médical, France) was used at 2.5 mg/mL.

A stock solution of Isopropyl β-D-thiogalactoside (IPTG) (Euromedex, Strasbourg, France), used for gene expression induction, was prepared at 100 mM.

Susceptibility testing

MICs of herbicides and quaternary ammonium were performed using broth microdilution method (11). Antimicrobial susceptibility tests were performed by disk diffusion method and interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (version 12.0). We used the E-test method to determine the MIC of antibiotics listed in Table 5 (bioMérieux), except for colistin MIC that was determined using UMIC microdilution method (Biocentric, Bandol, France).

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Efflux pump inhibition

To evaluate the role of efflux in GBF and paraquat tolerance, we measured their MICs with and without efflux pump inhibitor (EPI). We used the following concentration for EPIs, 10 mg/L of CCCP, 25 mg/L of PAβN and concentrations ranging from 2 to 256 mg/L of verapamil.

Plasmid curing

Elevated temperature (45°C) was used for plasmid curing of *Klebsiella sp.* strains (15). The curing of plasmid was controlled by the absence of the class II *aroA* and the *MFS-like* genes by RT-PCR. The PCR of the chromosomally encoded bla_{SHV} gene was used as a positive control (16).

Cloning and purification of the class II EPSPS

An electro-competent Escherichia coli BL21(DE3) was transformed with a commercial pET24a+ plasmid harboring the class II aroA synthetic gene (GenScript Biotech) optimized for E. coli and kanamycin-resistant gene as a selective marker. Transformant strains were selected on LB agar supplemented with 50 µg/mL of kanamycin and then grown in ZYP-5052 auto-inducing medium at 37°C for 3 hours. When the culture reached an OD600nm = 0.6, the temperature was reduced to 20°C and the culture was shaken for an additional 20 hours. Bacteria were collected using centrifugation and the resulting pellet was resuspended in 50 mM Tris pH 8, 300 mM NaCl. After an overnight incubation at -80°C, lysis was performed on thawed cells by adding 0.25 mg/mL lysosyme, 10 µg/mL DNAse I and 0.1 mM PMSF followed by 3 cycles of sonication on a Q700 sonicator (QSonica). After centrifugation (12,000 g, 20 min, 6°C), the filtered supernatant was loaded on a StrepTrap XT column (Cytiva) and purified using an AKTA avant system (Cytiva). The produced EPSPS protein was checked for purity by SDS-PAGE and protein expression was confirmed by performing

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MALDI-TOF on the obtained bands. Protein concentrations were evaluated using a Nanodrop 2000c spectrophotometer (Thermo Scientific). This purified enzyme is used in the enzymatic activity assay.

Characterization of the class II EPSPS activity

The activity of the class II EPSPS is measured by quantifying the inorganic phosphate (Pi) production, following the method described by Griffin et al. (11,17).

Transcriptomic analysis

The expression of *aroA* (class I and class II), *MFS-like*, and pumps (*acrA/B*-TolC), with and without herbicide (GBF and paraquat) and antibiotic (ciprofloxacin) pression, was measured by quantitative real-time PCR. RNA extraction was performed using Quick-RNA™ Miniprep Kit (Zymo Research Corp., Irvine, California (CA), US). Extracted RNA was quantified with NanoDrop™ (Labtech, Ortenberg, Germany). The housekeeping gene *rpoB* was chosen as a reference gene for relative quantification (18). Real-time reverse transcriptase PCR (RT-qPCR) was carried out using LightCycler® 96 one-step RT-qPCR (Roche Diagnostics GmbH, Mannheim, Germany). PCR amplification was performed in duplicate as follows: 10 min at 50°C; 30 s at 95°C; and then 45 cycles of 5s at 95°C and 30 s at 60°C. The relative RNA expression level was calculated using the ΔΔCt method.

Whole genome sequencing and bioinformatics analysis

The DNAs of the 11 isolates were extracted using EZ1. Then, the genomes have been sequenced using MiSeq (Illumina Inc., San Diego, CA, US) in a paired-end strategy. In addition, four isolates (Q6071, Q6073, Q6065 and Q8259) were also sequenced in a long-read strategy using Gridion (Oxford Nanopore Technologies Inc., United Kingdom) to reconstitute plasmids.

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Raw reads were assembled using the hybrid assembler Unicycler (version 0.4.8) after performing quality assessment using FASTQC (Galaxy Version 0.74). Subsequently, the assembly quality was verified using QUAST (Galaxy Version 5.2.0), and contaminations were eliminated before genome annotation by PROKKA (Galaxy Version 1.14.6). Abricate (Galaxy Version 1.0.1) was used to search for antibiotic resistance genes using the databases available (Resfinder, ARG-ANNOT and CARD), virulence genes (VFDB) and plasmid-encoding genes (Plasmidfinder). Species identification was carried out using Type Strain Genome Server (TYGS, https://tygs.dsmz.de/). The assignment of sequence types (ST) was determined by referring to Institut Pasteur MLST databases (available online, https://bigsdb.pasteur.fr/klebsiella/) specific to *K. pneumoniae* complex. The assignment of each ST of *K. aerogenes* and *Klebsiella oxytoca* was determined by referring to PubMLST database (https://pubmlst.org/). Classification of EPSPS protein sequences was performed using the *EPSPSClass* web server available at https://ppuigbo.me/programs/EPSPSClass/ (10,19). Mobile genetic elements were identified using IS finder (https://isfinder.biotoul.fr/, 13-June-2024) and Tn Central (https://tncentral.ncc.unesp.br/, version 2) databases. Phylogenetic tree was constructed using FastTree (version2.1.10) and visualized using Itol (version 6.8.1). Sequences comparison and genetic environment were visualized using Proksee (https://proksee.ca/) and Easyfig (version 2.2.5). BLASTp and BLASTn were used to search for the genes involved in glyphosate degradation: i) *gox* gene (GenBank accession: GU214711.1) encoding glyphosate oxidoreductase, ii) *thiO* gene (GenBank accession: KC831746.1) encoding glycine oxidase, and iii) *gat* gene (GenBank accession: AX543338.1) encoding glyphosate acetyltransferase (GNAT). Membrane transporter protein sequences were compared with the 3,572 sequences from the transport database (TransportDB, http://www.membranetransport.org/, version 2.0) (20), the 83 sequences of the transporter classification database (TCDB, https://www.tcdb.org/, 06-August-2024) (21), and 35 unique

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antiseptic resistance protein sequences that were found in plasmids in various *Enterobacteriacae* (22).

GenBank accession numbers

Genomic assemblies of bacteria used in this study have been deposited on NCBI dataset under the project number PRJNA108817 (Genome accession numbers: Q6071, JBBHEM000000000; Q6073, JBBHEL000000000; Q6075, JBBHEK000000000, Q6076, JBBHEJ000000000; Q6065, JBBHEI000000000; Q6066, JBBHEH000000000; Q6067, JBBHEG000000000; Q6068, JBBHEF000000000; Q8259, JBBHEE000000000; Q6223, JBBHED000000000; Q7925, JBBJBX000000000).

Genomic assemblies of bacteria used in this study have been deposited on NCBI dataset under the project number PRJNA108817 (Genome accession number: JBBHE**(D-M)**000000000 and JBBJBX000000000).

Results

Whole genome quality and data

Bioinformatics analysis confirmed the identification of two *K. pneumoniae,* four *K. aerogenes* and reclassified Q6073, Q8259 and Q7925 as *K. quasipneumoniae.* Q6075 as *K. variicola* and Q6076 as *K. ornithinolytica*. The average size of *K. pneumoniae* genome, including *K. quasipneumoniae* and *K. variicola*, was of 5.43 Mb with a mean GC content of 57.6. The average size of *K. aerogenes* genomes was 5.20 Mb with a GC content of 55.2 whereas the *K. ornithinolytica* genome was of 5.4 Mb with a GC content of 55.8. Genome coverage for all strains sequenced ranged between 22 and 90 for assemblies from MiSeq and between 323 - 954 for hybrid assembly. The number of contigs varies widely between two and 456 (Table 2). According to MLST analysis, *K. aerogenes* strains belonged to ST4, ST116, ST182 and ST241, while *K. pneumoniae* species belong to ST5808 and ST6544. ST981 and ST4627

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were respectively for *K. variicola* and *K. quasipneumoniae*. In addition, two new STs of *K. quaipneumoniae* and *K. ornithinolytica* strains were found.

Genomic support for glyphosate tolerance

Classification of EPSPS

As expected, we found a class I EPSPS sequence in the ten *Klebsiella* genomes. Class I EPSPS of *K. pneumoniae* strains were 100% similar to class I EPSPS of the type strain, while that of *K. quasipneumoniae*, *K. variicola*, *K. ornithinolytica*, *K. aerogenes* share 93% to 98% identity with the class I EPSPS of the *K. pneumoniae* ATCC 13883. Interestingly, four strains (one *K. pneumoniae*, two *K. quasipneumoniae* and one *K. aerogenes*) had an additional copy of the *aroA* gene encoding a class II EPSPS enzyme that normally confers high level tolerance to glyphosate. This class II sequence shares only 31-32 % identity and 93% coverage with the class I EPSPS sequences found in our study. In addition, *K. quasipneumoniae* Q8259 (with GBF MIC = 8 g/L) had an unclassified third copy of *aroA* gene, sharing only 26.54% and 23.58% of similarity with class I and class II respectively. As expected, the glyphosate susceptible *K. pneumoniae* Q6223 (GBF MIC = 4 g/L) and the type strain *K. pneumoniae* ATCC 13883 (GBF MIC = 8 g/L) each had only one copy of the class I *aroA* gene.

Degradation pathways of glyphosate

The search for the three genes (*gox*, *thiO* and *gat*) responsible for glyphosate degradation within the *Klebsiella spp.* genomes yielded no significant hits.

Characterization of the class II EPSPS

The four sequences of the class II EPSPS were the same, sharing 100% identity and 100% coverage. A BLASTp search on NCBI database found 87 hits with 100% coverage and 100%

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identity corresponding to sequences from six genera (*Klebsiella (n=65)*, *Escherichia (n=1)*, *Enterobacter (n=15)*, *Pseudomonas (n=1)*, *Pantoea (n=3)* and *Achromobacter (n=2)*) (Supplementary table 2). Among these hits, most of the strains were from the genus *Klebsiella* (65/87), and especially *K. pneumoniae* (41/87). Most of the strains (66/87) were isolated from various types of human clinical samples (blood, urine and rectal swab samples). The remaining were isolated from environmental (16/87) and undetermined samples (4/87). In terms of geographical distribution, the majority (52/87) originated from Asian countries (Singapore, China, Japan, Thailand, South Korea, and Malaysia). This AroA protein was found in only four complete genomes: an *Enterobacter sp*. R4-368 isolated from a *Jatropha Curcas* plant in Singapore (23), an *Enterobacter hormaechei* isolated from the nematode *Caenorhabditis elegans* in France (24), a *Pantoea stewartii* isolated from rice (*Oryza sativa*) in China (25), and a *Klebsiella sp.* isolated from breeding pond sediment in China. These 87 genomes carried between two to four *aroA* genes. The class I EPSPS was also present in all genomes except for *Pseudomonas sp. P818* which had two class II EPSPS. The additional copies included EPSPS sequences classified as non-determined found in certain genomes.

Construction of an EPSPS phylogenetic tree

We constructed a phylogenetic tree of 218 EPSPS sequences, including sequences identified in this work, from the 87 genomes found in NCBI, and from previously published sequences (Figure 1). As expected, Class I EPSPS clustered together with reference sequence and class I from other species. Conversely, the new class II EPSPS clustered with other sequences from the same class (including the *Agrobacterium sp.* CP4, *Coxiella burnetii*, *Lyngbya sp.* PCC 8106 and *Pseudomonas sp.* P818 class II EPSPSs). However, the four sequences of the new class II EPSPS sequences differed from the other class II EPSPS, sharing only 46.15% identity with the closest sequence, the CP4 EPSPS from Monsanto company (Accesssion number: Q9R4E4). Finally, the additional unclassified EPSPS found in the *K.*

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quasipneumoniae Q8259 grouped with unclassified EPSPS found within eight other *K. quasipneumoniae* genomes (K7, K27, K31, KP083, 2020-725, KML002, AM65-22ACA, and Fly-1170). Interestingly, these eight genomes also harbored a class I and class II AroA proteins.

Genetic environment of the class II EPSPS

We analyzed the genetic environment of this new *aroA* gene that was carried by an IncFIIK conjugative plasmid in the four *Klebsiella sp.* isolates. This gene was localized in a composite transposon (Tn) bounded by two insertion sequences IS*26* (IS*6* family) (Figure 2). This Tn had a GC% of around 61, with an average length of 14,932 bp, and was highly conserved between the four strains with an identity up to 97 %. The *aroA* gene was surrounded by a 236 amino acid hypothetical protein (Hp) and an adenylyl-sulfate kinase enzyme encoded by the *cysC* gene. This transposon also carried a gene coding for a protein from the membrane transporter family. A BLASTp comparison with 3,572 sequences of membrane transporter from the TransportDB database and 83 sequences of the TCDB, showed this transporter belongs to the major facilitator superfamily (MFS), including methyl viologen resistance protein (SmvA) and the antiseptic resistance protein (QacA). This MFS-like transporter shared from 90 to 98% coverage and from 32.71 and 54.81% identity with the ten most similar sequences found in both databases, which belong to bacteria of the genera *Corynebacterium*, *Pseudomonas*, *Bordetella*, *Salmonella*, *Staphylococcus*, and *Acinetobacter* (Table S1). Additionally, we compared this MFS-like protein with the 35 unique antiseptic resistance proteins previously found in plasmids of *Enterobacteriaceae* (22), that showed no significant similarity (Figure 3). The *MFS-like* gene was always associated with a *tetR* transcriptional regulator gene. An IS*21* was located into the transposon, downstream of the cluster of genes hp-*aroA*-*cysC* and upstream of the *MFS*-like gene. We identified a type-2 restriction enzyme EcoRIIR enzyme located between the *MFS-like* gene and the IS*6* element

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only in the Q6073 plasmid (pTKQ73). The Tn of Q8259 (pTKQ59), Q6065 (pTKA65) and Q7925 (pTKQ25) plasmids had an additional cluster of genes encoding for a cellulose synthase, a HlyD family efflux transporter (HlyD), an acyl-CoA dehydrogenase (Acyl-CoA) and a phosphopantetheine protein, located downstream the IS*21* element and upstream the Hp associated with the *aroA* gene.

The genetic environment of the novel *aroA* gene in the 73 previously published genomes on NCBI was similar to those observed in our genomes. The three genes *hp-aroA-cysC* were constantly found associated, except in the *Pantoea* MS1 genome. This gene was found in both chromosomes and plasmids. Interestingly, this cluster was located into the chromosomes of *Achromobacter sp.* MY14 (26), *Achromobacter* JD-1, and *E. hormaechei* CEnt1 (24). In *Achromobacter* genomes, the *aroA* gene lacked any flanking transposable elements in contrast with *Enterobacter* genome for which the *aroA* gene was flanked by two IS*6* elements and was associated with a hypothetical protein and the *cycC* gene. This suggests that the class II *aroA* gene, which is associated with the *hp* and *cycC* genes, can be initially present in the chromosome of the MY14 or JD-1 strains, and then captured by IS*6* elements and transferred to the chromosome of CEnt1 *E. hormaechei*. Alternatively, the cluster of genes *hp-aroA-cysC* might have been in *Enterobacter* initially, flanked with IS elements, and then transferred to the chromosome of *Achromobacter*, where it lost the IS elements which is a possible event during transposition. Additionally, as we found this cluster in various transposons and in different types of plasmids, we could suggest that this transposon disseminated among different species specially *Klebsiella* and *Enterobacter*. We also identified this cluster of genes, including a truncated *cysC* gene, in a *Pseudomonas sp.* strain P818 isolated from glyphosate-polluted soil in China. However, the contig was small and it was not possible to identify if this sequence was located into the chromosome or a plasmid. For five genomes, we were able to localize the Tn in IncF type plasmids, the other sequences being too small to

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locate them. The additional cluster of four genes (HlyD – Acyl-CoA) was found in only three other genomes (*P. stewartii* HR3, *Enterobacter kobei* 13426 and *K. pneumoniae* SC-YY39) but the cellulose synthase was absent in the *P. stewartii* MS1 strain.

Screening of food samples for the new class II EPSPS and the CP4 EPSPS

We suggested this *aroA* gene has been acquired in human bacteria from food, so we looked for the presence of this gene by PCR in food samples collected from supermarket in France. From the 159 food samples, most of them (57%) were from Asian origin and especially from Thailand (26%). Of these 159 samples tested, six were positive for the class II *aroA* gene, while none were positive for the CP4 *aroA* gene. The six positive samples were all imported from Asia (five from Thailand and one from Laos). After inoculating these positive samples on selective media, we only isolated one *K. pneumoniae* (Q7925) that carried the class II *aroA* gene. This strain, isolated from a Gian Dai (*Boesenbergia pandurata*) plant in Thailand, exhibited a GBF-MIC of 16 g/L and a wild-type antibiotic profile. In addition, this strain had a paraquat-MIC of 2,048 µg/mL. After WGS, the genome was assembled into 40 contigs with a GC% of 58.07%. This strain belonged to ST 4799. We found the constitutive class I EPSPS (100% coverage, 100% identity), and the novel class II EPSPS that was identical to those found in human fecal samples from Thailand. The class II EPSPS was located on a composite transposon similar to that of *K. quasipneumoniae* Q8259 (coverage 100%, identity 100%) but it was carried by a different conjugative IncFIB plasmid (141,794 bp length) (Figure 4).

Paraquat and glyphosate may be a substrate of plasmid genes

The plasmid of the plant-origin *K. quasipneumoniae* (pTKQ25) was cured after several culture at 45°C. Cured strains (Q7925-C1 and Q7925-C2) were negative by RT-PCR for the class II *aroA* and the *MFS-like* genes confirming the loss of transposon, but positive for the chromosomally encoded beta-lactamase bla_{SHV}. Testing the susceptibility of 27 antibiotics

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different families showed no difference in MICs between the cured isolates and the Q7925 (Table 5). We measured herbicides and quaternary ammonium compounds (Table 5) and observed a 32-fold decreases for paraquat (from 2,048 to 64 mg/L), suggesting that genes carried by this plasmid confers paraquat tolerance to *Klebsiella.*

Identification of an efflux mechanism for paraquat

GBF and paraquat MICs, in the absence and presence of pump inhibitors, were tested for the strain Q7925 and its two cured strains (C1 and C2) (Table 4). In the presence of CCCP, we observed a 16-fold decreased for GBF MIC decreased in the cured strains and a 32-fold decreased in the non-cured strain. There was no change in the GBF MIC when PAβN was added, nor verapamil.

For the non-cured strain, we observed a 4-fold decrease of paraquat MIC with CCCP and a 2-fold decrease with PAβN. Conversely, for the plasmid-cured strains, a 2-fold increase in paraquat MIC was observed with CCCP and a 4-fold increase with PAβN.

Transcriptomic analysis

Evaluation of glyphosate inhibition activity on the novel class II EPSPS

Curing the plasmid did not lead into a significant decrease of glyphosate MIC. However, this may be due to the presence of the class I EPSPS that generally confer MIC around 8 g/L in *Klebsiella.* To evaluate the ability of glyphosate to inhibit EPSPS, we express the class II EPSPS sequence in an *E. coli* and purified the protein.

Discussion

The extra copy of EPSPS found in our Klebsiella genomes belong to a class II EPSPS that is not usually found in strains of this genera. This class of EPSPS proteins were usually found in

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399 strains from genera of Agrobacterium, Pseudomonas, xxx. Many studies reveals the
400 importance of these class II EPSPS as efficient proteins used in the production of genetically
401 modified plants with glyphosate resistance as a trait. Bacterial strains originated for these
402 class II EPSPS were usually isolated from environment contaminated or treated with
403 glyphosate (27,28). These results were consistent with our findings, as our strains are isolated
404 from farmers that work in farms in the Nan province in Thailand, a province known for its big
405 agriculture works and the huge use of pesticides like glyphosate, and as the Pseudomonas
406 P818 strain found in NCBI, harboring this class II EPSPS, was isolated from glyphosate
407 polluted soil in China and was considered by Cao et *al.* (29). In this case we can say the
408 glyphosate exposure in such environments can exerts selective pressure, allowing tolerant
409 bacteria to survive.

410 In addition to the class II EPSPS, the identified *MFS-like* gene, coding for an MFS
411 transporter, was found in the pAMBL1 plasmid (KP873172) of a *Pseudomonas aeruginosa*
412 strain carrying a $bla_{\text{VIM-1}}$ gene in a Tn402-like transposon (30), and in the Tn6006 of an
413 Enterobacter cloacae JKB7 strain (Tn Central database) (figure S1). It was also similar to the
414 *qacA* of *Staphylococcus aureus* and *smvA* genes that are genes coding for resistance to
415 antiseptics (31). This transporter have an active role in the efflux of toxic compounds,
416 including antibiotics and herbicides, from bacterial cells. Efflux mechanisms are implicated in
417 antibiotic resistance (32) and also pesticides resistance (33), particularly MFS transporters
418 were found to be responsible not only for paraquat resistance (34) and glyphosate tolerance
419 (35), but also for antibiotic resistance (36). As the plasmid curing show the role of the plasmid
420 harboring both class II EPSPS and the MFS transporter in paraquat tolerance, it suggest that
421 this MFS is potentially implicated in this tolerance. In addition paraquat has been extensively
422 used in Thailand, particularly in rice and other crops, exerting selective pressure similar to

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423 that of glyphosate (2). The presence of both glyphosate and paraquat tolerance in our strain
424 suggest the co-selection due to the simultaneous use of multiple herbicides.

425 The findings in our study were with a big interest as four of the *Klebsiella spp.* strains were
426 isolated from humans that work in farms in Thailand, that use glyphosate and paraquat in
427 agriculture, and one isolated from a ginger plant "Gian Dai" originated from the same
428 country. Noted that this plant that we collected from a market in Marseille, France, belongs to
429 *Zingiberaceae* family, which are a family of plants used in traditional medicine in many
430 Asiatic countries (37)

431 In addition the transposon harboring the class II and the MFS was present in the chromosome
432 and Inc plasmids. It was documented that the presence of IncF as a plasmid replicon in
433 association with mobile genetic elements (like IS26) is a frequency of spreading antibiotic
434 resistance, here the importance of studying the emergence of plasmids harboring pesticide
435 and/or biocide resistance genes in clinical settings and also in the environment that is usually
436 the origin of these genes. The transposons found were more or less evolved, which is
437 characteristic of transposons and mobile genetic elements that can evolve and acquire
438 resistance genes; this phenomenon is well-known in the field of transposition (38). IS6
439 elements were associated with this transposons in our strains and strains found in NCBI
440 database. These elements have a big role in the spread of antibiotic resistance and is clinically
441 important group of insertion sequences including IS26 (39).

442 Furthermore, the dissemination of the *aroA* gene in association with a *MFS-like* gene, via the
443 mechanism of transposition, is occurring among various bacterial family such as
444 *Enterobacteriaceae, Pseudomonadaceae, and Burkholderiaceae,* isolated from environmental
445 and predominantly from clinical samples across different continents. This phenomenon is
446 particularly notable in strains associated with human infections, such as *K. pneumoniae,* and

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highlights the widespread distribution and potential transfer of these genetic elements among bacterial populations.

In conclusion, our study identified a novel transposon carrying both the *aroA* gene, and a MFS transporter associated with paraquat efflux in *Klebsiella spp*. strains isolated from a polluted region in Thailand. The co-location of these genes on conjugative IncF plasmids suggests a potential co-selection mechanism driven by the heavy usage of both herbicides and pesticides in the area, raising concerns regarding pesticide tolerance, food safety, and human health. The discovery of these genes on a composite transposon and their presence across multiple plasmid types and the chromosome suggest the potential for widespread dissemination through both transposition and conjugation. These findings raise concerns about the emergence of multiple antimicrobial resistance in environmental bacteria and highlight the importance of monitoring agricultural and industrial pollutants that could exacerbate the spread of such genetic elements. The One Health implications of this study emphasize the need for continued vigilance in managing environmental contaminants and their role in the evolution of bacterial tolerance mechanisms.

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Transparency declaration

The authors declare no conflict of interest.

Authors' contributions

SM, SAB and JMR: designed and conceived the study. AH, HZ, TV, LP: performed the experiments and interpreted the data. AH, HZ, TV, LP, SAB and JMR drafted the manuscript. AH, SM, SAB, and JMR: made critical corrections and revisions.

All authors have read and approved the final version of the manuscript.

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605 group of insertion sequences including IS26. Mob DNA. 2021 Dec 23;12(1):11.

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Figure 1: Phylogenetic classification of 218 EPSPS protein sequences. The phylogenetic tree was constructed with FastTree and visualized using Itol. A: a simplified phylogenetic tree with collapsed branches, B: Detailed hylogenetic tree of class I, class II and ND EPSPS protein sequences found in this study and EPSPS sequences from different classes cited in the bibliography. Class II EPSPS indicated by a red strip designed the new class II EPSPS found in our study. EPSPS classes found in our study are in bold. Class I EPSPS : Zm: Zea mays, Gm: Glycine max, Cp: Colwellia psychrerythrea, Vc: Vibrio cholerae serotype O1, Ec: Escherichia coli, Pl: Photorhabdus luminescens; class II EPSPS : Cb: Coxiella burnetii, Lygna: Lyngbya sp. PCC 8106, CP4: Agrobacterium sp.CP4; class III EPSPS : Bv: Brevundimonas vesicularis; class IV EPSPS : Sd: Streptomyces davawensis JCM 4913, DGT-

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28: Streptomyces sviceus, DGT-31: Streptomyces flavogriseus, DGT-32: Streptomyces roseosporus, DGT-33: Streptomyces griseus. 37
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Supplementary data:

Table S1: List of the total of 159 various types of food samples collected from different regions in this study

Table S2: Bacterial strains harboring the class II *aroA* found in our study and by BLAST analysis in NCBI database. Strains found in our study are highlighted in red; The contig size correspond for the size of the contig were the class II *aroA* gene is located; ND: not determined.

Table S3: Comparison of the MFS-like, found in the five *Klebsiella spp.* strains, with the top ten most similar sequences found in commun in the TransportDB and TCDB databases.

Figure S1: A schematic representation compares the transposon found in the pTKQ59 plasmid with other transposons associated with MFS transporters from the Tn Central

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database. The MFS-like transposon identified in our study was 100% similar (both in coverage and identity) to Tn6006 from *Enterobacter cloacae* JKB7 (EU591509.1). In contrast, MFS transporters in the Tns of *Cupriavidus necator* strain NH9 (CP017760), *Pseudomonas sp.* B13 (AJ431260), and *Rhodococcus erythropolis* strain NI86 (NZ_JABBPH010000003) show no significant similarity to our MFS-like.

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663 **Tables and figures**

664 **Table 1:** Primers used in Real-time PCR and Standard PCR to detect the class II *aroA* and
665 CP4 *aroA* genes.

PCR type	Primer		Sequence (5'- 3') [a]	Hybridation temperature	Amplicon length
Real-time PCR	Class II *aroA*	F	GATGAGGATCACCGGCACG	62°C	164 bp
		R	CCACCATGGTTTCCGTGTAG	62°C	
		P	GCATCACTTACGATTTGCCCGTG	70°C	
	CP4 *aroA*	F	CAAGTCGATCTCCCACCGG	62°C	168 bp
		R	CCGACGCCATCGATGATCC	62°C	
		P	CGGTCTCGCGAGCGGTGAAAC	70°C	
Standard PCR	Class II *aroA*	F	GGTTACCACGGTTCGGAAGT	62°C	1126 bp
		R	CTCCGCATCCACGACTGTTA	62°C	
	CP4 *aroA*	F	GGATCCGTAAGGAAGGCGAC	60.25°C	1082 pb
		R	GATCATCGTGGCATCGTCCA	60.25°C	

a: F, forward; R, reverse; P, probe.

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667 **Table 2:** Genomic analysis of all sequenced *Klebsiella spp.* strains. Presence (+) and absence (-) of different classes of *aroA* genes. ST: Sequence
668 type.

Strain	Code	Sample type	Host	Origin	WGS technology	ST	Coverage	Nb of contig	Size (bp)	GC %	Antibiotic Resistance genes	Class I *aroA*	Class II *aroA*	ND *aroA*
K. pneumoniae	Q6223	Rectal swab	Human	Thailand	Illumina MiSeq	5808	22x	453	5,313,116	57.66	*oqxBgb, oqxA, fosA6,* $bla_{SHV-187}$	+	-	-
K. pneumoniae	Q6071	Rectal swab	Human	Thailand	Hybrid	6544	90x; 737x	3	5,465,030	57.27	*oqxBgb, oqxA, fosA5,* $bla_{SHV-187}$	+	+	-
K. quasipneumoniae	Q7925	Plant	Environment	Thailand	Illumina MiSeq	4799	41x	40	5,436,548	58.07	*oqxBgb, oqxA, fosA6,* $bla_{OKP-A-11}$	+	+	-
K. quasipneumoniae	Q6073	Rectal swab	Human	Thailand	Hybrid	New	60x; 685x	2	5,494,926	57.77	*oqxBgb, oqxA, fosA6,* $bla_{OKP-A-11}$	+	+	-
K. quasipneumoniae	Q8259	Rectal swab	Human	Thailand	Hybrid	4627	63x; 323x	2	5,297,204	57.86	*oqxBgb, oqxA, fosA6,* $bla_{OKP-B-2}$	+	+	+
K. variicola	Q6075	Rectal swab	Human	Thailand	Illumina MiSeq	981	89x	53	5,579,407	57.43	*oqxBgb, oqxA, fosA6,* bla_{LEN-24}	+	-	-
K. ornithinolytica	Q6076	Rectal swab	Human	Thailand	Illumina MiSeq	New	73x	80	5,398,208	55.83	*oqxBgb, oqxA, fosA5,* bla_{ORN1a}	+	-	-
K. aerogenes	Q6065	Rectal swab	Human	Thailand	Hybrid	4	53x; 954x	4	5,297,018	55	*oqxBgb, oqxA, fosA5, ampH, ampC*	+	+	-
K. aerogenes	Q6066	Rectal swab	Human	Thailand	Illumina MiSeq	241	63x	69	4,905,684	55.21	*oqxBgb, oqxA, fosA5, ampH, ampC*	+	-	-
K. aerogenes	Q6067	Rectal swab	Human	Thailand	Illumina MiSeq	116	62x	85	4,895,463	55.19	*oqxBgb, oqxA, fosA5, ampH, ampC*	+	-	-
K. aerogenes	Q6068	Rectal swab	Human	Thailand	Illumina MiSeq	182	77x	67	4,981,827	55.29	*oqxBgb, oqxA, fosA5, ampH, ampC*	+	-	-

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669 **Table 3:** MICs of herbicides (N = 6) and antiseptics (N = 3) tested for all strains in this study.

	Herbicides (N = 6)						Antiseptics (N = 3)		
Strain	**GBF**	**PARA**	**ABA**	**ATR**	**CYPR**	**BUTA**	**BKC**	**CHX**	**TRC**
ATCC 13883	8	16	>1024	>1024	>1024	>1024	8	4	8
Q6223	4	64	>1024	>1024	>1024	>1024	16	4	8
Q6075	16	64	>1024	>1024	>1024	>1024	16	16	8
Q6076	16	64	>1024	>1024	>1024	>1024	16	16	4
Q6066	16	32	>1024	>1024	>1024	>1024	16	16	8
Q6067	16	32	>1024	>1024	>1024	>1024	16	16	8
Q6068	16	64	>1024	>1024	>1024	>1024	16	8	8
Q6071*	**16**	**512**	>1024	>1024	>1024	>1024	**16**	**8**	8
Q6073*	**16**	**1024**	>1024	>1024	>1024	>1024	**16**	**8**	8
Q8259*	**8**	**512**	>1024	>1024	>1024	>1024	**16**	**4**	8
Q6065*	**16**	**1024**	>1024	>1024	>1024	>1024	**16**	**8**	8
Q7925*	**16**	**2048**	>1024	>1024	>1024	>1024	**16**	**8**	8
Q7925-C1	16	64	>1024	>1024	>1024	>1024	16	8	8
Q7925-C2	16	64	>1024	>1024	>1024	>1024	16	8	8

670 *Strains with plasmids that harbor the class II EPSPS; **GBF**: glyphosate-based formulation;
671 **PARA**: paraquat; **ABA**: abamectin; **ATR**: atrazine; **CYPR**: cypermethrin; **BUTA**: butachlor;
672 **BKC**: benzalkonium chloride; **CHX**: chlorhexidine; **TRC**: triclosan ; GBF-MIC in g/L ; MIC
673 of all other molecules in µg/mL.
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675 **Table 4**: GBF and paraquat MICs tested in the presence and the absence of pump inhibitors
676 (CCCP, PAβN and verapamil) for the strain Q7925 and the associated plasmid-cured strains
677 (Q7925-C1 and Q7925-C2).

Molecules tested	Q7925	Q7925-C1	Q7925-C2
GBF	16	16	16
GBF + CCCP	1	1	1
GBF + PAβN	16	16	16
GBF+ Verapamil	16	16	16
Paraquat	2048	64	64
Paraquat + CCCP	512	128	128
Paraquat + PAβN	1024	256	256
Paraquat + Verapamil	2048	64	64

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679 **Table 5** : Herbicide, antiseptic and antibiotic susceptibility tested for non-cured (Q7925) and
680 cured strains (Q7925-C1 and Q7925-C2). GBF-MIC in g/L ; MIC of all other molecules in
681 µg/mL.

			MIC		
Group of molecules	**Molecules**	**Code**	**Q7925**	**Q7925-C1**	**Q7925-C2**
Herbicides	**GBF-Radikal green**	GBF	16	16	16
	Paraquat	PARA	**2048**	**64**	**64**
	Abamectin	ABA	>1024	>1024	>1024
	Atrazine	ATRA	>1024	>1024	>1024
	Cypermethrin	CYPR	>1024	>1024	>1024
	Butachlor	BUTA	>1024	>1024	>1024
Antiseptics	**Benzalkonium chloride**	BKC	16	16	16
	Chlorhexidine	CHX	8	8	8
	Triclosan	TRC	8	8	8
Antibiotics	**Amoxicillin**	AC	>256	>256	>256
	Amoxicillin-clavulanic acid	AAC	1.5	1.5	1
	Cefepime	PM	0.023	0.023	0.023
	Piperacillin-tazobactam	P/T	1.5	1.5	1.5
	Benzylpenicillin	PG	>32	>32	>32
	Ceftriaxone	TX	0.047	0.047	0.047
	Ertapenem	ETP	0.004	0.004	0.004
	Imipenem	IPM	0.19	0.19	0.19
	Fosfomycin	FF	6	6	4
	Trimethoprim-sulfamethoxazole	TS	0.094	0.094	0.094
	Amikacin	AK	1.5	1.5	1.5
	Ciprofloxacin	CI	0.023	0.023	0.023
	Tetracycline	TC	1.5	1.5	1.5
	Colistin	CS	0.25	0.5	0.125
	Gentamicin	GM	0.38	0.38	0.38
	Doxycycline	DC	2	2	2
	Minocycline	MC	2	2	2
	Tigecycline	TGC	0.38	0.5	0.5
	Oxacillin	OX	>256	>256	>256
	Ofloxacin	OF	0.19	0.25	0.19
	Levofloxacin	LE	0.064	0.094	0.094
	Moxifloxacin	MX	0.064	0.064	0.064
	Chloromphenicol	CL	3	3	3
	Ceftazidime	TZ	0.125	0.125	0.125
	Cefotaxime	CTX	0.032	0.032	0.047
	Kanamycin	KM	1.5	1.5	1.5
	Tobramycin	TM	0.5	0.25	0.5

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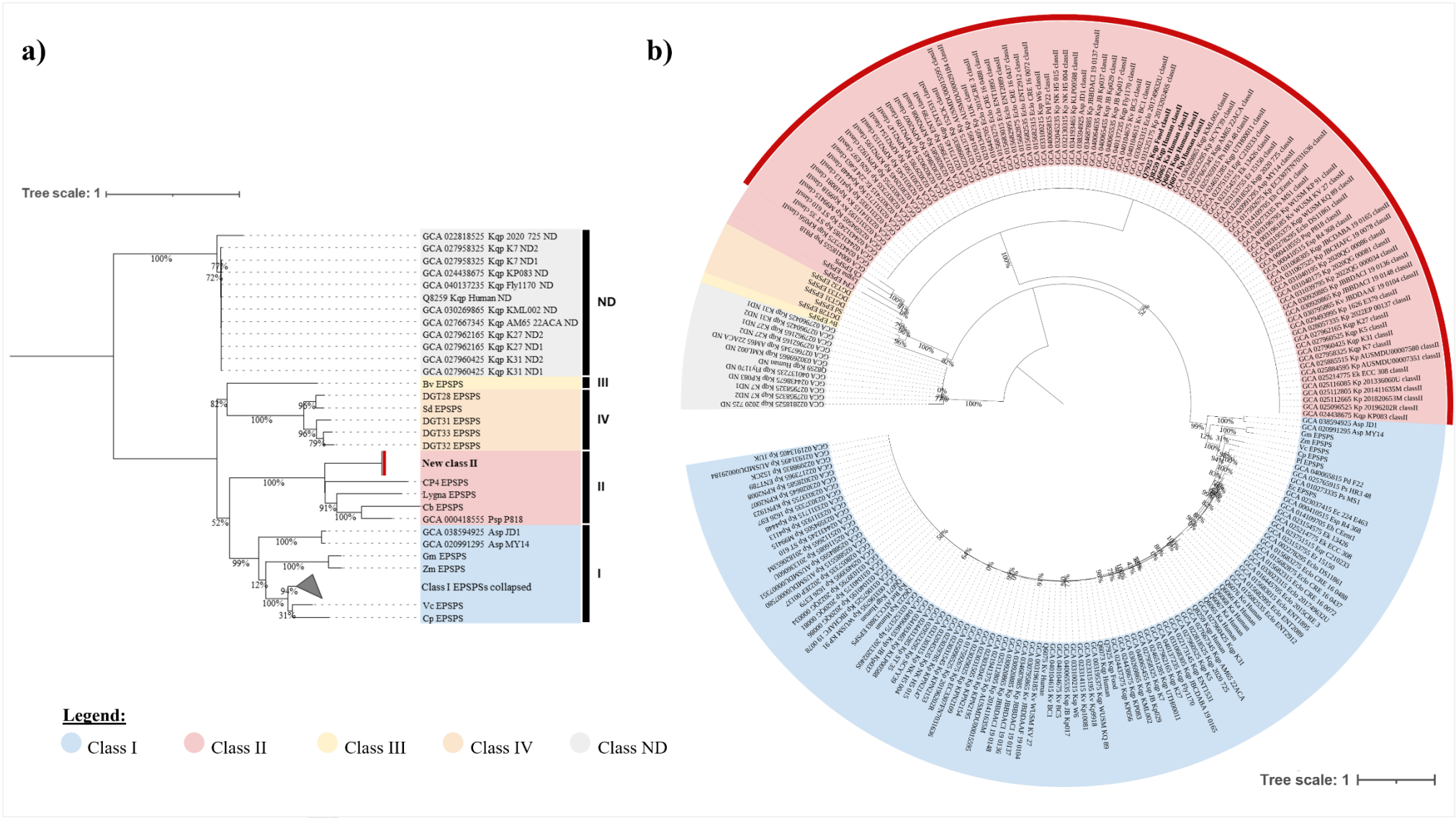
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Q8259 Kqp Human ND
GCA 030269865 Kqp KML002 ND
GCA 027667345 Kqp AM65 22ACA ND
GCA 027962165 Kqp K27 ND2
GCA 027962165 Kqp K27 ND1
GCA 027960425 Kqp K31 ND2
GCA 027960425 Kqp K31 ND1
Bv EPSPS
DGT28 EPSPS
Sd EPSPS
DGT31 EPSPS
DGT33 EPSPS
DGT32 EPSPS
New class II
CP4 EPSPS
Lygna EPSPS
Cb EPSPS
GCA 000418555 Psp P818
GCA 038594925 Asp JD1
GCA 020991295 Asp MY14
Gm EPSPS
Zm EPSPS
Class I EPSPSs collapsed
Vc EPSPS
Cp EPSPS
ND
III
IV
II
I
Legend:
Class I
Class II
Class III
Class IV
Class ND
b)
Tree scale: 1

682

Figure 1: Phylogenetic classification of 218 EPSPS protein sequences. The phylogenetic tree was constructed with FastTree and visualized using Itol. A: a simplified phylogenetic tree with collapsed branches, B: Detailed phylogenetic tree of class I, class II and ND EPSPS protein sequences found in this study and EPSPS sequences from different classes cited in the bibliography. Class II EPSPS indicated by a red strip designed the new class II EPSPS found in our study. EPSPS classes found in our study are in bold. Class I EPSPS : Zm: *Zea mays*, Gm: *Glycine max*, Cp: *Colwellia psychrerythrea*, Vc: *Vibrio cholerae* serotype O1, Ec: *Escherichia coli*, Pl: *Photorhabdus luminescens*; class II EPSPS : Cb: *Coxiella burnetii*, Lygna: *Lyngbya* sp. PCC 8106, CP4: Agrobacterium sp.CP4; class III EPSPS : Bv: Brevundimonas vesicularis; class IV EPSPS : Sd: *Streptomyces davawensis* JCM 4913, DGT-28: *Streptomyces sviceus*, DGT-31: *Streptomyces flavogriseus*, DGT-32: *Streptomyces roseosporus*, DGT-33: *Streptomyces griseus*.

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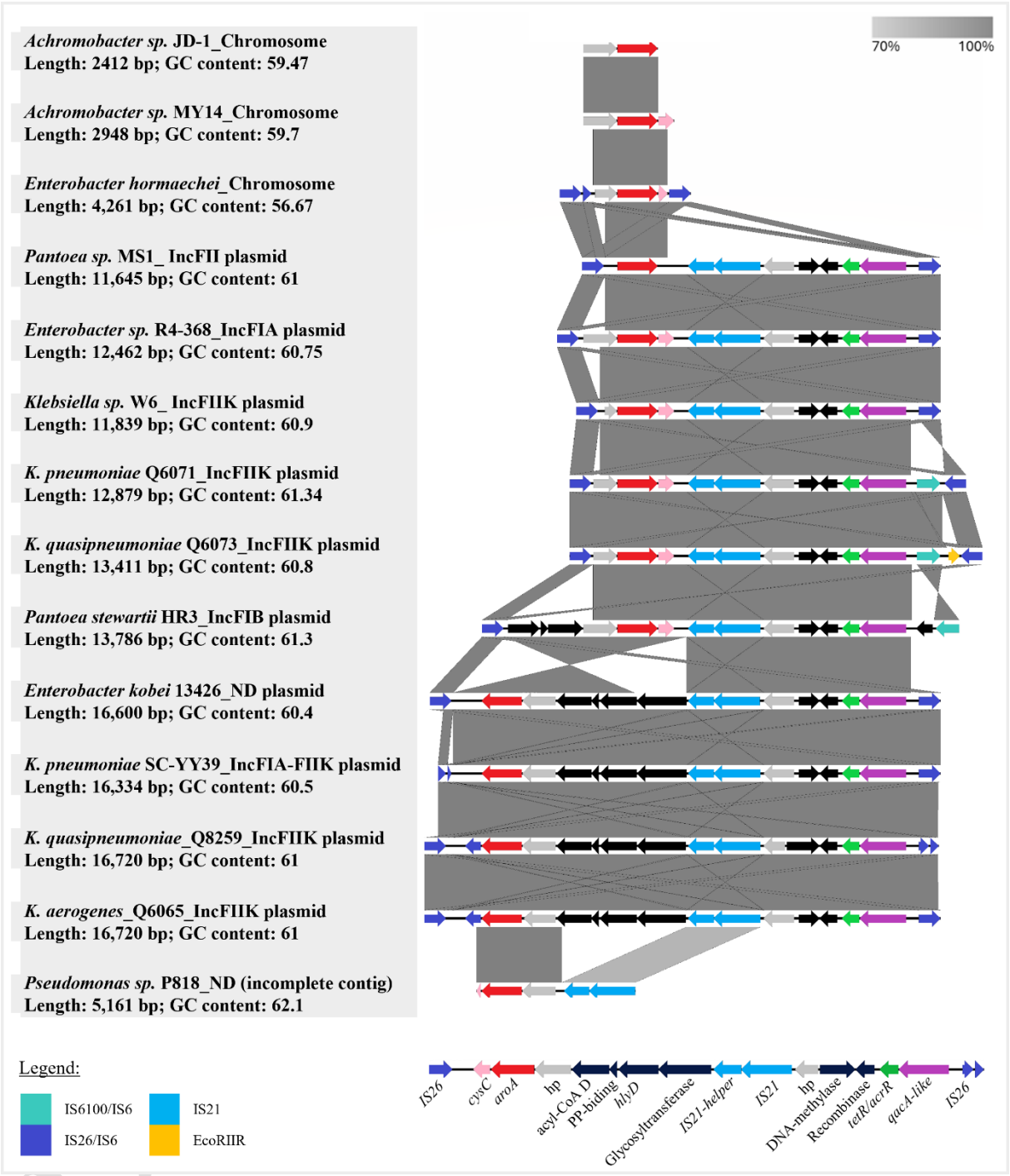

Figure 2: Genetic environment of the mobile *aroA* gene within the transposon of the four *Klebsiella* strains isolated in this work and comparison with genetic environment of this same gene previously published. hp: hypothetical protein, Acyl-CoA D: Acyl-CoA dehydrogenase and PP-biding: phosphopantetheine-binding protein, ND: Not determined.

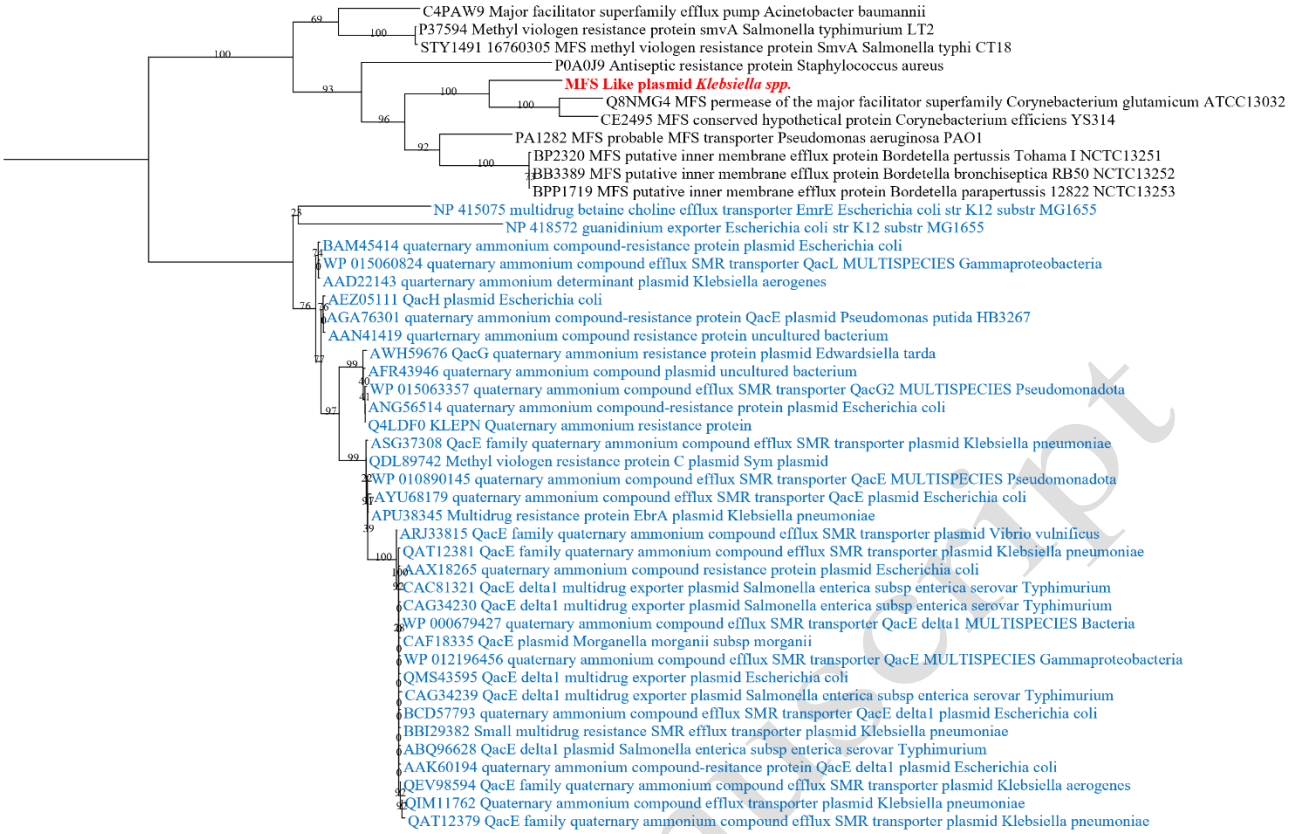

Figure 3: Phylogenetic analysis of the MFS-like. Protein sequences in black represents the ten similar sequences from the TransportDB and the TCDB databases, while sequences in blue represent the 35 unique sequences from Slipski et al.

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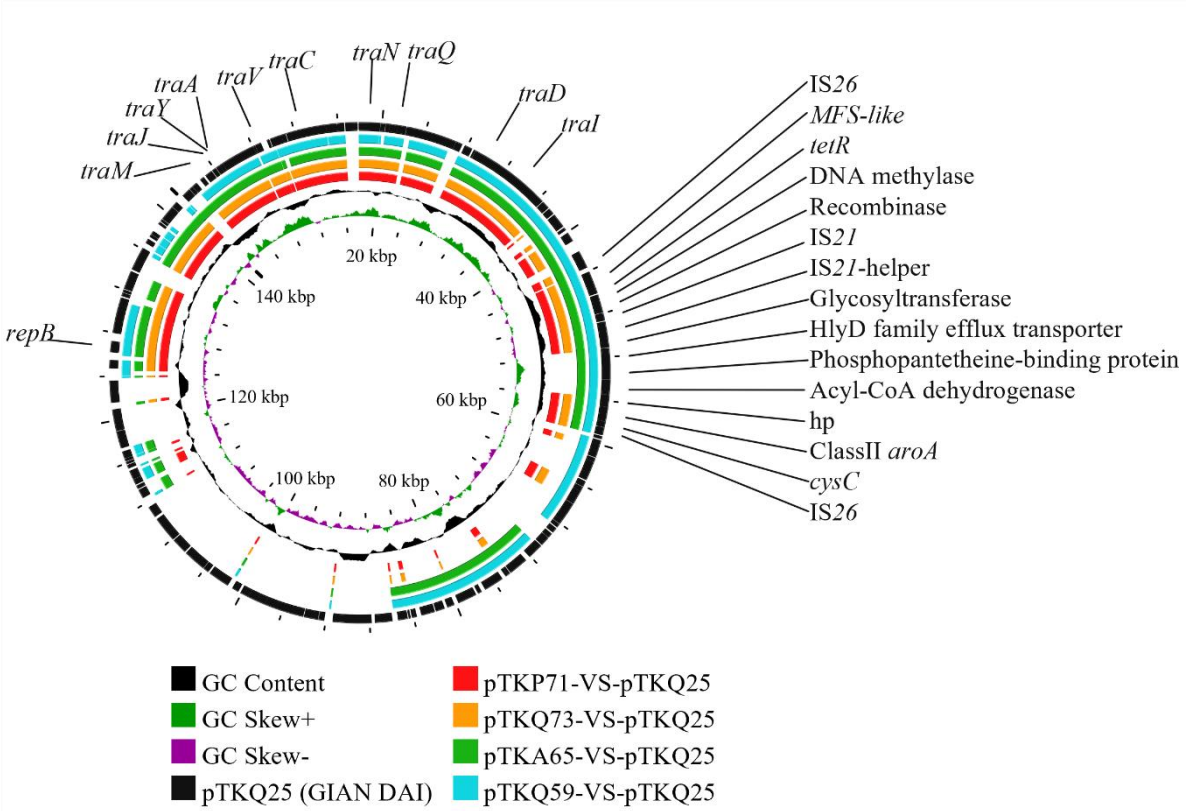

Figure 4: Comparison of the IncFIB plasmid of the *K. quasipneumoniae* strain (pTKP25) isolated from the food sample GIAN DAI with the other four plasmids of the strains isolated from the human fecal samples from Thailand (PTKP71, pTKQ73, pTKQ59 and pTKA65).

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Supplementary data:

Table S1: List of the total of 159 various types of food samples collected in this study

Food sample	Brand	Country origin	Color	Expiry date	Sampling date
Eggplant	Lao agriculture trading co	Thailand	Light green	ND	5/11/2022
Glutinous rice flour	Cock brand	Thailand	White	8/16/2023	5/11/2022
Thai rice	Saint Eloi	Thailand	White	3/8/2024	5/11/2022
Thai rice full	Universal rice co	Thailand	Light brown	8/19/2023	5/11/2022
Thai rice vermicilli Bio	Autour du riz	Thailand	White	8/24/2023	5/11/2022
Thai rice 1-2 full	Autour du riz	Thailand	White	8/4/2023	5/11/2022
Thai rice 1-2 full tagliatelle	Autour du riz	Thailand	White	8/5/2023	5/11/2022
Thai rice full	Compagne du riz	Thailand	Light brown	12/2/2023	5/11/2022
Thai rice Bio	Carrefour	Thailand	White	2/3/2022	5/12/2022
Jasmine Rice	Magic Chef	Thailand	White	10/2023	5/25/2022
Thai Jasmine Rice	Kaset	Thailand	White	11/18/2023	5/25/2022
Soybeans	Le petit chinois	Thailand	Dark brown	10/2023	5/25/2022
Canned corn	Deli	Thailand	Yellow	ND	5/25/2022
Canned corn	Al wadi	Thailand	Yellow	5/9/2024	5/25/2022
Green eggplant (*Solanum torvum*)	Top Thai-Paris Store	Thailand	Green	ND	6/16/2022
Lemongrass (*Cymbogon citratus*)	Siris's Trading-Paris Store	Thailand	Green	ND	6/16/2022
Guichai leaf (*Allium tuberosum*)	Top Thai-Paris Store	Thailand	Green	ND	6/16/2022
Sadao leaf (*Azadirachta indica*)	Siris's trading-Paris Store	Thailand	Green	ND	6/16/2022
Spring onion chive (*Allium ascalonicum*)	Top Thai-Paris Store	Thailand	Green	ND	6/16/2022
Baby corn (*Zea mays*)	Top Thai-Paris Store	Thailand	Yellow	ND	6/16/2022
Gian Dai (*Boesenbergia pandurata*)	Siris's Trading-Paris Store	Thailand	Light yellow	ND	6/16/2022
Jackfruit (*Artocarpus heterophyllus*)	Top Thai-Paris Store	Thailand	Yellow	ND	6/16/2022

Poivre vert	Paris Store	Thailand	Black	ND	6/16/2022
Peking duck sauce	Lee brand	Thailand	Brown	12/31/2024	6/16/2022
Sweet chilin sauce	Thai dancer	Thailand	Brown-red	12/6/2023	6/16/2022
Red bean curd	EDM	Thailand	Red	2/18/2023	6/16/2022
Rice vermicelli	Wai wai	Thailand	White-yellow	12/20/2023	6/16/2022
Rice vermicelli	Eaglobe	Thailand	White	1/30/2024	6/16/2022
Rice vermicelli M	PSP	Thailand	White	01/2024	6/16/2022
Rice noodle	Thai flavor	Thailand	White-yellow	08/2023	6/16/2022
Dried barley	Thai top choice	Thailand	Light brown	02/2023	6/16/2022
Haricot Mungo Décortiqué	Thai top choice	Thailand	Brown	10/2023	6/16/2022
Complete rice	Golden Butterfly	Thailand	Light brown	2024	6/16/2022
Long fragrant rice	Golden Butterfly	Thailand	White	7/31/2022	6/16/2022
Thai hom mali rice	Golden phoenix	Thailand	White	9/1/2023	6/16/2022
White thai rice	Golden phoenix	Thailand	White	8/1/2023	6/16/2022
Hom mali complete bio rice	Golden phoenix	Thailand	White	8/1/2023	6/16/2022
Tomyum shrimp creamy flavor	YumYum	Thailand	Light brown	8/27/2022	6/16/2022
Fruit de Dragon	Paris Store	Thailand	Magenta	ND	6/16/2022
Young coconut meat	Twin elephant & earth	Thailand	White	11/19/2022	6/16/2022
Pineapple slices in light syrup	PSP	Thailand	Yellow	11/10/2023	6/16/2022
Tamarind drink	Teptip	Thailand	Light yellow	11/9/2023	6/16/2022
Black mushrooms	Intinéraire des Saveurs	China	Black and white	3/21/2023	5/11/2022
Vinegar sauce (sushi)	ND	China	Light brown	ND	5/14/2022
Fine brown bulgur	Shine Star	China	Dark brown	02/2023	5/25/2022
Chili sauce	Sauce piment	China	Red-orange	6/30/2024	6/16/2022
Hoi sin sauce	Amoy	China	Brown	04/2023	6/16/2022
Sweet and sour sauce	Lee Kum Kee	China	Brown	9/19/2023	6/16/2022

Sauce mirin	Paris Store	China	Light yellow	7/31/2023	6/16/2022
Spicy soybean curd	Eaglobe	China	Light yellow	10/31/2023	6/16/2022
Mung bean vermicelli	Long Kou Vermicelli	China	White	12/31/2023	6/16/2022
Mung bean vermicelli	PSP	China	White	12/31/2025	6/16/2022
Dried noodles	Central Asia Pacific Limited	China	White-yellow	12/31/2023	6/16/2022
Soy bean	Eaglobe	China	Light brown	12/31/2023	6/16/2022
White somen noodles	Eaglobe	China	White	6/18/2023	6/16/2022
Red Bean	Eaglobe	China	Red	12/31/2023	6/16/2022
White sesame	Eaglobe	China	White	2023	6/16/2022
Arbouse in Light sirop	PSP	China	Red	5/25/2024	6/16/2022
Grass jelly	Eagle-Coin	China	Black	4/13/2023	6/16/2022
Soy sauce	Kikkoman	Japan	Black	ND	5/11/2022
Green tea	ND	Japan	Dark green	ND	5/14/2022
Sesame oil	ND	Japan	Light brown	ND	5/14/2022
Nouilles sauce	Olivier	Japan	Orange	ND	5/14/2022
Sashimi soy sauce	Yamasa	Japan	Brown	11/29/2023	6/16/2022
Chili oil	La-Yu	Japan	Brown	10/27/2022	6/16/2022
Sesame seed oil	Kuki	Japan	Brown	7/6/2023	6/16/2022
Kimchi preparation sauce	Ebara Kimchi	Japan	Brown	9/7/2022	6/16/2022
Breadcrumbs	Queen's Chef	Japan	White	07/2022	6/16/2022
Mixed grains rice	ND	Taiwan	Multicolored	4/15/2022	5/11/2022
Spicy soybean curd	Hwa Nan	Taiwan	Light yellow	3/3/2024	6/16/2022
Fermented chili beancurd	Hong Yang	Taiwan	Light yellow	11/30/2023	6/16/2022
Grass jelly drink	Taisun	Taiwan	Brown	6/19/2023	6/16/2022
Golden mushrooms (*Flammulina velutips*)	Eaglobe	Taiwan	White	1/14/2024	6/16/2022
Fresh rice vermicelli	Thuan Phong	Vietnam	White-yellow	1/10/2024	6/16/2022

Rice vermicelli	Binh Tay	Vietnam	White	4/10/2023	6/16/2022
Dried rice spaghetti	Vinawang	Vietnam	White	11/2023	6/16/2022
Glutinous rice	Vinawang	Vietnam	White	10/3/2024	6/16/2022
Caspe banana flower	New Thai	Laos	Yellow	ND	6/16/2022
Dap ca leaf (*Houttuynia cordata*)	Siris's trading-Paris Store	Laos	Green	ND	6/16/2022
Red pepper	New Thai	Laos	Red	ND	6/16/2022
Black sugar syrup	Nok Cha Won	South Korea	Brown	6/22/2023	6/16/2022
Beef Bulgogi Marinade	Daesang	South Korea	Brown	6/22/2022	6/16/2022
Korean style instant noodle	Ottogi	Korea	Light brown	7/19/2022	6/16/2022
Super spicy noodles	ND	Korea	Light brown	8/11/2022	6/16/2022
Rice vermicelli	Eaglobe	Cambodia	White-yellow	1/31/2025	6/16/2022
Long rice	Golden butterfly	Cambodia	White	12/31/2022	6/16/2022
Oriental instant noodles	Koka	Singapore	Light brown	08/2023	6/16/2022
Basmati rice	-	Himalaya	White	ND	5/14/2022
Basmati rice	Taj	India	White	3/1/2023	5/12/2022
Basmati rice	Auchan	Pakistan	White	9/6/2023	5/14/2022
Soy Proteins	Graine d'or	France	Light brown	2/15/2023	5/11/2022
Potato	Casino	France	Yellow	ND	5/11/2022
Basmati rice	Carrefour	France	White	9/1/2022	5/12/2022
Corn (canned)	Saint Eloi	France	Yellow	7/1/2025	5/12/2022
Nems sauce	Suzi Wan	France	Light orange	12/23/2022	5/12/2022
Sweet soy sauce	Bozen	France	Black	1/1/2023	5/12/2022
Salted soy sauce	Bozen	France	Black	1/1/2023	5/12/2022
Salted soy sauce	Carrefour	France	Black	2/11/2023	5/12/2022
Wholemeal oats	Crownfield	France	Light brown	7/29/2022	5/12/2022
Quinoa	Super U	France	Light brown	5/19/2024	5/12/2022
Sweet soy sauce	Casino	France	Black	4/7/2023	5/12/2022

Salted soy sauce	Côté sushi	France	Black	ND	5/12/2022
Salted soy sauce	Enjoy sushi	France	Black	1/1/2024	5/12/2022
Pad Thai sauce	Suzi Wan	France	Black	7/10/2023	5/12/2022
Red beets (steamed)	Betty loire	France	Dark red	7/27/2022	5/14/2022
Potato	Lidl	France	Yellow	ND	5/14/2022
Corn	Ebly	France	Dark brown	5/12/2022	5/14/2022
Sweet soy sauce	Suzi Wan	France	Black	ND	5/14/2022
Salted (43%) soy sauce	Suzi Wan	France	Black	ND	5/14/2022
Salted soy sauce	Suzi Wan	France	Black	ND	5/14/2022
Oatmeal BIO	Terre et Céréales	France	Light yellow	ND	5/14/2022
Fish sauce	Carrefour	France	Dark yellow	ND	5/14/2022
Canned corn	Casino	France	Yellow	ND	5/25/2022
Rapeseed oil (Colza)	Vita Dór	France	Yellow	ND	5/25/2022
Mung bean sprouts	Chen Da Xing	France	Light yellow	ND	6/16/2022
Potato	Paris Store	France	Yellow	ND	6/16/2022
Green mint	Paris Store	France	Green	ND	6/16/2022
Long purple eggplant	Paris Store	Spain	Purple	ND	6/16/2022
Black eggplant	Paris Store	Spain	Black	ND	6/16/2022
Nectarine	Paris Store	Spain	Red	ND	6/16/2022
Butternut	Paris Store	Spain	Orange	ND	6/16/2022
Pure basmati rice	Tilda	Spain	White	11/1/2022	6/16/2022
Olives vertes	creSpo	Spain	Green	6/7/2024	6/16/2022
Basmati rice	Bon-Ri	Italy	White	ND	5/12/2022
Cannelloni	Barilla	Italy	Yellow	ND	5/14/2022
Bunch onion	Paris Store	Italy	Green	ND	6/16/2022
Sushi rice	Shinode	Italy	White	10/3/2024	6/16/2022
White corn flour	MR distribution	Italy	White	10/12/2022	6/16/2022
Chickpea flour Bio	ND	EU	Light yellow	ND	5/14/2022

	Nouilles powder	Paris Store	EU	Light brown	ND	5/14/2022
	Corn cake Bio	Regain	Czech Republic	Yellow	2/24/2023	5/11/2022
	Sweet soy sauce	Kikkoman	Netherlands	Black	1/7/2024	5/11/2022
	Aroma flavor	Maggi	Switzerland	Dark brown	ND	5/14/2022
	Wheat	Deroni	Bulgaria	Light yellow	04/2022	5/25/2022
	Vine tomato	Paris Store	Belgium	Red	ND	6/16/2022
	Wheat	Local	Algeria	Light yellow	ND	4/6/2022
	Soybeans	Local	Algeria	Red	ND	4/6/2022
	Rice	Local	Algeria	White	ND	4/6/2022
	Corn grain maize	Local	Algeria	Yellow	ND	4/6/2022
	Big red pepper	Local	Algeria	Red	ND	4/6/2022
	Small red pepper	Local	Algeria	Red	ND	4/6/2022
	Mini potato	Local	Algeria	Yellow	ND	4/6/2022
	Tomato	Local	Algeria	Red	ND	4/6/2022
	Potato	Local	Algeria	Yellow	ND	4/6/2022
	Garlic	Local	Algeria	White	ND	4/6/2022
	Beetroot	Local	Algeria	Red	ND	4/6/2022
	Canned corn	Local	Algeria	Yellow	ND	4/6/2022
	Onion	Local	Algeria	Light yellow	ND	4/6/2022
	Local cereals	Saloum business company	Senegal	White and brown	ND	5/11/2022
	Tomato	Paris Store	Maroc	Red	ND	6/16/2022
	Red sweet potato	Paris Store	Honduras	Red	ND	6/16/2022
	Purple sweet potato	Paris Store	Honduras	Purple	ND	6/16/2022
	Sweet potato	Paris Store	Honduras	Yellow	ND	6/16/2022
	Suriname long rice	Riz du monde	South of America	White	2/11/2024	6/16/2022
	Chia seeds	ND	Nicaragua	Black	ND	5/14/2022

	Sesame tahini	ND	Turkey	Yellow	ND	5/14/2022
	Coarse bulgur	Bashan	Turkey	Light brown	09/2022	5/25/2022
	Rice	Bashan	Turkey	white	3/30/2024	5/25/2022
	Round rice	Éco +	ND	White	11/22/2021	5/14/2022

	Asia		Europe		Africa		America		Turkey		ND : Non determined

Table S2: Bacterial strains harboring the class II *aroA* found in our study and by BLAST analysis in NCBI database.

Species	Strain	Genome assembly accession	Contig size (bp)	Type of sample	Host	Host disease	Country	Collection date	Reference
Pantoea stewartii	HR3-48	GCA_025765915.1	333,832	*Oryza sativa* cell culture	Environment	ND	China	ND	Zhang *et al.*, 2022
Pantoea stewartii	MS1	GCA_010273335.1	54,366	Jackfruit pulp and rags	Environment	Bronzing disease	Malaysia	2017	Zulperi *et al.*, 2017
Pantoea dispersa	F-22	GCA_040065815.1	11,159	Agricultural field	Environment	ND	Mexico	2021	-
Escherichia coli	224/E463	GCA_023037415.1	10,335	Cell culture	ND	ND	Singapore	ND	-
Pseudomonas sp.	P818	GCA_000418555.1	22,341	Glyphosate-polluted soil	Environment	ND	China	2010	Cao *et al.*, 2013
Achromobacter sp.	MY14	GCA_020991295.1	676,505	Soil	Environment	ND	China	2020	Mu *et al.*, 2022
Achromobacter sp.	JD-1	GCA_038594925.1	676,505	Farm land soil	Environment	ND	China	2020	-
Klebsiella sp.	W6	GCA_033100215.1	124,877	Tissue sample	Breeding pond sediment	ND	China	2020	-
Klebsiella sp.	JB_Kp037	GCA_040064635.1	7,580	Water	Environment	ND	South Korea	2022	-
Klebsiella sp.	JB_Kp029	GCA_040065455.1	7,580	Water	Environment	ND	South Korea	2022	-
Klebsiella sp.	JB_Kp017	GCA_040065535.1	7,580	Water	Environment	ND	South Korea	2022	-
Klebsiella aerogenes	Q6065	-	167,785	Rectal swab	Human	None	Thailand	2018	This study
Klebsiella pneumoniae	Q6071	-	141,542	Rectal swab	Human	None	Thailand	2018	This study
K. pneumoniae	EC3307-F-N703-1-636	GCA_017592675.1	7,580	ND	ND	ND	ND	ND	-
K. pneumoniae	SC-YY39	GCA_029523295.1	123,257	Blood	Human	ND	China	2018	-
K. pneumoniae	WUSM_KP_91	GCA_003196795.1	10,152	Urine	Human	Infection	USA	2017	Potter *et al.*, 2018
K. pneumoniae	2022EP-00137	GCA_028057335.2	9,993	Urine	Human	ND	USA		-
K. pneumoniae	2022QG-000034	GCA_031039795.1	9,995	Wound drainage	Human	ND	USA	2022	-
K. pneumoniae	2020QG-00086	GCA_031040195.1	9,996	Urine	Human	ND	USA	2022	-
K. pneumoniae	2020QG-00081	GCA_031040175.1	9,996	Urine	Human	ND	USA	2020	-
K. pneumoniae	1UK	GCA_021913405.1	15,326	ND	Human	None	Thailand	2018	-
K. pneumoniae	AUSMDU00029184	GCA_021931495.1	11,143	Urine	Human	ND	Australia	2019	-
K. pneumoniae	AUSMDU00015595	GCA_021941375.1	15,249	Rectal Swab	Human	ND	Australia	2018	-
K. pneumoniae	152CK	GCA_022098835.1	10,203	ND	Human	None	Thailand	2018	-
K. pneumoniae	ENT789	GCA_022173965.1	14,665	Rectal Swab	Human	ND	Singapore	2013	-

K. pneumoniae	KPN2007	GCA_023028645.1	16,041	Urine	Human	ND	Australia	2020	-
K. pneumoniae	KPN2008	GCA_023028585.1	16,068	Blood Venous	Human	ND	Australia	2020	-
K. pneumoniae	KPN2109	GCA_023029025.1	15,246	Urine	Human	ND	Australia	2020	-
K. pneumoniae	KPN2147	GCA_023029785.1	15,250	Urine Catheter	Human	ND	Australia	2020	-
K. pneumoniae	KPN2154	GCA_023031505.1	15,246	Urine	Human	ND	Australia	2020	-
K. pneumoniae	KPN2153	GCA_023031545.1	15,457	Blood Peripheral	Human	ND	Australia	2020	-
K. pneumoniae	KPN2195	GCA_023032045.1	15,247	Urine	Human	ND	Australia	2020	-
K. pneumoniae	KPN1923	GCA_023033755.1	29,323	Urine	Human	ND	Australia	2020	-
K. pneumoniae	1626/E97	GCA_023037335.1	14,664	Cell culture	ND	ND	Singapore	ND	-
K. pneumoniae	Kp4448	GCA_023311715.1	4,092	Clinical sample	Human	Klebsiella infection	USA	2017	-
K. pneumoniae	M99415	GCA_023594505.1	15,251	Rectal Swab	Human	ND	Australia	2019	-
K. pneumoniae	ST-35/patient 166	GCA_024431285.1	10,203	Blood	Human	ND	USA	ND	-
K. pneumoniae	ST-610/patient 164	GCA_024431245.1	10,203	Blood	Human	ND	USA	ND	-
K. pneumoniae	20196202R	GCA_025096525.1	15,138	Respiratory	Human	ND	Singapore	2019	-
K. pneumoniae	201820653M	GCA_025112665.1	15,052	Gastrointestinal	Human	ND	Singapore	2018	-
K. pneumoniae	201411635M	GCA_025112805.1	2,405	Wound	Human	ND	Singapore	2014	-
K. pneumoniae	201336060U	GCA_025116085.1	14,664	Urine	Human	ND	Singapore	2013	-
K. pneumoniae	AUSMDU00007351	GCA_025884595.1	15,248	ND	Human	ND	Australia	2017	-
K. pneumoniae	AUSMDU00007580	GCA_025885515.1	15,249	ND	Human	ND	Australia	2017	-
K. pneumoniae	Kp4113	GCA_023311935.2	4,092	Rectal swab	Human	Klebsiella infection	USA	2017	-
K. pneumoniae	1626/E379	GCA_029493995.1	14,665	Cell culture	ND	ND	Singapore	ND	-
K. pneumoniae	JBBDACI-19-0148	GCA_030920865.1	14,663	Drained fluid	Human	ND	Japan	2020	-
K. pneumoniae	JBBDACI-19-0136	GCA_030920885.1	14,663	Pleural effusion	Human	ND	Japan	2020	-
K. pneumoniae	JBCHAFC-19-0078	GCA_031067525.1	10,865	Arterial blood	Human	ND	Japan	2020	-
K. pneumoniae	KLP00588	GCA_034193465.1	9,995	Wound	Human	ND	USA	2021	-
K. pneumoniae	JBBDACI-19-0137	GCA_034687885.1	14,663	Drained fluid	Human	ND	Japan	2020	-
K. pneumoniae	201320240S	GCA_031525175.1	14,873	Rectal Swab	Human	ND	Singapore	2013	-
K. pneumoniae	NK_H5_015	GCA_032045235.1	16,029	Blood	Human	Diseased	Norway	2017	-
K. pneumoniae	NK_H5_004	GCA_032130315.1	15,227	Blood	Human	Diseased	Norway	2017	-

Klebsiella quasipneumoniae	Q6073	-	137,338	Rectal swab	Human	None	Thailand	2018	This study
K. quasipneumoniae	Q8259	-	145,614	Rectal swab	Human	None	Thailand	2018	This study
K. quasipneumoniae	Q7925	-	138,155	Gian Dai	Environment	ND	Thailand	2022	This study
K. quasipneumoniae	KML002	GCA_030269865.1	15,293	Urine	Human	Urinary tract infection	Japan	2020	-
K. quasipneumoniae	2020-725	GCA_022818525.1	7,580	Bivalve molluscs	Molluscs	ND	Norway	2020	Håkonsholm *et al.*, 2020
K. quasipneumoniae	UTH00011	GCA_024651285.1	16,559	Blood	Human	Bacteremia	Japan	2019	-
K. quasipneumoniae	AM65-22ACA	GCA_027667345.1	11,042	Fecal material	Human	ND	China	2014	-
K. quasipneumoniae	WUSM_KQ_89	GCA_003195375.1	14,823	Urine CVS	Human	Infection	USA	2017	Potter *et al.*, 2018
K. quasipneumoniae	ENT1531	GCA_022173145.1	10,312	Rectal Swab	Human	ND	Singapore	2014	-
K. quasipneumoniae	KP056	GCA_024437375.1	13,315	Parsley	Environment	ND	Guadeloupe	2018	-
K. quasipneumoniae	KP083	GCA_024438675.1	15,467	Lettuce	Environment	ND	Guadeloupe	2018	-
K. quasipneumoniae	K7	GCA_027958325.1	15,251	Urine	Human	ND	Singapore	2020	-
K. quasipneumoniae	K5	GCA_027960525.1	10,520	Urine	Human	ND	Singapore	2020	-
K. quasipneumoniae	K31	GCA_027960425.1	14,667	Urine	Human	ND	Singapore	2020	-
K. quasipneumoniae	K27	GCA_027962165.1	11,143	Tissue	Human	ND	Singapore	2020	-
K. quasipneumoniae	JBCDABA-19-0165	GCA_031068305.1	14,873	Urine	Human	ND	Japan	2019	-
K. quasipneumoniae	Fly-1170	GCA_040137235.1	124,856	Fly	Environment	ND	China	2023	-
Klebsiella variicola	WUSM_KV_27	GCA_003196185.1	10,229	Urine	Human	Infection	USA	2017	Potter *et al.*, 2018
K. variicola	Kp10081	GCA_023314115.1	15,248	Clinical sample	Human	Klebsiella infection	USA	2017	-
K. variicola	Kp9918	GCA_023315195.2	15,248	Rectal swab	Human	Klebsiella infection	USA	2018	-
K. variicola	JBDDAAF-19-0104	GCA_030795865.1	14,667	ND	Human	ND	Japan	2019	-
K. variicola	BC-1	GCA_040104615.1	220,750	ND	ND	ND	Thailand	2021	-
K. variicola	BC-5	GCA_040104675.1	68,049	Mushroom	Environment	ND	Thailand	2021	-
Enterobacter cloacae	DS11861	GCA_002278295.1	10,918	Fecal	Human	None	Singapore	2014	-
E. cloacae	ENT1895	GCA_015683015.1	11,143	Rectal Swab	Human	ND	Singapore	2015	-
E. cloacae	CRE-16-0072	GCA_015682315.1	10,887	Rectal Swab	Human	ND	Singapore	2016	-
E. cloacae	ENT2912	GCA_015682535.1	15,074	Rectal Swab	Human	ND	Singapore	2015	-
E. cloacae	CRE-16-0437	GCA_015682875.1	10,661	Rectal Swab	Human	ND	Singapore	2016	-
E. cloacae	CRE-16-0488	GCA_015683275.1	10,680	Rectal Swab	Human	ND	Singapore	2016	-

E. cloacae	ENT2089	GCA_015682995.1	11,143	Rectal Swab	Human	ND	Singapore	2015	-
E. cloacae	2015CRE_3	GCA_016445705.1	11,143	Long-Term Care Facility	Human	Mass screening	Singapore	2015	-
E. cloacae	201749632U	GCA_033023315.1	9,263	Urine	Human	ND	Singapore	2017	-
Enterobacter kobei	13426	GCA_023154575.1	92,611	Urine	Human	ND	China	2013	Liao *et al*., 2022
E. kobei	ECC-308	GCA_025214775.1	15,281	Clinical sample	Human	ND	China	2019	-
Enterobacter hormaechei	CEent1	GCA_014109705.1	4,803,493	Cell culture	Nematode	ND	USA	2013	Dirksen *et al*., 2020
Enterobacter roggenkampii	15150	GCA_023153755.1	53,027	Urine	Human	ND	China	2013	Liao *et al*., 2022
Enterobacter quasiroggenkampii	C210233	GCA_023751515.1	86,649	NA	Human	ND	China	2021	-
Enterobacter sp.	R4-368	GCA_000410515.1	116,007	*Jatropha curcas* L.	Environment	ND	Singapore	ND	Madhaiyan *et al*., 2013

Strains found in our study are highlighted in red; The contig size correspond for the size of the contig were the class II aroA gene is located; ND: not determined.

Table S3: Comparison of the MFS-like, found in the five *Klebsiella spp.* strains, with the top ten most similar sequences found in common in the TransportDB and TCDB databases.

Strain	**Ascension**	**Protein annotation**	**Length (amino acid)**	**Position of the PS50850 domain**	**Coverage (%)**	**Identity (%)**
Corynebacterium efficiens YS-314	CE2495	MFS conserved hypothetical protein	531	63-528	97	54.81
Corynebacterium glutamicum ATCC13032	Q8NMG4	MFS permease of the major facilitator superfamily	494	19-487	98	52.93
Pseudomonas aeruginosa PAO1	PA1282	MFS probable MFS transporter	501	9-491	91	46.97
Bordetella bronchiseptica RB50 NCTC-13252	BB3389	MFS putative inner membrane efflux protein	499	9-488	91	44.25
Bordetella pertussis Tohama I NCTC-13251	BP2320	MFS putative inner membrane efflux protein	499	9-488	91	44.03
Bordetella parapertussis 12822 NCTC-13253	BPP1719	MFS putative inner membrane efflux protein	499	9-488	91	44.03
Salmonella typhi CT18	STY1491	MFS methyl viologen resistance protein SmvA	495	7-493	93	35.36
Salmonella typhimurium LT2	STM1574	MFS methyl viologen resistance protein SmvA	495	7-493	93	35.15
Staphylococcus aureus	P0A0J9	Antiseptic resistance protein QacA	514	20-508	90	34.14
Acinetobacter baumannii	C4PAW9	Major facilitator superfamily efflux pump SmvA	492	7-491	93	32.71

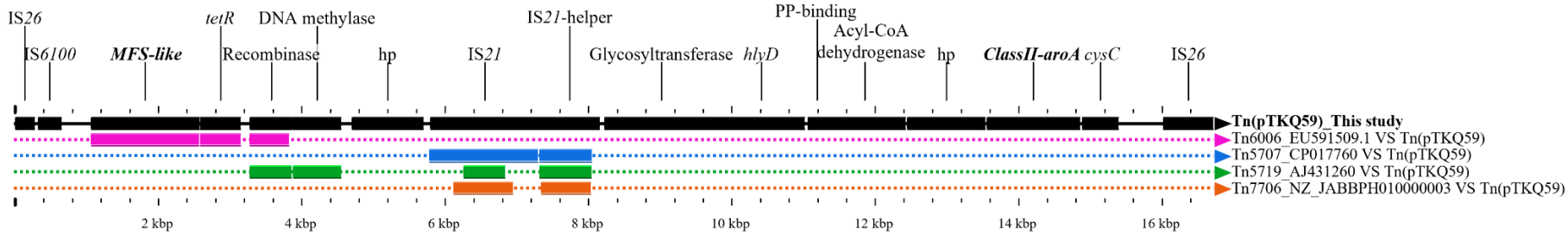

Figure S1: A schematic representation compares the transposon found in the pTKQ59 plasmid with other transposons associated with MFS transporters from the Tn Central database. The MFS-like transposon identified in our study was 100% similar (both in coverage and identity) to Tn6006 from *Enterobacter cloacae* JKB7 (EU591509.1). In contrast, MFS transporters in the Tns of *Cupriavidus necator* strain NH9 (CP017760), *Pseudomonas sp.* B13 (AJ431260), and *Rhodococcus erythropolis* strain NI86 (NZ_JABBPH010000003) show no significant similarity to our MFS-like.

Article 3

Does 5-enolpyruvylshikimate-3-phosphate synthase class correlate with bacterial sensitivity to glyphosate formulations in vitro, or are other mechanisms involved?

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En cours de préparation

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Résumé

L'enzyme EPSPS est la cible du glyphosate dans la voie du shikimate, une voie indispensable à la synthèse des acides aminés, donc à la survie des plantes. Les différentes classes de cette enzyme, qui se distinguent par leur affinité pour le glyphosate, ont été étudiées, initialement dans un but de bioingénierie pour le développement de plantes génétiquement modifiées résistantes au glyphosate. Ainsi, la classe I possède l'affinité la plus élevée pour le glyphosate par rapport aux autres classes (II, III et IV), ce qui permet de différencier ces classes et de classer les organismes qui les possèdent comme tolérants ou résistants au glyphosate. Lors de nos premiers travaux, nous avons observé une discordance entre les classes aroA identifiées dans nos souches et les CMI observées. La définition de la tolérance au glyphosate n'est pas encore claire dans la littérature. Il existe peu de travaux qui ont déterminé la tolérance des microorganismes selon leur concentration minimale inhibitrice (CMI) au glyphosate. De plus, aucune étude n'a combiné la classification in silico des enzymes EPSPS et la tolérance in vitro déterminée par la CMI. L'objectif de cette étude est de réaliser une approche combinée impliquant des analyses génomiques et bioinformatiques ainsi qu'une évaluation in vitro, en déterminant les CMI de deux formulations de glyphosate. La première est un glyphosate sous forme de sel d'isopropylamine (IPA), et la seconde est un herbicide à base de glyphosate (HBG) contenant 360 g/L de sel d'isopropylamine, ainsi qu'un ammonium quaternaire dont les informations spécifiques et la formule n'ont pas été fournies. Nous avons choisi de tester deux formulations plutôt qu'une seule, car plusieurs formulations ont été utilisées dans la littérature, et il a été observé que la composition de la formulation affecte les valeurs de CMI. Cette étude regroupe un total de 60 souches bactériennes appartenant à 57 espèces différentes (35 analysées et 25 en cours d'analyse), provenant des collections DSMZ, CIP, ATCC et CSUR. Les CMI-HBG et CMI-IPA de chaque souche ont été déterminées par la méthode de microdilution en milieu liquide, selon les directives du Clinical and Laboratory Standards Institute (CLSI). Les

souches ont été séquencées par séquençage à haut débit (Illumina MiSeq) et les EPSPS ont été classées. Nous avons également recherché trois gènes impliqués dans la dégradation du glyphosate (*gox*, *gat* et *thio*). Cinq souches ont montré les CMIs les plus basses (< 0,125 g/L), tandis que 14 avaient des CMIs intermédiaires. Les CMIs les plus élevées, atteignant jusqu'à 8 g/L pour le GBH et 32 g/L pour l'IPA, ont principalement été observées chez les Enterobacteriaceae et *Pseudomonas* spp. Nous avons identifié 16 souches possédant des enzymes de classe I et 17 des enzymes de classe II. Aucune souche testée ne possédait les gènes de dégradation du glyphosate. Il n'existe pas de corrélation entre les valeurs de CMI et les classes d'EPSPS, ce qui démontre que la tolérance au glyphosate chez les microorganismes ne dépend pas uniquement de la classe de l'enzyme, et que d'autres mécanismes participent à la réponse bactérienne. Nous proposons ainsi de standardiser la terminologie utilisée, en restreignant le terme "affinité" pour les études axées uniquement sur l'enzyme EPSPS, et "sensibilité" ou "tolérance" pour les études impliquant des microorganismes.

Title: **Does 5-enolpyruvylshikimate-3-phosphate synthase class correlate with bacterial sensitivity to glyphosate formulations *in vitro*, or are other mechanisms involved?**

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Abstract

The EPSPS enzyme is the molecular target of glyphosate and is usually associated to bacterial sensitivity or tolerance. Class 1 EPSPS confers sensitivity, while class 2 provides increased tolerance. Bacterial responses are assessed through bioinformatic analyses or in vitro MIC testing, but no study has combined both approaches to standardize the definition. To address this gap, a combined approach involving genomic, bioinformatic analyses, and in vitro MIC evaluation for IPA and GBH was conducted on various bacterial species. Sixty bacterial strains were studied, (35 fully analysed and 25 still under analysis), sourced from DSMZ, CIP, ATCC, and CSUR collections. The MICs for IPA and GBH were determined following Clinical and Laboratory Standards Institute (CLSI) guidelines for aerobic and anaerobic bacteria. Whole genome sequencing and EPSPS enzyme classification were conducted. Five strains showed the lowest MICs (< 0.125 g/L), while 14 displayed intermediate MICs. The highest MICs, reaching up to 8 g/L for GBH and 32 g/L for IPA, were primarily observed in *Enterobacteriaceae* and *Pseudomonas spp.*. We identified 16 strains possessing class I enzymes and 17 containing class II enzymes, but no correlation was highlighted between MIC values and EPSPS enzyme classes. This study demonstrates that glyphosate tolerance in microorganisms is not solely dependent on the class of the EPSPS enzyme, as other mechanisms can influence bacterial responses. As it it a growing filed, we believe it is becoming necessary to standardize the terminology used. We suggest using the terminology "affinity" when referring to studies focused solely on the EPSPS enzyme and "sensitivity" or "tolerance" for studies involving microorganisms.

Keywords: Bacteria, EPSPS class, GBH, IPA, MICs.

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Introduction

Glyphosate was patented as an herbicide in 1971 (US Patent No. 3799758), and its large-scale production began in 1974 (Mesnage & Antoniou, 2017). After its patent expired, in 2000, it became more accessible, significantly reducing its price (Benbrook, 2016). This non-selective herbicide quickly became the most widely used and effective herbicide globally (Valavanidis, 2018). Today, glyphosate remains the most used herbicide worldwide, with an annual consumption expected to reach 920,000 tons by 2025 (Ferrante et al., 2023). It is extensively used in the agricultural sector, particularly in conjunction with genetically modified crops resistant to glyphosate (Danne et al., 2019). In Europe, where the use of transgenic crops is more limited, glyphosate is primarily employed in no-till farming practices, where it is applied before sowing to eliminate weeds and desiccate cereals, legumes, and seeds before harvest (Leino et al., 2021). It is also used in urban areas, including industrial sites, public parks, and infrastructure (Benbrook, 2016).

Glyphosate specifically targets the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), which plays a role in the sixth step of the shikimate pathway. This metabolic pathway, highly conserved in archaea, bacteria, apicomplexans, algae, fungi, and plants, is crucial to produce chorismate, a precursor of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) as well as vitamins like folic acid and menaquinone (Hertel et al., 2021; Schönbrunn et al., 2001). EPSPS catalyses the reaction between phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) to produce EPSP. Glyphosate acts as a competitive inhibitor, mimicking the carbocation state of PEP and binding to the enzyme to form a stable, non-covalent EPSPS-S3P-glyphosate complex (Achary et al., 2020). This blocks protein synthesis and leads to the

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63 accumulation of toxic intermediate metabolites, causing the death of plants and exposed
64 microorganisms, thus giving glyphosate antimicrobial properties (Walsh et al., 2023).

65 Species containing class I EPSPS sequences (alpha and beta) are considered as susceptible to
66 glyphosate, whereas those with class II, III, and IV sequences are thought to confer resistance
67 (Yuan et al., 2022). Class I EPSPS enzymes, naturally found in all eukaryotes and in several
68 bacterial species such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhimurium*,
69 are relatively susceptible to glyphosate inhibition. In contrast, class II EPSPS enzymes confer
70 resistance. They are found only in bacteria, including *Staphylococcus aureus*, *Agrobacterium* sp.
71 strain CP4 and *Pseudomonas* sp (Achary et al., 2020). A genomic meta-analysis of thousands of
72 EPSPS sequences in bacteria revealed that 57% of the species studied possess class I enzymes,
73 suggesting they are susceptible to glyphosate (29% class I alpha and 28% class I beta). In
74 contrast, class II enzymes confer glyphosate resistance in 32% of bacteria, while 8% of
75 sequences remain unclassified. Class III and IV EPSPS enzymes, associated with glyphosate
76 resistance, are relatively rare in nature, representing less than 5% of sequences. For example,
77 class IV sequences, found only in a single clade of actinobacteria, account for just 0.25%, while
78 class III enzymes represent only 3% (Leino et al., 2021). Members of Class II, the first of which
79 was identified as coming from *Agrobacterium* sp. strain CP4, have been the basis of
80 bioengineering trials for the development of genetically modified glyphosate-resistant plants
81 (Funke et al., 2006) .

82 Most studies on bacterial resistance to glyphosate have focused on analysing EPSPS for
83 bioengineering and biotechnological purposes. However, upon examining bacterial tolerance to
84 this herbicide, we found no clear correlation between the EPSPS class and the glyphosate
85 minimum inhibitory concentrations (MICs). Additionally, the few available MIC studies are

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often difficult to compare due to methodological differences, variations in the GBH composition tested, and the frequent lack of genomic analysis of the different EPSPS classes. We addressed this gap by investigating the correlation through a combination of genomic and bioinformatics approaches, alongside *in vitro* assessment of the MIC for GBH and isopropylamine salt of glyphosate (IPA) across various bacterial species.

Material and methods

Determination of MIC for IPA and GBH

This study focused on 60 bacterial strains (35 have already been analyzed, 25 are currently under analysis). These strains were sourced from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), the Collection de l'Institut Pasteur (CIP), the American Type Culture Collection (ATCC), as well as the Collection de Souches de l'Unité des Rickettsies (CSUR), isolated from clinical samples in Marseille, France. All strains were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) (Microflex®, Brüker Daltonics, Bremen, Germany) (Table 1). The MICs were determined in 96-well plates using the broth microdilution method, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI), M07-A9, Vol. 29, NO. 2 for aerobic bacteria, and M11-A8, Vol. 29, NO. 2 for anaerobic bacteria. We used pure IPA in a solid paste (CAS: 38641-94-0, purity 98%) (Combi Blocks Inc, California, USA), as well as a commercial glyphosate-based herbicide (GBH) (Radikal, Fitogal, Spain, expiration date: 30/04/2028), a concentrated solution containing 360 g/L of IPA. This formulation also includes a quaternary ammonium as a co-formulant, although its exact nature and concentration are not disclosed by the manufacturer. A stock solution of 256 g/L was prepared for both agents by dissolving the salt and diluting the GBH solution in sterile cation-adjusted Mueller-Hinton broth (CAMHB, Merck KGaA,

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Darmstadt, Germany) for aerobic bacteria, and in sterile Brucella broth supplemented with 5% lysed horse blood, 5 µg/mL hemin, and 1 µg/mL Vitamin K1 for anaerobic conditions. The MIC was defined as the lowest concentration at which no visible bacterial growth was observed. It was determined both visually and by adding 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Aldrich®, St Louis, Missouri, USA), followed by an additional two-hour incubation period to allow for proper MIC reading. The experiment was performed in triplicate and repeated independently.

Whole genome sequencing and EPSPS classification

The genomes of the 35 bacterial isolates were sequenced using MiSeq technology (Illumina Inc., San Diego, CA, USA) in a paired-end strategy, after DNA extraction with the EZ1 DNA Tissue Kit (Qiagen GmbH, Germany). Raw reads were assembled using Unicycler (version 0.4.8) following a quality check with FASTQC (Galaxy Version 0.74). The assembly quality was subsequently evaluated using QUAST (Galaxy Version 5.2.0) before annotating the genome with PROKKA (Galaxy Version 1.14.6). EPSPS protein sequences were classified using the EPSPSClass web server available at "https://ppuigbo.me/programs/EPSPSClass/". Alignment of the EPSPS sequences was performed using Muscle (MEGA version 11). Then, a phylogenetic tree was generated with FastTree (version 2.1.10) and visualized using Itol (version 6.8.1). Phylogenetic analysis was conducted using the EPSPS sequences of the 35 strains analyzed in our study and the four reference sequences from each known EPSPS class: Class I (accession number: Q9KRB0), Class II (accession number: Q83E11), Class III (accession number: CAR82018.1), and Class IV (accession number: CCK29908.1).

Statistical analysis

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The relationship between GBH and IPA MICs and EPSPS classes was assessed using non-linear methods. Spearman's correlation coefficient was calculated for each pair of variables to identify associations, and hypothesis tests were performed to evaluate statistical significance at a 5% level (P value < .05) using RStudio (Posit Software, PBC, Boston, Massachusetts, USA).

Glyphosate degradation genes

The BLASTp tool was employed to search for the three genes associated with glyphosate degradation. The first one is the *gat* gene (GenBank accession: AX543338.1) encoding glyphosate acetyltransferase (GNAT), the second is the *gox* gene (GenBank accession: GU214711.1) encoding glyphosate oxidoreductase, and the third is the *thiO* gene (GenBank accession: KC831746.1) encoding glycine oxidase.

Results

MIC values of GBH and IPA across tested strains:

Among the 35 strains analyzed, the lowest MICs were observed in five Gram-positive bacterial strains, including *Lactiplantibacillus plantarum*, *Corynebacterium propinquum*, and three *Streptococcus* spp., with values ranging from 0.003 to 0.03 g/L for GBH and from 0.01 to 0.125 g/L for IPA. MIC values between 0.25 and 2 g/L for GBH and 2 to 8 g/L for IPA were found in 14 strains, encompassing Gram-positive and Gram-negative bacteria. For Gram-positive strains, such as *Bacillus subtilis*, *Enterococcus casseliflavus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus hirae*, *Staphylococcus aureus*, and *Streptomyces cattleya*, MICs ranged from 0.5 to 1 g/L for GBH and 2 to 4 g/L for IPA. Among Gram-negative non-fermenting bacilli, except for *Brevundimonas paucivorans* with a MIC of 0.25 g/L for GBH and 2 g/L for IPA, strains like *Achromobacter xylosoxidans* (n=2), *Acinetobacter baumannii*, *Burkholderia*

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153 *cenocepacia*, and *Ochrobactrum pseudogrignonense* displayed MICs ranging between 1 to 2 g/L
154 for GBH and 4 to 8 g/L for IPA. The highest MICs, ranging from 4 to 8 g/L for GBH and 8 to 32
155 g/L for IPA, were found in 14 strains, primarily from the *Enterobacteriaceae* family and
156 *Pseudomonas* spp. Notably, two strains, *Pseudomonas aeruginosa* and *Klebsiella aerogenes*,
157 exhibited high tolerance, with MICs of 16 g/L for GBH and between 16 and 64 g/L for IPA. A
158 highly significant correlation was observed between IPA and GBH MICs, with a correlation
159 coefficient of 0.9581 and a P-value of 1.75*10 -19. All bacterial strains exhibited greater
160 tolerance to IPA, consistently showing higher MICs compared to GBH for the same strain
161 (Figure 1).

162 **EPSPS classification**

163 The classification of EPSPS enzymes from the 35 isolates is as follows: 16 isolates belong to
164 class I, 17 to class II, and one *Streptomyces cattleya* isolate possesses both class I and IV EPSPS.
165 No class III EPSPS enzymes were identified, and the EPSPS enzyme in *B. paucivorans* remains
166 unclassified. Most enterobacterial strains (n=11) carrying a class I EPSPS exhibited MICs greater
167 than 4, while others carrying the class II gene, such as *L. plantarum* and *Streptococcus mitis*, had
168 very low MICs (IPA 0.01 g/L, GBH 0.003 g/L) and (IPA 0.06 g/L, GBH 0.007 g/L), respectively.
169 No correlation was observed between IPA MIC and EPSPS class, nor between GBH MIC and
170 EPSPS class, with a correlation coefficient of -0.4826 and a P-value of 0.00332 for each pair.

171 **Degradation genes:**

172 None of the three genes involved in glyphosate degradation (*gat*, *gox*, *thiO*) were detected in the
173 genomes of any of the 35 isolates analyzed.

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Discussion

As reported in the literature, this study highlights that EPSPS classes I and II are the most found in bacteria, whereas classes III and IV are rarer (Leino et al., 2021). It has been noted that approximately 8% of EPSPS enzymes in bacteria remain unclassified, as is the case with the EPSPS enzyme in *B. paucivorans*, which was not identified in our study.

We also observed an increased bacterial tolerance to glyphosate in its IPA form compared to the GBH formulation, with a median MIC of 8 g/L for IPA (3;16). In contrast, the median MIC for GBH was 2 g/L (1;4). This suggests that the adjuvants present in GBH formulations, though often considered "inert, and inactive " may significantly impact microorganisms (Gandhi et al., 2021; Mesnage et al., 2019). In our case, the manufacturer did not disclose the specific adjuvant used in the GBH formulation, which could have serious consequences for the environment, the organisms exposed, and even the users of these pesticides. This lack of transparency raises further concerns about the potential environmental and health impacts (Mesnage et al., 2019).

Our results also revealed that enterobacteria, despite possessing a class I EPSPS enzyme, are among the most tolerant to both IPA and GBH. This increased tolerance could be attributed to additional mechanisms, such as the structure of their outer membrane, which limits the entry of toxic molecules, or the diversity of their efflux pumps, which actively expel various compounds, including pesticide (Gaurav et al., 2023). Bacteria have a predominance of efflux pumps from the Resistance-Nodulation-Division (RND) superfamily, which are primarily found in Gram-negative bacteria and are involved in pathogenesis, virulence, stress response, as well as the well-known function of antimicrobial resistance (Auda et al., 2020; Huang et al., 2022).

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Strains from *Pseudomonas* spp. showed significantly higher glyphosate tolerance than other bacteria with class II EPSPS enzymes. *Pseudomonas* spp. are also known for their highly developed efflux systems, including multiple efflux pumps. For example, twelve RND superfamily systems capable of secreting antibiotics or antimicrobial products have been functionally described in these species and other efflux pump families (Yamasaki et al., 2023). This suggests that glyphosate tolerance is not solely dependent on the EPSPS enzyme class and can also be enhanced through efflux pump overexpression and synergistic action with other resistance mechanisms (Auda et al., 2020). The interaction between the EPSPS class and other cellular mechanisms may increase glyphosate tolerance in certain bacterial strains.

Although class II EPSPS is generally associated with resistance, some strains have shown high sensitivity to glyphosate, confirming that other intrinsic or external (environmental) factors can influence this tolerance, making certain strains more sensitive despite the presence of class II EPSPS. This sensitivity could be due to the absence of other resistance mechanisms, such as efflux pumps, or to disruptions in genetic regulation affecting EPSPS expression. Additionally, laboratory culture conditions differ from environmental conditions and may impact the enzyme's efficiency, while unknown metabolic or structural factors could also play a role (Motta et al., 2018).

It is challenging to compare glyphosate MIC data across studies due to the need for standardized procedures and the often-incomplete reporting of formulation compositions (Pöppe et al., 2019). Additionally, different definitions of tolerance exist, one based on bioinformatic analyses (Leino et al., 2021), EPSPS kinetic enzymatic evaluation (Griffin et al., 2021; Priestman et al., 2005), and the other based on MIC measurements (Bote et al., 2019; Nielsen et al., 2018; Pöppe et al., 2019).

These findings highlight the need to standardize the terminology surrounding "glyphosate tolerance," which can lead to confusion in the literature. It would be more appropriate to use

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the term "affinity" for studies focused solely on the EPSPS enzyme and "sensitivity" or "tolerance" for studies involving microorganisms. This distinction is critical as research on the impact of glyphosate and GBHs on microbial biodiversity and bacterial sensitivity is increasing.

The strength of this study lies in its combined approach, analysing bacterial sensitivity to glyphosate through EPSPS class classification and integrating genomic analysis with MIC testing for GBH and IPA. While this work represents a valuable advancement in understanding bacterial responses to glyphosate, future research should include a broader range of bacterial species and explore non-EPSPS-related resistance mechanisms, such as efflux pumps and degradation pathways.

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Conflict of interest statement: The authors declare that they have no competing interests.

Data availability:

The data presented in this study are openly available in NCBI in Bioproject XXXX

Authors' contributions

LH, JMR and SAB designed and conceived the study. HZ, AH performed the experiments and interpreted the data. LH, HZ, AH drafted the manuscript. HZ, HA, LH, JMR, and SAB made critical corrections and revisions.

All authors read and approved the final version of the manuscript.

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Reference

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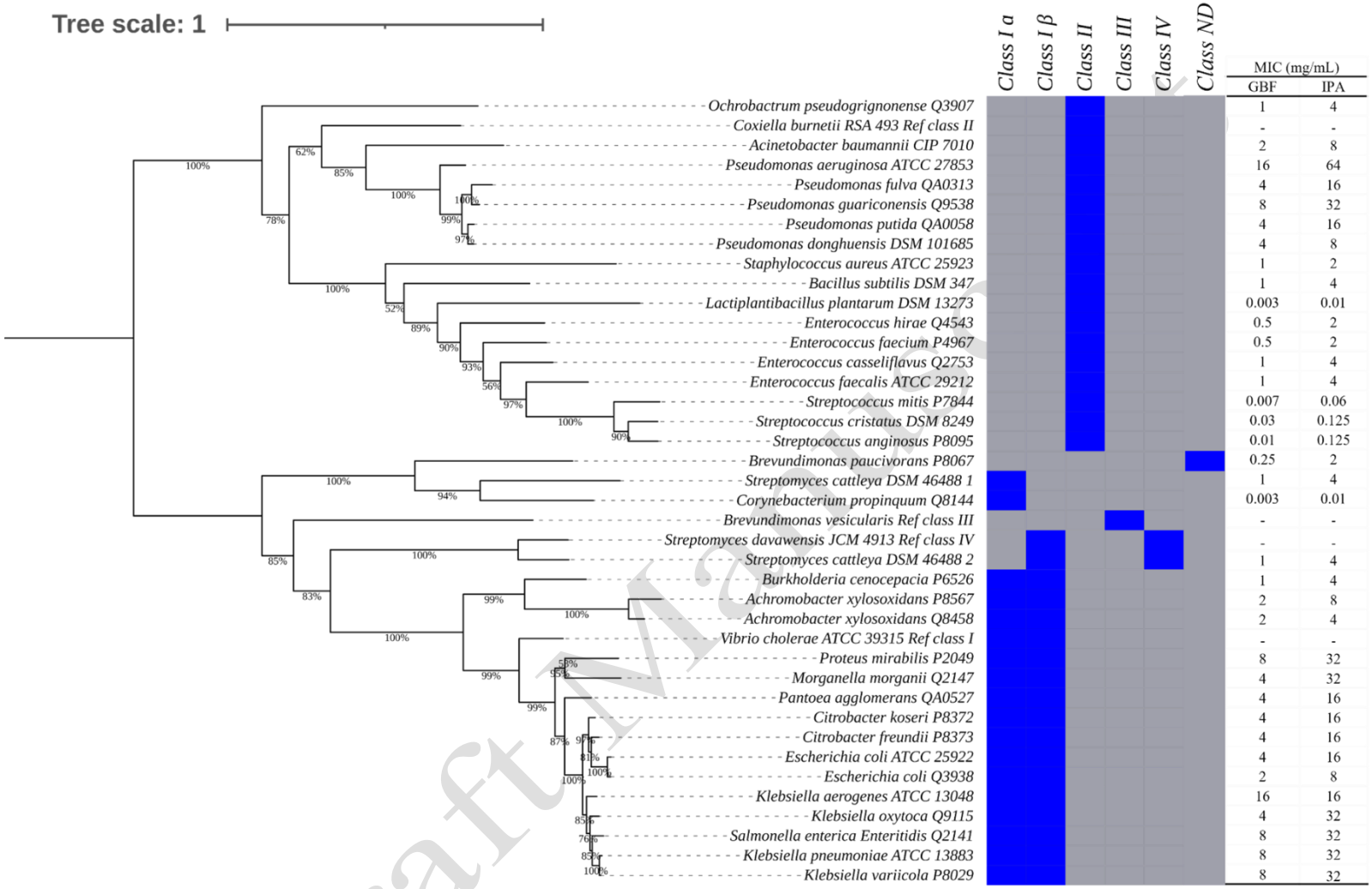

Figure 1: Phylogenetic tree of 40 EPSPS protein sequences from 39 bacterial strains, including 35 from this study and 4 reference strains from the ppuigbo EPSPSClass database, with an associated heatmap showing EPSPS class distribution (gray: absence, blue: presence of class-specific amino acid markers), and MIC values for glyphosate-based formulation (GBF) and isopropylamine salt (IPA).

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Chapitre III

Y-a-t-il une corrélation entre tolérance au glyphosate et résistance aux antibiotiques ? Exemple chez les bactéries du groupe ESKAPEE

Avant-propos

Le glyphosate est un herbicide dont les propriétés antimicrobiennes peuvent modifier les microbiotes environnementaux et du tube digestif des animaux (31). Comme nous l'avons déjà vu dans les chapitres précédents, le glyphosate possède une action antimicrobienne. De plus, nous avons identifié (**Article 2**) un gène de résistance au glyphosate porté par des plasmides de type IncF, plasmides qui sont également souvent porteur de gènes de résistance au glyphosate. Enfin, nous avons vu que la résistance au glyphosate était médiée par des pompes d'efflux chez les bactéries (**Article 1**) dont certains antibiotiques sont des substrats (41). Nous avons donc émis l'hypothèse que le glyphosate, herbicide massivement utilisé dans le monde, pouvait jouer un rôle dans la sélection et/ou la dissémination de la résistance aux antibiotiques.

Dans un premier temps, nous avons recherché la présence de souches bactériennes présentant un niveau de tolérance au glyphosate dans le tractus digestif de sujets sains exposés au glyphosate. Nous nous sommes concentrés sur l'isolement des bactéries Gram-négatives de prélèvements rectaux réalisés chez des fermiers thaïlandais ayant différents niveaux d'exposition au glyphosate (**Article 4**). Nous avons isolé des souches présentant des niveaux de tolérance au glyphosate ≥7 mg/L, sans différence significative entre le groupe exposé et non exposé au glyphosate. Nous avons évalué le niveau de sensibilité de ces souches aux antibiotiques utilisés en médecine humaine, mais n'avons, là non plus, pas mis en évidence de différence significative. En revanche, nous avons identifié six souches d'*Escherichia coli* productrices de β-lactamases à spectre étendu (BLSE) et/ou résistantes à la colistine chez des fermiers exposés au glyphosate, et ce en l'absence d'utilisation de ces antibiotiques. Bien qu'ils ne soient pas significatifs, ces résultats préliminaires doivent être corroborés par des études supplémentaires.

Dans un deuxième temps, nous avons évalué l'effet antibiotique du glyphosate en association avec d'autres antibiotiques. Nous avons mesuré l'effet que pouvait avoir la combinaison du glyphosate avec des antibiotiques utilisés en médecine humaine sur des bactéries pathogènes pour l'homme et fréquemment multirésistantes, ces deux agents pouvant être retrouvé à des concentrations non négligeables dans l'environnement et dans le tube digestif humain. L'objectif était d'observer si le glyphosate pouvait augmenter le niveau de résistance de ces pathogènes. Pour cela, nous avons sélectionné des souches appartenant aux bactéries Gram-négatives du groupe ESKAPEE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp.*, *Escherichia coli*). Nous avons ainsi montré qu'une formulation de glyphosate (HBG, Herbicide Based Glyphosat Radikal Green, Spain) testée présentait des effets additifs, synergiques ou antagonistes en fonction de l'association et de la bactérie testée. Nous avons notamment observé un effet synergique entre l'HBG et les carbapénèmes sur les souches produisant des métallo-β-lactamases (MBL). Nous avons aussi observé un effet synergique sur des souches d'*A. baumannii* en association avec la colistine. En revanche, nous avons observé un effet antagoniste en cas d'association avec les fluoroquinolones. Ces résultats nous ont permis de mettre en évidence le rôle du glyphosate dans la modulation de la résistance aux antibiotiques, en raison de ses propriétés chélatrices (**Article 5**).

Article 4

Antibiotic resistance in glyphosate tolerant Gram-negative bacteria isolated from Thai farmers exposed and not exposed to Glyphosate

Aïcha Hamieh, Hanane Zerrouki, Linda Hadjadj, Chuanphot Thinphovong, Anamika Kritiyakan, Kittipong Chaisiri, Serge Morand, Jean-Marc Rolain,* and Sophie Alexandra Baron*.

Soumis dans Microbial Pathogenesis (Impact Factor: 3.3)

Résumé

Le glyphosate, un herbicide largement utilisé, possède des propriétés antimicrobiennes qui pourraient influencer la diversité des microbiotes. Bien que son rôle dans la sélection de la résistance aux antibiotiques chez les bactéries reste encore peu exploré, des études suggèrent que l'exposition répétée au glyphosate pourrait entraîner une co-sélection, favorisant l'apparition de souches bactériennes tolérantes au glyphosate et potentiellement résistantes à divers antibiotiques (39).

L'objectif de notre étude était d'évaluer l'impact de l'exposition au glyphosate sur la tolérance au glyphosate et la résistance aux antibiotiques des bactéries Gram-négatives (BGN) isolées du tube digestif d'agriculteurs thaïlandais. Les échantillons fécaux ont été prélevés chez des agriculteurs travaillant dans des fermes situées dans la province de Nan, une région connue pour son utilisation intensive d'herbicides, et notamment de glyphosate. L'exposition au glyphosate a été évaluée à l'aide de tests de cholinestérase, un biomarqueur couramment utilisé pour mesurer l'exposition aux pesticides organophosphorés, y compris le glyphosate. Les échantillons analysés, regroupés par niveau d'exposition au glyphosate (Exposés / Non Exposés), ont été cultivés sur géloses MacConkey (McK) avec ou sans un herbicide à base de glyphosate (HBG) à une concentration de 7 g/L. La sensibilité aux antibiotiques des souches isolées a été évaluée à l'aide de la méthode de diffusion en milieu gélosé (méthode des disques), tandis que la concentration minimale inhibitrice (CMI) du glyphosate a été déterminée par la méthode de microdilution en milieu liquide.

Au total, 547 BGN ont été isolés à partir de 112 échantillons fécaux humains. *Escherichia coli*, *Klebsiella pneumoniae* et *Citrobacter freundii* sont les espèces bactériennes les plus retrouvées sur milieu contenant du HBG. Les CMI HBG varient de 2 g/L à 16 g/L. L'espèce *K. pneumoniae* présentait la CMI médiane la plus élevée (16 g/L). Aucune différence de tolérance au

glyphosate n'a été observée entre les groupes Exposés et Non Exposés. De même, les taux de résistance aux antibiotiques testés n'étaient pas significativement différents entre les groupes Exposés et Non Exposés. En revanche, six souches d'*E. coli* résistantes à la colistine (*mcr* positifs) et/ou produisant une bêta-lactamase à spectre étendu (BLSE) ont été isolées du groupe exposé au glyphosate, tandis qu'une seule souche de *K. pneumoniae* résistante à la colistine a été isolée dans un échantillon non exposé au glyphosate. De plus, une enquête menée dans les villages d'où proviennent les échantillons, y compris ces souches, a révélé qu'aucun de ces villages n'utilise d'antibiotiques, en particulier la colistine et les céphalosporines. Notre travail présente plusieurs limites. Tout d'abord, le test utilisé pour définir l'exposition au glyphosate est qualitatif, il aurait été préférable d'adopter des méthodes plus sensibles, telles que la chromatographie liquide à haute performance (High-Performance Liquid Chromatography, HPLC), pour une mesure plus précise de l'exposition au glyphosate permettant de discriminer les deux groupes de manière plus précise. De plus, l'utilisation de glyphosate étant importante en Thaïlande, ces fermiers peuvent avoir acquis des bactéries résistantes au glyphosate via l'alimentation, sans être directement exposés au glyphosate (de même que le portage de bactéries multirésistantes (BMR) sans administration préalable d'antibiotique). De plus, il aurait été pertinent de tester divers milieux de culture, et non de se limiter aux BGNs, afin d'obtenir un panorama complet de la diversité microbienne chez ces agriculteurs, qu'ils soient exposés ou non à cet herbicide. Ce travail nécessite des explorations complémentaires pour évaluer le rôle du glyphosate dans la résistance aux antibiotiques.

Microbial Pathogenesis

Antibiotic resistance in glyphosate tolerant Gram-negative bacteria isolated from Thai farmers exposed and not exposed to Glyphosate.

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	Sophie Baron
Abstract:	Background: Glyphosate, a widely used herbicide, has the potential to impact microbial diversity, but its influence on antibiotic resistance (AR) in bacteria remains understudied. Objectives: The objective of our study was to evaluate the impact of glyphosate exposure on glyphosate tolerance and AR in Gram-negative bacteria isolated from the digestive tracts of Thai farmers. Methods: Human faecal samples from Thailand, grouped by level of glyphosate exposure, were cultured on MacConkey (McK) agar with or without 7 g/L of a glyphosate-based formulation (GBF). Antibiotic susceptibility and glyphosate minimum inhibitory concentration (MIC) of isolated strains were assessed using the disk diffusion and broth microdilution methods, respectively. Results: A total of 547 GNB were isolated from 112 human faecal samples. GBF medium predominantly selected Escherichia coli, Klebsiella pneumoniae, and Citrobacter freundii. GBF MICs ranged from 2 g/L to 16 g/L. K. pneumoniae species had the highest median MIC (16 g/L). AR rates were not significantly different between the exposed and not exposed groups. In contrast, six mobile colistin resistance (MCR)- and/or extended spectrum beta lactamase (ESBL)-producing E. coli strains were isolated from glyphosate-exposed group, while only one colistin-resistant K. pneumoniae strain was isolated from a sample which was not exposed to glyphosate. Conclusions: This study highlighted the putative effect of glyphosate selection of clinically relevant pathogens, and the possible link between colistin and/or cephalosporin resistance and glyphosate exposure by the isolation of colistin-resistant and/or ESBL-producing strains from glyphosate-exposed farmers. These results encourage further investigations of the influence of glyphosate on AR.
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Declaration of interests

☐The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Aix Marseille Univ, APHM, MEPHI
IHU - Méditerranée Infection

Marseille, 11/09/2024

Microbial Pathogenesis

Dear Editor,

Please find enclosed our **short communication** entitled "**Antibiotic resistance in glyphosate tolerant Gram-negative bacteria isolated from Thai farmers exposed and not exposed to Glyphosate**".

The widespread use of glyphosate in agriculture and its potential ecological implications have raised concerns about its wider effects on the microbiota, particularly the human microbiota. We evaluated the level of glyphosate tolerance and antibiotic resistance on GNB isolated from fecal sample in a population of Thai farmers that are exposed or not to glyphosate. Our study encompassed a total of 547 GNB strains isolated from 112 human fecal samples. Glyphosate based formulation predominantly selected *Escherichia coli*, *Klebsiella pneumoniae*, and *Citrobacter freundii*. In our study, the isolation of colistin-resistant and/or ESBL-producing strains from glyphosate-exposed farmers raises interesting questions about a potential association between glyphosate exposure and acquisition of mobile genetic elements including colistin and/or cephalosporin resistance.
Investigating the consequences of glyphosate on human, animal and environmental health is crucial, especially when the re-authorization of this herbicide is currently under evaluation in the European Union. We believe that our manuscript, aligns perfectly with the mission and scope of the Journal of Microbial Pathogenesis as a short communication, and we are excited about the opportunity to contribute our work to your esteemed journal.

This work has not been published previously, and it is not under consideration for publication elsewhere. Please note that we previously submitted this manuscript in Internation Journal of Antimicrobial Agents under the number IJAA-D-24-00349, and in Journal of Global Antimicrobial Resistance under the number JGAR-D-24-00455. All co-authors have seen and approved the manuscript and have complied with the stipulations of the instructions to Authors. There are no conflicts of interest.

Thank you for your time and consideration.

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Sincerely yours,

Jean-Marc ROLAIN and Sophie Alexandra BARON

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Proposed title: Antibiotic resistance in glyphosate tolerant Gram-negative bacteria isolated from Thai farmers exposed and not exposed to Glyphosate.

Running title: Antibiotic resistance in glyphosate tolerant GNB.

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Abstract

Background: Glyphosate, a widely used herbicide, has the potential to impact microbial diversity, but its influence on antibiotic resistance (AR) in bacteria remains understudied.

Objectives: The objective of our study was to evaluate the impact of glyphosate exposure on glyphosate tolerance and AR in Gram-negative bacteria isolated from the digestive tracts of Thai farmers.

Methods: Human faecal samples from Thailand, grouped by level of glyphosate exposure, were cultured on MacConkey (McK) agar with or without 7 g/L of a glyphosate-based formulation (GBF). Antibiotic susceptibility and glyphosate minimum inhibitory concentration (MIC) of isolated strains were assessed using the disk diffusion and broth microdilution methods, respectively.

Results: A total of 547 GNB were isolated from 112 human faecal samples. GBF medium predominantly selected *Escherichia coli*, *Klebsiella pneumoniae*, and *Citrobacter freundii*. GBF MICs ranged from 2 g/L to 16 g/L. *K. pneumoniae* species had the highest median MIC (16 g/L). AR rates were not significantly different between the exposed and not exposed groups. In contrast, six mobile colistin resistance (MCR)- and/or extended spectrum beta lactamase (ESBL)-producing *E. coli* strains were isolated from glyphosate-exposed group, while only one colistin-resistant *K. pneumoniae* strain was isolated from a sample which was not exposed to glyphosate.

Conclusions: This study highlighted the putative effect of glyphosate selection of clinically relevant pathogens, and the possible link between colistin and/or cephalosporin resistance and glyphosate exposure by the isolation of colistin-resistant and/or ESBL-producing strains from glyphosate-exposed farmers. These results encourage further investigations of the influence of glyphosate on AR.

Keywords: glyphosate, glyphosate-based formulation, antibiotic resistance, Thailand, One Health.

1. Introduction

N-(phosphonomethyl)glycine, also known as glyphosate, is a non-selective herbicide used for pre-planting and post-harvest clearing [1], with global annual use estimated at 700 000 tons [2]. Widely used, glyphosate poses potential health risks, such as cancer and respiratory disorders, for farmers and consumers [3,4]. Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme in the shikimate pathway essential for the synthesis of amino acids and plant growth [1]. This enzyme is also present in bacteria, which can either be sensitive (class I EPSPS), or tolerant (class II, III and IV) to glyphosate. This activity led the Monsanto Company to patent glyphosate as a human antibiotic, antifungal and antiparasitic (Patent No.: US 7771736, 2010). Its role in the selection of antibiotic resistance genes and/or bacteria has been questioned. Antibiotic resistance (AR) has emerged and spread worldwide with the massive use of antibiotics in human, agriculture, and environmental health. Horizontal gene transfer in microbiomes promotes this spread. Other determinants may also be involved, such as poor sanitation, lack of hygiene, climate change, and cross-resistance with heavy metals [5,6]. Few studies have explored the role of pesticides, including glyphosate, in AR emergence and microbial ecosystem changes [7,8]. To date, studies have shown that glyphosate does not directly increase AR but stimulates the exchange of mobile genetic elements between bacteria.

In Thailand, agriculture is the main economic development sector, leading to the massive use of pesticides [9]. Many studies reveal high levels of pesticides in food products [9] and human sera [10]. Simultaneously, AR has become a public health issue in the country, prompting governmental initiatives to monitor and control it. This study aimed to evaluate the impact of glyphosate exposure on AR and tolerance to a glyphosate-based formulation (GBF) in Gram-negative bacteria (GNB) isolated from the digestive tract of Thai farmers.

2. Materials and methods

2.1 Human Ethical approval

The collection of human biological specimens and data for this research study was approved by the Mahidol University Ethics Committee of the Faculty of Tropical Medicine under certification number MUTM-2018-035-01. The Ethics Committee of Faculty of Tropical Medicine certified that this project complied with the Declaration of Helsinki, the ICH Guidelines for Good Clinical Practice and other international guidelines for human research Protection.

2.2 Sampling and study population

Sampling was conducted between 2018 and 2019 from farmers aged between 16 and 70, raising poultry and/or pigs, in eight villages of the Nan Province, Thailand (Figure S1). Blood samples and rectal swabs were collected from each farmer. Rectal swabs were sent to the IHU Méditerranée Infection, Marseille, France, in compliance with the relevant transportation regulations and stored at -80°C for further analysis.

2.3 Glyphosate exposure

Glyphosate exposure was evaluated by measuring the concentration of acetyl cholinesterase enzyme (AChE) in capillary blood samples of each farmer using field kits, as previously described [11]. This test classified the individuals into four categories: normal, safe, at risk, and unsafe (Table S1). Due to our small study population, we divided it into “not exposed (NE)” (normal and safe), and “exposed (E)” (at risk and unsafe) groups.

2.4 Microbiological analyses

Faecal samples were enriched in Tryptic Soy broth (bioMérieux, Marcy L’Etoile, France), incubated 24h at 37°C, and then plated on MacConkey agar (McK) (Becton Dickinson, Le Pont de Claix, France) with and without GBF (Radikal green®, Fitogal, Spain). This GBF

contains 360 g/L of isopropylamine salt and a quaternary ammonium compound. Bacterial isolates were identified using Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF, Bruker Daltonik, Bremen, Germany). If two colonies from the same sample shared the same identification, resistance profile and GBF minimum inhibitory concentration (MIC), there were considered as one clone.

Broth microdilution method was used to determine GBF MIC [12]. Antimicrobial susceptibility tests were performed using the disk diffusion method and interpreted according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). MIC determination was performed using the E-test method (bioMérieux, France) for imipenem and ertapenem, and UMIC microdilution method (Biocentric, Bandol, France) for colistin. Real-time PCR was performed to detect genes encoding colistin resistance (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5* and *mcr-8*) in colistin-resistant strains, and genes encoding extended spectrum beta-lactamases (ESBLs) ($bla_{CTX\text{-}M\text{-}A}$, $bla_{CTX\text{-}M\text{-}B}$ and bla_{TEM}) in strains showing an ESBL profile [13].

2.5 Statistical analysis

Statistical analyses were performed using Welch two-sample t-test and Fisher's Exact Test, at a significance level of 5% (P value < .05) using RStudio (Posit Software, PBC, Boston, Massachusetts, United States).

3. Results

3.1 Samples and glyphosate exposure

Overall, 112 farmers were included in this study. Of the 112 sera samples collected, glyphosate exposure was measured as being "normal" for 26 farmers (23.21%), "safe" for 27 farmers (24.11%), "at risk" for 54 farmers (48.21%), and "unsafe" for five farmers (4.46%).

For further analysis, the study population was divided into two groups: "not exposed (NE)" (N=53), and "exposed (E)" (N=59).

3.2 Bacterial species identification

A total of 547 GNB (362 on McK and 185 on McK + GBF) were isolated from the 112 faecal samples (Table 1). From NE group, 17 species (eight genera) were isolated, whereas 23 species (11 genera) were isolated from the E group (Figure S2). Most species isolated in both groups were *Escherichia coli*, *Citrobacter freundii* and *Klebsiella pneumoniae*, with a frequency of 60.2%, 9.24% and 12.8% in the NE group and 54.7%, 17.1% and 8% in the E group, respectively.

3.3 Glyphosate based formulation MIC

GBF MICs ranged from 2 g/L to 16 g/L with a median of 8 g/L (Table 1). The median MIC of *E. coli* and *C. freundii* was 8 g/L, while that of *K. pneumoniae* was 16 g/L. We observed no significant difference between the median MIC of the E group (8 g/L) and the NE group (8 g/L).

3.4 Antibiotic susceptibility

We compared the level of AR for each antibiotic in the two groups (E and NE) (Supplementary file 2). Due to the low number of strains, we focused our analysis on the three most frequent species (*E. coli*, *K. pneumoniae* and *C. freundii*). We observed no significant differences for each antibiotic-bacteria pair between the two groups (Table 2). However, four colistin-resistant *E. coli* (two with *mcr-1* and two with both *mcr-1* and *mcr-3* genes), including one ESBL-producer, were isolated from four samples of the E group from three villages. One colistin-resistant *K. pneumoniae* (non-*mcr* mediated) was isolated from a sample of the NE group (Table S2). Three ESBL-producing *E. coli*, including two with the $bla_{CTX\text{-}M\text{-}A}$ gene and one with both the $bla_{CTX\text{-}M\text{-}A}$ and bla_{TEM} genes (also resistant to colistin with the *mcr-1* and

mcr-3 genes) were isolated from three samples of the E group (Table S2). We found no use of antibiotics by the farmers in villages 4 and 5, while some farmers in village 1, work on nearby farms using Moxlistin (amoxicillin 120 mg + colistin 1 000 000 units), as there are no farms in their village.

4. Discussion

The widespread use of glyphosate around the world has increased the need to study the potential effect of it on plants, humans, and bacteria [14]. Many questions remain about the role of glyphosate in the selection of AR.

In our study, the median GBF MIC (8 g/L) found was equivalent to that found in studies that used a GBF containing isopropylamine salt and the same method [12]. There is currently no consensus to determine glyphosate bacterial MICs, that makes studies difficult to compare. In addition, comparing MIC values between McK and McK-GBF revealed differences in GBF diffusion between liquid and solid media [15]. Strains with a MIC of 4 g/L were isolated on McK-GBF, whereas those with an MIC of 16 g/L were not found on McK-GBF.

Enterobacteria exhibit a higher level of resistance than *Acinetobacter*, which have a type II EPSPS (tolerant), when Enterobacteria possess a type I EPSPS (sensitive). Initial studies aimed at optimizing crop cultivation and pesticide use, led to the selection of genes involved in glyphosate resistance. However, real-world scenarios involve other strain-level factors that can influence glyphosate resistance, such as efflux mechanisms. It seems essential to clarify the definition of "sensitivity" and "tolerance" to glyphosate to take into account both phenotypic and genotypic aspects.

Finally, we failed to correlate AR in Enterobacteria with human exposure to glyphosate. The main limitation of our study is the limited ability of the serological test to distinguish glyphosate-exposed from non-exposed people. The small number of samples as well as the

limited number (N=16) of antibiotics tested are also limitations. It would be interesting to test a wider range of molecules used in veterinary medicine and in the environment. Interestingly, MCR-producing colistin-resistant and/or ESBL-producing strains were isolated in four people belonging to the E group and came from three villages. Colistin has been widely used in veterinary medicine in farms, especially in southeast Asia [16]. In our study, these villages used neither colistin nor cephalosporins to treat their livestock. The information may be lacking, as farmers do not want to reveal they use antibiotics, and/or may not be aware of it. It is interesting to see that subjects carrying these resistant bacteria had all been exposed to glyphosate. These findings support the need for further research into the influence of glyphosate on AR.

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Author contributions

Aïcha Hamieh: Formal analysis, Investigation, Writing - Original Draft; **Hanane Zerrouki:** Formal analysis, Investigation; **Linda Hadjadj:** Formal analysis, Investigation; **Chuanphot Thinphovong:** Investigation; **Anamika Kritiyakan:** Investigation; **Kittipong Chaisiri:** Investigation; **Serge Morand**: Conceptualization, Methodology, Validation, Funding acquisition; **Jean-Marc Rolain**: Conceptualization, Methodology, Validation, Writing - Review & Editing, Supervision, Funding acquisition; **Sophie Alexandra Baron**: Conceptualization, Methodology, Validation, Writing - Review & Editing, Supervision.

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Transparency declarations

Informed consent was obtained from all subjects involved in the study. All authors have read and approved the final manuscript. The authors declare no conflict of interest.

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Legend of Table and Figure

Table 1: The distribution of glyphosate-based formulation (GBF) MICs for a total of 547 strains isolated on MacConkey and MacConkey supplemented with 7 g/L of GBF in the Not Exposed (NE) and Exposed (E) groups.

Table 2: P-values of Fisher's Exact Test calculated to assess the independence between the rate of resistance for each antibiotic and the degree of exposure (NE and E) for *K. pneumoniae*, *E. coli* and *C. freundii* strains.

Supplementary file 1:

Table S1: Glyphosate exposure categories based on acetyl cholinesterase enzyme (AChE) concentration levels in capillary blood samples.

Table S2: MCR- and ESBL-producing strains isolated in this study.

Figure S1: Distribution of sampling sites in Saenthong, Thawangpha, Nan Province, Thailand (Farm Resist Project); red circle: village; blue circle: farm using Moxlistin.

Figure S2: Bacterial species selection in the presence of a glyphosate. All the strains isolated on MacConkey + Glyphosate based formulation (McK + GBF) were also isolated on MacConkey medium (McK). The visual, generated using Cytoscape software (version 3.8.2), indicates that only a few species were able to grow in the medium supplemented with GBF, out of all the isolated strains.

Supplementary file 2: Antibiotic resistance profiles of bacterial species isolated (on McK and McK-GBF) from farmers with different glyphosate exposure.

Table 1: The distribution of glyphosate-based formulation (GBF) MICs for a total of 547 strains isolated on MacConkey and MacConkey supplemented with 7 g/L of GBF in the Not Exposed (NE) and Exposed (E) groups.

		GBF MIC (g/L)								
Species isolated on MacConkey agar		**NE (n= 53 Samples)**				**E (n= 59 Samples)**				
Genus	**Species**	**≤ 4**	**8**	**16**	**Total NE**	**≤ 4**	**8**	**16**	**Total E**	**Total**
Acinetobacter	*A. bereziniae*	-	-	-	0	1	-	-	1	**1**
	A. pittii	-	-	-	0	1	-	-	1	**1**
	A. radioresistens	-	1	-	1	-	-	-	0	**1**
Citrobacter	*C. braakii*	2	-	-	2	-	-	-	0	**2**
	C. freundii	3	6	-	9	8	14	1	23	**32**
	C. pasteuri	-	-	1	1	-	-	-	0	**1**
	Citrobacter sp	-	-	-	0	-	1	-	1	**1**
Enterobacter	*E. cloacae*	-	-	4	4	-	1	2	3	**7**
Escherichia	*E. coli*	19	90	1	110	17	100	1	118	**228**
	E. fergusonii	-	1	-	1	-	2	-	2	**3**
	E. hermannii	-	-	-	0	1	-	-	1	**1**
	E. massiliensis	-	2	-	2	-	-	-	0	**2**
Hafnia	*H. alvei*	-	-	-	0	1	1	-	2	**2**
Klebsiella	*K. aerogenes*	-	-	3	3	-	-	2	2	**5**
	K. oxytoca	-	3	-	3	-	5	-	5	**8**
	K. pneumoniae	-	7	5	12	-	2	5	7	**19**
	K. variicola	-	-	1	1	-	-	-	0	**1**
Morganella	*M. morganii*	-	9	1	10	-	6	-	6	**16**
Proteus	*P. mirabilis*	-	3	2	5	2	4	1	7	**12**
	Proteus group (P. hauseri, P. penneri,P. vulgaris)	-	5	-	5	1	6	-	7	**12**
Providencia	*P. alcalifaciens*	-	2	-	2	-	3	-	3	**5**
	P. rettgeri	-	-	-	0	-	1	-	1	**1**
Raoultella	*R. ornithinolytica*	-	-	-	0	-	-	1	1	**1**
	Total	**24**	**129**	**18**	**171**	**32**	**146**	**11**	**191**	**362**
Species isolated on MacConkey agar supplemented with 7 g/L GBF										
Genus	**Species**	**≤ 4**	**8**	**16**	**Total NE**	**≤ 4**	**8**	**16**	**Total E**	**Total**
Citrobacter	*C. freundii*	1	13	-	14	1	27	-	28	**42**
Enterobacter	*E. cloacae*	-	-	1	1	-	2	3	5	**6**
Escherichia	*E. coli*	2	35	3	40	8	35	2	45	**85**
Klebsiella	*K. aerogenes*	-	-	3	3	-	-	4	4	**7**
	K. oxytoca	-	-	-	0	-	1	1	2	**2**
	K. pneumoniae	-	5	15	20	1	6	10	17	**37**
	K. variicola	-	-	-	0	-	-	1	1	**1**
Proteus	*P. mirabilis*	-	-	-	0	-	3	2	5	**5**
	Total	**3**	**53**	22	**78**	**10**	**74**	**18**	**107**	**185**
Total species isolated in the two media		**27**	**182**	**35**	**249**	**42**	**220**	**29**	**298**	**547**

Table 2: P-values of Fisher's Exact Test calculated to assess the independence between the rate of resistance for each antibiotic and the degree of exposure (NE and E) for *K. pneumoniae*, *E. coli* and *C. freundii* strains.

Species			**AMX**	**AMC**	**TPZ**	**CRO**	**FEP**	**KF**	**ETP**	**IPM**	**CN**	**AK**	**CIP**	**SXT**	**FF**	**F**	**DO**	**CT**
Klebsiella pneumoniae	**NE (n = 32)**	Nb of R	-	0	0	0	0	0	0	0	0	0	3	0	32	6	1	1
		Frequency	-	0	0	0	0	0	0	0	0	0	0.09	0	1.00	0.19	0.03	0.03
(N = 56)	**E (n = 24)**	Nb of R	-	1	1	0	0	1	0	0	0	0	0	2	23	3	2	0
		Frequency	-	0.04	0.04	0	0	0.04	0	0	0	0	0	0.08	0.96	0.13	0.08	0
	Fisher's exact test	***p-value***	-	0.429	0.429	NA	NA	0.429	NA	NA	NA	NA	0.252	0.179	0.429	0.718	0.571	1
Escherichia coli	**NE (n = 150)**	Nb of R	68	18	0	1	1	10	0	0	3	0	5	31	1	9	32	0
(N = 313)		Frequency	0.45	0.12	0	0.01	0.01	0.07	0	0	0.02	0	0.03	0.21	0.01	0.06	0.21	0
	E (n = 163)	Nb of R	77	23	2	4	4	19	0	0	5	0	3	38	4	8	41	4
		Frequency	0.47	0.14	0.01	0.02	0.02	0.12	0	0	0.03	0	0.02	0.23	0.02	0.05	0.25	0.02
	Fisher's exact test	***p-value***	0.821	0.618	0.499	0.373	0.373	0.172	NA	NA	0.725	NA	0.487	0.588	0.373	0.804	0.504	0.1239
Citrobacter freundii	**NE (n = 23)**	Nb of R	-	-	0	0	0	17	0	0	0	0	0	0	2	1	0	0
(N = 72)		Frequency	-	-	0	0	0	0.74	0	0	0	0	0	0	0.09	0.04	0	0
	E (n = 49)	Nb of R	-	-	2	1	0	33	0	0	0	0	0	2	1	2	3	0
		Frequency	-	-	0.04	0.02	0	0.67	0	0	0	0	0	0.04	0.02	0.04	0.06	0
	Fisher's exact test	***p-value***	-	-	1	1	NA	0.784	NA	NA	NA	NA	NA	1	0.238	1	0.547	NA

Antibiotics : AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; TZP, piperacillin/tazobactam; CRO, ceftriaxone; FEP, cefepime; KF, cefalotine; ERT, ertapenem; IMP, imipenem; CN, gentamicin; AK, amikacin; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; FF, fosfomycin; F, nitrofurantoïne; DO, doxycycline and CT, colistin;

E : exposed ; NA: not available ; NE : Not exposed ; Nb of R: number of resistant strains.

Supplementary file 1:

Table S1: Glyphosate exposure categories based on acetyl cholinesterase enzyme (AChE) concentration levels in capillary blood samples.

Glyphosate exposure group	Glyphosate exposure category	AChE concentration (units / mL)
Not exposed	Normal	> 100
	Safe	87.5 - 100
Exposed	At risk	75 - 87.4
	Unsafe	< 75

Table S2: MCR- and ESBL-producing strains isolated in this study.

Species	Code	Village	Glyphosate status	GM	MIC-Gly (g/L)	ESBL phenotype	MIC-CT (mg/L)	AR genes
E. coli	1021	1	E (at risk)	McK-Gly	8	Yes	-	$bla_{CTX\text{-}M\text{-}A}$
E. coli	1042	1	E (at risk)	McK-Gly	8	No	4	*mcr-1*
E. coli	4103	4	E (at risk)	McK	16	No	4	*mcr-1*, *mcr-3*
E. coli	5062	5	E (unsafe)	McK-Gly	8	Yes	8	*mcr-1*, *mcr-3*, $bla_{CTX\text{-}M\text{-}A}$, bla_{TEM}
E. coli	5089	5	E (at risk)	McK-Gly	8	Yes	-	$bla_{CTX\text{-}M\text{-}A}$
E. coli	5111	5	E (at risk)	McK-Gly	8	No	8	*mcr-1*
K. pneumoniae	1022	1	NE (normal)	McK-Gly	16	No	4	-

GM: growth medium; MIC: Minimum Inhibitory Concentration; GBF: Glyphosate-based formulation; CT: colistin; AR: Antibiotic resistance; McK: MacConkey agar medium.

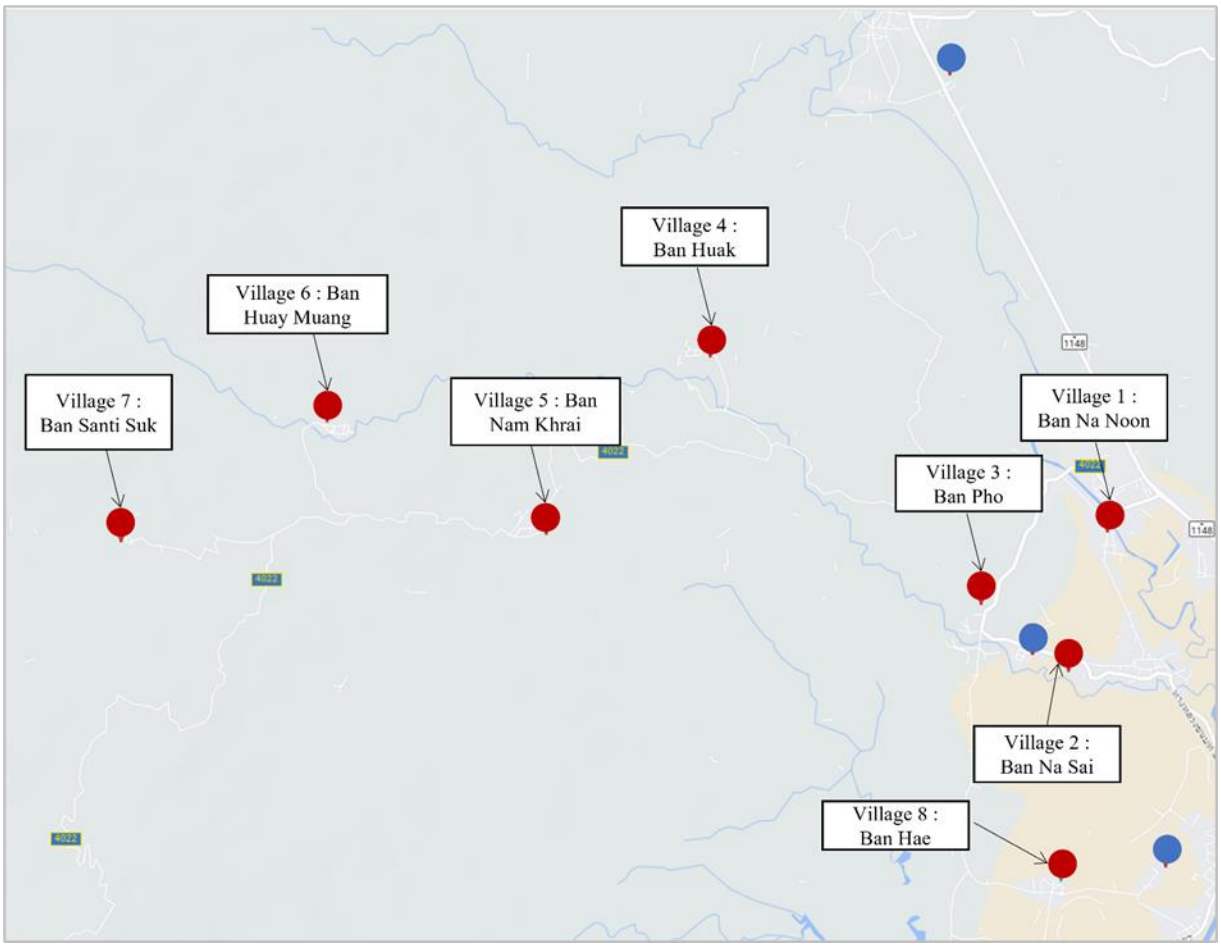
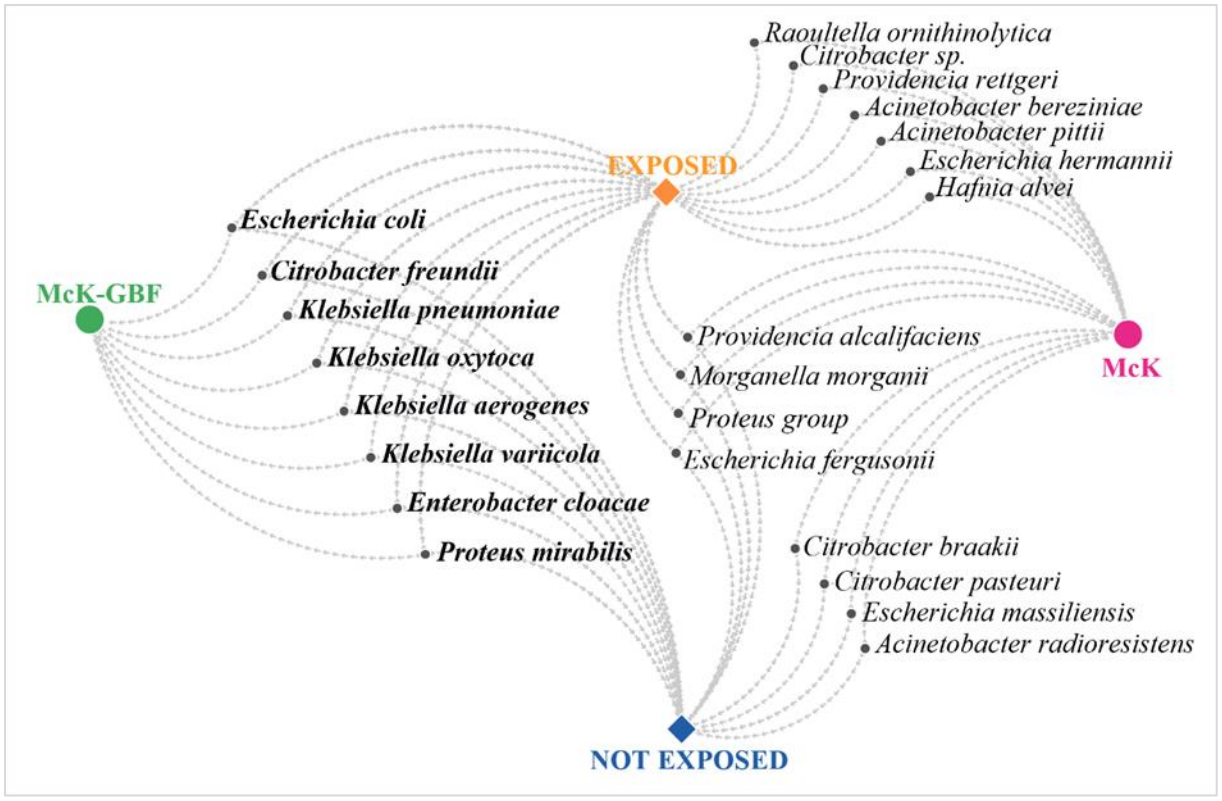


Figure S1: Distribution of sampling sites in Saenthong, Thawangpha, Nan Province, Thailand (Farm Resist Project); red circle: village; blue circle: farm using Moxlistin.



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Supplementary file 2:

Antibiotic resistance profiles of bacterial species isolated (on McK and McK + GBF) from farmers with different glyphosate exposure.

Non-fermenting GNB																				
Farmer code	**Glyphosate exposure**	**Growth media**	***Species***	**GBF-MIC (g/L)**	**TIC**	**TCC**	**TPZ**	**ATM**	**CAZ**	**FEP**	**MER**	**IPM**	**CN**	**AK**	**CIP**	**SXT**	**FF**	**RA**	**DO**	**CT**
1012-B	NE (Safe)	McK	*Acinetobacter radioresistens*	8	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
1028-C	E (At risk)	McK	*Acinetobacter pittii*	2	S	S	R	R	S	R	S	S	S	S	S	R	S	S	R	S
1110-E	E (At risk)	McK	*Acinetobacter bereziniae*	4	S	S	S	R	R	S	S	S	S	S	S	R	S	S	R	S
Enterobacterales																				
Farmer code	**Glyphosate exposure**	**Growth media**	***Species***	**GBF-MIC (g/L)**	**AMX**	**AMC**	**TPZ**	**CRO**	**FEP**	**KF**	**ETP**	**IPM**	**CN**	**AK**	**CIP**	**SXT**	**FF**	**F**	**DO**	**CT**
1003-A	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
1003-B	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1003-C	NE (Safe)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1003	NE (Safe)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
1005	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1005	NE (Safe)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1006-A	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1006-B	NE (Safe)	McK	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1006-C	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
1006-D	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
1006-E	NE (Safe)	McK	*Escherichia fergusonii*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
1006-A	NE (Safe)	McK + GBF	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1006-B	NE (Safe)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
1007-A	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1007-B	NE (Safe)	McK	*Klebsiella pneumoniae*	8	S	S	S	S	S	S	S	S	S	S	S	S	R	R	S	S
1007-C	NE (Safe)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S
1007-D	NE (Safe)	McK	*Citrobacter freundii*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1007-A	NE (Safe)	McK + GBF	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	R	S
1007-B	NE (Safe)	McK + GBF	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1012-A	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1012-C	NE (Safe)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	R	S	S	R	S	S	R	s
1012-A	NE (Safe)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
1012-B	NE (Safe)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1013-A	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
1013-B	NE (Safe)	McK	*Proteus penneri*	8	R	S	S	S	S	R	S	S	S	S	S	S	S	R	S	R
1013	NE (Safe)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1015-A	NE (Safe)	McK	*Citrobacter freundii*	4	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1015-B	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1015-C	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	R	S	S	S	S	S	R	S	S	R	s
1015-A	NE (Safe)	McK + GBF	*Citrobacter freundii*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1015-B	NE (Safe)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1018	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1018	NE (Safe)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1019-A	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
1019-B	NE (Safe)	McK	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	R	S	R	R	S	S
1019-A	NE (Safe)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	R	S	R	R	S	S
1019-B	NE (Safe)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	R	S	R	S	S	S
1022-A	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1022-B	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
1022-C	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	s
1022-A	NE (Safe)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S

1022-B	NE (Safe)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R
1022-C	NE (Safe)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1024	NE (Safe)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1025	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1025-A	NE (Safe)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
1025-B	NE (Safe)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
1026-A	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
1026-B	NE (Safe)	McK	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1026-C	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
1026-D	NE (Safe)	McK	*Citrobacter braakii*	4	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1026	NE (Safe)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	s
1029-A	NE (Safe)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
1029-B	NE (Safe)	McK	*Escherichia coli*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	R	R	S
1029-C	NE (Safe)	McK	*Proteus mirabilis*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R
2012-A	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2012-B	NE (Normal)	McK	*Citrobacter freundii*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2012-C	NE (Normal)	McK	*Escherichia coli*	8	R	R	S	R	S	R	S	S	S	S	R	S	S	S	S	S
2012-D	NE (Normal)	McK	*Citrobacter pasteurii*	16	R	R	S	S	S	R	S	S	S	S	S	S	R	S	S	S
2012-E	NE (Normal)	McK	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	R	S	S
2012-A	NE (Normal)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
2012-B	NE (Normal)	McK + GBF	*Klebsiella pneumoniae*	32	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
2012-C	NE (Normal)	McK + GBF	*Klebsiella pneumoniae*	32	R	S	S	S	S	S	S	S	S	S	S	S	R	R	S	S
3001-A	NE (Normal)	McK	*Klebsiella variicola*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
3001-B	NE (Normal)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
3001-C	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3001	NE (Normal)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
4012-A	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4012-B	NE (Normal)	McK	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
4012-C	NE (Normal)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	R	S	S	R	S
4012-D	NE (Normal)	McK	*Citrobacter freundii*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4012-A	NE (Normal)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4012-B	NE (Normal)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
4140-A	NE (Normal)	McK	*Enterobacter cloacae*	16	R	R	S	S	S	R	S	S	S	S	S	S	R	S	S	S
4140-B	NE (Normal)	McK	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	R	S	S
4140-C	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4140	NE (Normal)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
6010-A	NE (Normal)	McK	*Citrobacter braakii*	4	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
6010-B	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6010-C	NE (Normal)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6010-D	NE (Normal)	McK	*Klebsiella aerogenes*	16	R	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S
6010-E	NE (Normal)	McK	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
6010-F	NE (Normal)	McK	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6010	NE (Normal)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
7004-A	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7004-B	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
7004-C	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
7004-D	NE (Normal)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	R	S	S	S	s
7004	NE (Normal)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
7023-A	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
7023-B	NE (Normal)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
7023-C	NE (Normal)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	s
7023-D	NE (Normal)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7023-E	NE (Normal)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7023-F	NE (Normal)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
7023	NE (Normal)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S

5026-A	NE (Normal)	McK	*Escherichia massiliensis*	8	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S
5026-B	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
5026-C	NE (Normal)	McK	*Escherichia coli*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
5026-D	NE (Normal)	McK	*Morganella morganii*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	R	S	R
5026	NE (Normal)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	S	S	S
7022-A	NE (Normal)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7022-B	NE (Normal)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	R	S	S	S	S	R	S	S
7022-C	NE (Normal)	McK	*Providencia alcalifaciens*	8	S	R	S	S	S	S	S	S	S	S	S	S	S	R	R	R
7022-D	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
7022-A	NE (Normal)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
7022-B	NE (Normal)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8003-A	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8003-B	NE (Normal)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	R	S	R	S	s
8003-C	NE (Normal)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	R	S	S	S	S
8003-D	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8003	NE (Normal)	McK + GBF	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	R	S	S	S	S
8014	NE (Normal)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
8014	NE (Normal)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
1051	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1051	NE (Normal)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1056-A	NE (Safe)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1056-B	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1056-C	NE (Safe)	McK	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1056-A	NE (Safe)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1056-B	NE (Safe)	McK + GBF	*Enterobacter cloacae*	16	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
2002-A	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2002-B	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2002-C	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2002	NE (Safe)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2015-A	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
2015-B	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2015-C	NE (Safe)	McK	*Klebsiella aerogenes*	16	R	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S
2015-A	NE (Safe)	McK + GBF	*Klebsiella aerogenes*	32	R	R	S	S	S	R	S	S	S	S	S	S	R	S	S	S
2015-B	NE (Safe)	McK + GBF	*Klebsiella aerogenes*	16	R	R	S	S	S	R	S	S	S	S	S	S	R	S	S	S
3006-A	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3006-B	NE (Safe)	McK	*Morganella morganii*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	R	R	R
3006-C	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3006-A	NE (Safe)	McK + GBF	*Escherichia coli*	16	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3006-B	NE (Safe)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
3002-A	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3002-B	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3002-C	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
3002-D	NE (Safe)	McK	*Enterobacter cloacae*	16	R	R	S	S	S	R	S	S	S	S	S	S	S	R	S	S
3002-A	NE (Safe)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3002-B	NE (Safe)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3002-C	NE (Safe)	McK + GBF	*Klebsiella pneumoniae*	32	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
4004-A	NE (Safe)	McK	*Escherichia massiliensis*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4004-B	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4004-C	NE (Safe)	McK	*Klebsiella aerogenes*	32	R	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S
4004-D	NE (Safe)	McK	*Klebsiella oxytoca*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
4004-E	NE (Safe)	McK	*Enterobacter cloacae*	16	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
4004-A	NE (Safe)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4004-B	NE (Safe)	McK + GBF	*Escherichia coli*	16	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4004-C	NE (Safe)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
5016-A	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

5016-B	NE (Safe)	McK	*Proteus vulgaris*	8	R	S	S	S	S	R	S	S	S	S	S	S	S	R	S	R
5016-A	NE (Safe)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
5016-B	NE (Safe)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5016-C	NE (Safe)	McK + GBF	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S
6001-A	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
6001-B	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6001-C	NE (Safe)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6001-D	NE (Safe)	McK	*Citrobacter freundii*	4	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
6001-A	NE (Safe)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
6006-A	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6006-B	NE (Safe)	McK	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
6006-C	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
6006-D	NE (Safe)	McK	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
6006-E	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6006-A	NE (Safe)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6006-B	NE (Safe)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
7001-A	NE (Safe)	McK	*Providencia alcalifaciens*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	R	R	R
7001-B	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7001-C	NE (Safe)	McK	*Klebsiella oxytoca*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
7001-D	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	R	R	S	S	R	S
7001-E	NE (Safe)	McK	*Morganella morganii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	R	R
7001-F	NE (Safe)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7001-G	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
7001-A	NE (Safe)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
7019-A	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7019-B	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	R	R	S	S	R	S
7019-C	NE (Safe)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7019-D	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
7019-E	NE (Safe)	McK	*Enterobacter cloacae*	16	R	R	S	S	S	R	S	S	S	S	S	S	S	R	S	S
7019-F	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7019-A	NE (Safe)	McK + GBF	*Escherichia coli*	16	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
7019-B	NE (Safe)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
7019-C	NE (Safe)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7020-A	NE (Safe)	McK	*Morganella morganii*	8	R	R	S	S	S	R	S	R	S	S	S	S	R	R	S	R
7020-B	NE (Safe)	McK	*Klebsiella oxytoca*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7020-C	NE (Safe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
7020-D	NE (Safe)	McK	*Citrobacter freundii*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7020-E	NE (Safe)	McK	*Morganella morganii*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	R	S	R
7020-A	NE (Safe)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7020-B	NE (Safe)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8002-A	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8002-B	NE (Safe)	McK	*Escherichia coli*	8	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S
8002-A	NE (Safe)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	R	S	S	S	S	S	S	S	S	R	S
8002-B	NE (Safe)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1057-A	NE (Normal)	McK	*Proteus mirabilis*	16	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R
1057-B	NE (Normal)	McK	*Morganella morganii*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	R	S	R
1057-C	NE (Normal)	McK	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1057	NE (Normal)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	R	S	S	S	S	S	R	S	R	R	s
1069-A	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1069-B	NE (Normal)	McK	*Proteus vulgaris*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	R	S	R
1069-C	NE (Normal)	McK	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	R	S	S
1069	NE (Normal)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1071-A	NE (Normal)	McK	*Escherichia coli*	8	R	S	S	S	S	R	S	S	S	S	S	S	S	S	R	s
1071-B	NE (Normal)	McK	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1071-C	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

1071-D	NE (Normal)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	R	S	R	R	S	S	R	S
1071-E	NE (Normal)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
1071-A	NE (Normal)	McK + GBF	*Escherichia coli*	8	S	S	S	S	R	S	S	S	S	S	R	S	S	S	S	S
1071-B	NE (Normal)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1071-C	NE (Normal)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1072-A	NE (Normal)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1072-B	NE (Normal)	McK	*Proteus penneri*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	R	S	R
1072-C	NE (Normal)	McK	*Proteus mirabilis*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R
1074	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1074-A	NE (Normal)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
1074-B	NE (Normal)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1122-A	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
1122-B	NE (Normal)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1122-C	NE (Normal)	McK	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	s
1122-A	NE (Normal)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1122-B	NE (Normal)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1122-C	NE (Normal)	McK + GBF	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1152	NE (Normal)	McK	*Proteus mirabilis*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R
1152	NE (Normal)	McK + GBF	*Citrobacter freundii*	4	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1154-A	NE (Normal)	McK	*Morganella morganii*	16	R	R	S	S	S	R	S	S	S	S	S	S	R	R	R	R
1154-B	NE (Normal)	McK	*Proteus mirabilis*	16	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R
1154-C	NE (Normal)	McK	*Escherichia coli*	16	R	R	S	S	S	R	S	S	S	S	S	S	S	R	S	S
1164-A	NE (Normal)	McK	*Morganella morganii*	8	R	R	S	S	S	R	S	S	R	S	S	S	R	R	R	R
1164-B	NE (Normal)	McK	*Morganella morganii*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	R	S	R
1164-C	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1063-A	NE (Normal)	McK	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	R	S	R	S	S
1063-B	NE (Normal)	McK	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
1063-C	NE (Normal)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1063-D	NE (Normal)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S
1063-E	NE (Normal)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1063-F	NE (Normal)	McK	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1063-A	NE (Normal)	McK + GBF	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1063-B	NE (Normal)	McK + GBF	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1079-A	NE (Normal)	McK	*Klebsiella pneumoniae*	8	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1079-B	NE (Normal)	McK	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1079-C	NE (Normal)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1079-D	NE (Normal)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1079-A	NE (Normal)	McK + GBF	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1079-B	NE (Normal)	McK + GBF	*Klebsiella aerogenes*	16	R	R	S	S	S	R	S	S	S	S	S	S	S	R	S	S
1199-A	NE (Normal)	McK	*Proteus vulgaris*	8	R	S	S	S	S	R	S	S	S	S	S	S	S	R	S	R
1199-B	NE (Normal)	McK	*Morganella morganii*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	R	R	S
1199-C	NE (Normal)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
1009-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1009-B	E (At risk)	McK	*Citrobacter freundii*	4	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1009-C	E (At risk)	McK	*Proteus mirabilis*	4	R	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R
1009	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1017-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
1017-B	E (At risk)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	R	R	S
1017-C	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1017	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1021-A	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
1021-B	E (At risk)	McK	*Citrobacter freundii*	4	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1021-A	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1021-B	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1021-C	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	R	S	S	S	S

1021-D	E (At risk)	McK + GBF	*Escherichia coli*	8	R	R	S	R	R	R	S	S	S	S	S	S	S	S	S	S
1023-A	E (At risk)	McK	*Escherichia coli*	4	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1023-B	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1023-C	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
1023-A	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	4	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1023-B	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1027	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1027-A	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	s
1027-B	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1028-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1028-B	E (At risk)	McK	*Citrobacter freundii*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1028-D	E (At risk)	McK	*Enterobacter cloacae*	8	S	S	S	S	S	R	S	S	S	S	S	S	R	S	S	S
1028	E (At risk)	McK + GBF	*Citrobacter freundii*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2025-A	E (At risk)	McK	*Enterobacter cloacae*	16	R	R	S	S	S	R	S	S	S	S	S	S	R	S	S	S
2025-B	E (At risk)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2025-C	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
2025	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
2093-A	E (Unsafe)	McK	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
2093-B	E (Unsafe)	McK	*Klebsiella aerogenes*	16	R	R	S	S	S	R	S	S	S	S	S	S	R	S	S	S
2093-C	E (Unsafe)	McK	*Escherichia coli*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	s
2093-D	E (Unsafe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
2093-A	E (Unsafe)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2093-B	E (Unsafe)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
2093-C	E (Unsafe)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
3039-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3039-B	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
3039-C	E (At risk)	McK	*Proteus mirabilis*	4	R	S	S	S	S	R	S	S	S	S	S	S	S	R	S	R
3039-A	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S
3039-B	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	R	S
4075-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4075-B	E (At risk)	McK	*Hafnia alvei*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4075-C	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4075-A	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
4075-B	E (At risk)	McK + GBF	*Klebsiella aerogenes*	32	R	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S
4078-A	E (Unsafe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4078-B	E (Unsafe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
4078-C	E (Unsafe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
5062-A	E (Unsafe)	McK	*Escherichia coli*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	s
5062-B	E (Unsafe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
5062-C	E (Unsafe)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	R	S	R	S	S	S	S	S
5062-D	E (Unsafe)	McK	*Klebsiella pneumoniae*	16	R	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S
5062-E	E (Unsafe)	McK	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
5062-F	E (Unsafe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5062-G	E (Unsafe)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
5062-H	E (Unsafe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5062	E (Unsafe)	McK + GBF	*Escherichia coli*	8	R	R	S	R	R	R	S	S	R	S	S	R	S	S	R	R
6018-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6018-B	E (At risk)	McK	*Citrobacter freundii*	16	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
6018-C	E (At risk)	McK	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
6018-D	E (At risk)	McK	*Escherichia coli*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
6018	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
6024	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7010-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7010-B	E (At risk)	McK	*Klebsiella oxytoca*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
7010	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S

5015-A	E (At risk)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5015-B	E (At risk)	McK	*Citrobacter freundii*	4	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5015-C	E (At risk)	McK	*Morganella morganii*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	R	R	S
5015	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
5041-A	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
5041-B	E (At risk)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	R	R	S
5041-C	E (At risk)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	R	S	S	S	S	R	S	S
5041-D	E (At risk)	McK	*Escherichia coli*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	R	S
5041-E	E (At risk)	McK	*Proteus hauseri*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	R	S	R
5041-A	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
5041-B	E (At risk)	McK + GBF	*Proteus mirabilis*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R
5105-A	E (Unsafe)	McK	*Escherichia coli*	16	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	s
5105-B	E (Unsafe)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5105-C	E (Unsafe)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5105-A	E (Unsafe)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
5105-B	E (Unsafe)	McK + GBF	*Proteus mirabilis*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R
7077-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
7077-B	E (At risk)	McK	*Escherichia coli*	8	R	R	R	S	S	R	S	S	S	S	R	R	S	S	S	S
7050	E (At risk)	McK	*Proteus mirabilis*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R
7050	E (At risk)	McK + GBF	*Proteus mirabilis*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R
8009-A	E (At risk)	McK	*Proteus hauseri*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	R	S	R
8009-B	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s
8009-C	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
8009	E (At risk)	McK + GBF	*Escherichia coli*	8	R	R	R	S	S	R	S	S	S	S	S	S	S	S	R	s
8039-A	E (At risk)	McK	*Citrobacter freundii*	8	R	R	R	S	S	R	S	S	S	S	S	S	S	S	S	S
8039-B	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8039-C	E (At risk)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8039-D	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8039-A	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
8039-B	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8093-A	E (Unsafe)	McK	*Escherichia coli*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	s
8093-B	E (Unsafe)	McK	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
8093-C	E (Unsafe)	McK	*Escherichia coli*	8	S	R	S	S	S	R	S	S	S	S	S	S	S	S	S	s
8093-D	E (Unsafe)	McK	*Citrobacter freundii*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8093	E (Unsafe)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1044-A	E (At risk)	McK	*Citrobacter freundii*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1044-B	E (At risk)	McK	*Klebsiella oxytoca*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1044-C	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1044-D	E (At risk)	McK	*Enterobacter cloacae*	16	R	R	S	S	S	R	S	S	S	S	S	S	R	S	S	S
1044-E	E (At risk)	McK	*Hafnia alvei*	4	S	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1044-A	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1044-B	E (At risk)	McK + GBF	*Enterobacter cloacae*	16	R	R	S	S	S	R	S	S	S	S	S	S	R	R	S	S
1044-C	E (At risk)	McK + GBF	*Klebsiella oxytoca*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1067-A	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
1067-B	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1067-C	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1067	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2024	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2024	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2007-A	E (At risk)	McK	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2007-B	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
2007-C	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
2007-D	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3129-A	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
3129-B	E (At risk)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	R	S	S	R	S

3129-C	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3129-D	E (At risk)	McK	*Proteus hauseri*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
3129	E (At risk)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	R	S	S	S	S	S	S	R	R	S	s
4103-A	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	R	S	S	R	S	S	S	R
4103-B	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	R	S	S	S	S	S	S	R	R	S	s
4103	E (At risk)	McK + GBF	*Proteus mirabilis*	16	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R
4105-A	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4105-B	E (At risk)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	R	S	S	S	S
4105-C	E (At risk)	McK	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
4105	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
5086-A	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5086-B	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
5086-C	E (At risk)	McK	*Proteus vulgaris*	4	R	S	S	R	S	R	S	S	S	S	S	S	R	R	S	R
5086-D	E (At risk)	McK	*Escherichia fergusonii*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
5086-A	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
5086-B	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5086-C	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
5089-A	E (At risk)	McK	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
5089-B	E (At risk)	McK	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
5089-C	E (At risk)	McK	*Raoultella ornithinolytica*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
5089-D	E (At risk)	McK	*Citrobacter freundii*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5089-A	E (At risk)	McK + GBF	*Klebsiella oxytoca*	32	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
5089-B	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
5089-C	E (At risk)	McK + GBF	*Escherichia coli*	8	R	R	S	R	R	R	S	S	S	S	S	R	S	S	S	S
5089-D	E (At risk)	McK + GBF	*Citrobacter freundii*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5109-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5109-B	E (At risk)	McK	*Klebsiella aerogenes*	32	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
5109-C	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
5109-D	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
5109-F	E (At risk)	McK	*Escherichia fergusonii*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
5109-A	E (At risk)	McK + GBF	*Klebsiella aerogenes*	32	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
5109-B	E (At risk)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5109-C	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
5109-D	E (At risk)	McK + GBF	*Klebsiella aerogenes*	32	R	R	S	S	S	R	S	S	S	S	S	S	R	S	R	S
5111-A	E (At risk)	McK	*Citrobacter freundii*	4	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
5111-B	E (At risk)	McK	*Morganella morganii*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	R	S	R
5111-C	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
5111-D	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5111-A	E (At risk)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
5111-B	E (At risk)	McK + GBF	*Escherichia coli*	16	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	R
5111-C	E (At risk)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
6003-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6003-B	E (At risk)	McK	*Morganella morganii*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	R	S	R
6003-C	E (At risk)	McK	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
6003-A	E (At risk)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
6003-B	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
6003-C	E (At risk)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
6030-A	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
6030-B	E (At risk)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6030-C	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
6030-D	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6030-E	E (At risk)	McK	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6030	E (At risk)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
6033-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6033-B	E (At risk)	McK	*Providencia alcalifaciens*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	R	R	R

6033-C	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
6033-D	E (At risk)	McK	*Proteus hauseri*	8	R	R	S	S	S	R	S	S	S	S	S	R	R	R	R	R
6033-A	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
6033-B	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6034-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6034-B	E (At risk)	McK	*Providencia alcalifaciens*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	R	R	S
6034-C	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
6034-D	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
6034-E	E (At risk)	McK	*Klebsiella oxytoca*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6034-A	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
6034-B	E (At risk)	McK + GBF	*Escherichia coli*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6044-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6044-B	E (At risk)	McK	*Providencia alcalifaciens*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	R	R	R
6044-C	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
6044-D	E (At risk)	McK	*Proteus hauseri*	8	R	S	S	S	S	R	S	S	S	S	R	R	R	R	R	R
6044-A	E (At risk)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
6044-B	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
6044-C	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
8016-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8016-B	E (At risk)	McK	*Proteus penneri*	8	R	S	S	S	S	R	S	S	S	S	S	S	S	R	S	R
8066-A	E (At risk)	McK	*Citrobacter sp*	8	S	S	S	S	S	S	S	S	S	S	S	R	S	R	R	S
8066-B	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8066-C	E (At risk)	McK	*Klebsiella oxytoca*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
8066-D	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8077-A	E (At risk)	McK	*Klebsiella oxytoca*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
8077-B	E (At risk)	McK	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
8077-C	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8077-D	E (At risk)	McK	*Proteus mirabilis*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R
8077-A	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
8077-B	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8077-C	E (At risk)	McK + GBF	*Proteus mirabilis*	16	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R
8078-A	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8078-B	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
8078-C	E (At risk)	McK	*Klebsiella pneumoniae*	32	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
8078-D	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8078-A	E (At risk)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8078-B	E (At risk)	McK + GBF	*Escherichia coli*	16	R	R	S	S	S	S	S	S	S	S	S	R	S	S	R	S
8078-C	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	32	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
8084-A	E (At risk)	McK	*Providencia rettgeri*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R
8084-B	E (At risk)	McK	*Morganella morganii*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	R	R	R
8084-C	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
8084-D	E (At risk)	McK	*Citrobacter freundii*	4	S	S	S	S	S	S	S	S	S	S	S	R	S	R	R	S
8084-E	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8084-A	E (At risk)	McK + GBF	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8084-B	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8084-C	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1042-A	E (At risk)	McK	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1042-B	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1042-C	E (At risk)	McK	*Citrobacter freundii*	4	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1042-D	E (At risk)	McK	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
1042-E	E (At risk)	McK	*Escherichia coli*	4	R	R	S	S	S	S	S	S	S	S	S	R	S	S	R	S
1042-A	E (At risk)	McK + GBF	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	R
1042-B	E (At risk)	McK + GBF	*Klebsiella aerogenes*	16	R	R	S	S	S	R	S	S	S	S	S	S	R	R	S	S
1076-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1076-A	E (At risk)	McK + GBF	*Enterobacter cloacae*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	R	S	S

1076-B	E (At risk)	McK + GBF	*Enterobacter cloacae*	16	R	R	S	S	S	R	S	S	S	S	S	S	R	R	S	S
1084-A	E (At risk)	McK	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1084-B	E (At risk)	McK	*Proteus mirabilis*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R
1084	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	R	S	S
1086-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
1086-B	E (At risk)	McK	*Proteus mirabilis*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	R	S	R
1086-C	E (At risk)	McK	*Proteus mirabilis*	16	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R
1086-A	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1086-B	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1110-A	E (At risk)	McK	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1110-B	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1110-C	E (At risk)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1110-D	E (At risk)	McK	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	R	R	S
1110-A	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1110-B	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	R	R	R	R	S
1110-C	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	R	S	S
1112	E (At risk)	McK	*Morganella morganii*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	R	S	R
1125-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1125-B	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1125-C	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	R	R	S
1125-D	E (At risk)	McK	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
1125-A	E (At risk)	McK + GBF	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
1125-B	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1139	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1165-A	E (At risk)	McK	*Morganella morganii*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	R	S	R
1165-B	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
1131-A	E (At risk)	McK	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	R	R	S	S	R	S
1131-B	E (At risk)	McK	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1131-A	E (At risk)	McK + GBF	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
1131-B	E (At risk)	McK + GBF	*Escherichia coli*	4	R	R	S	S	S	R	S	S	S	S	S	R	S	S	R	S
1131-C	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	16	R	R	S	S	S	R	S	S	S	S	S	S	R	S	S	S
1131-D	E (At risk)	McK + GBF	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
1133-A	E (At risk)	McK	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S
1133-B	E (At risk)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1133-A	E (At risk)	McK + GBF	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1133-B	E (At risk)	McK + GBF	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1133-C	E (At risk)	McK + GBF	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1135	E (At risk)	McK	*Escherichia coli*	8	S	S	S	R	R	S	S	S	S	S	S	S	R	S	S	S
1174-A	E (At risk)	McK	*Citrobacter freundii*	4	S	S	R	R	S	S	S	S	S	S	S	S	R	S	S	S
1174-B	E (At risk)	McK	*Escherichia hermannii*	4	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
1174-C	E (At risk)	McK	*Citrobacter freundii*	4	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1174-A	E (At risk)	McK + GBF	*Citrobacter freundii*	4	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1174-B	E (At risk)	McK + GBF	*Enterobacter cloacae*	8	R	R	S	S	S	R	S	S	S	S	S	S	R	S	S	S
1182-A	E (At risk)	McK	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1182-B	E (At risk)	McK	*Escherichia coli*	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1182-C	E (At risk)	McK	*Escherichia coli*	4	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
1182-A	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1182-B	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1182-C	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1182-D	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1182-E	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S	S
1196-A	E (At risk)	McK	*Citrobacter freundii*	8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1196-B	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1196-A	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1196-B	E (At risk)	McK + GBF	*Klebsiella variicola*	16	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

1196-C	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1196-D	E (At risk)	McK + GBF	*Citrobacter freundii*	8	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S
1208-A	E (At risk)	McK	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1208-B	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	R	S	S	R	S	S	R	S
1208-C	E (At risk)	McK	*Escherichia coli*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1208-A	E (At risk)	McK + GBF	*Enterobacter cloacae*	16	R	R	S	S	S	R	S	S	S	S	S	S	S	R	S	S
1208-B	E (At risk)	McK + GBF	*Klebsiella pneumoniae*	8	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
1208-C	E (At risk)	McK + GBF	*Escherichia coli*	8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

GNB: Gram-Negative Bacteria, E: Exposed, NE: Not Exposed, McK: MacConkey, GBF: Glyphosate-based formulation, MIC: Minimum Inhibitory Concentration R: resistant, S: susceptible.

Antibiotic panel used for Enterobacterales: AMX: Amoxicillin, AMC: Amoxicillin/clavulanic acid, TPZ: Piperacillin/tazobactam, CRO: Ceftriaxone, FEP: Cefepime, KF: Cefalotine, ETP: Ertapenem, IPM: Imipenem, CN: Gentamicin , AK: Amikacin, CIP: Ciprofloxacin , SXT: Trimethoprim/sulfamethoxazole, FF: Fosfomycin , F: Nitrofurantoïne, DO: Doxycycline, CT: Colistin. **Antibiotic panel used for non-fermenting GNB**: TIC: Ticarcillin, TCC: Ticarcillin/ clavulanic acid, TPZ: Piperacillin/tazobactam, ATM: Aztreonam, CAZ: Ceftazidime, FEP: Cefepime, MER: Meropeneme, IPM: Imipenem, CN: Gentamicin, AK: Amikacin, CIP: Ciprofloxacin, SXT: Trimethoprim/sulfamethoxazole, FF: Fosfomycin, RA: Rifampicin, DO: Doxycycline, CT: Colistin

Article 5

The effect of combinations of glyphosate-based herbicide with various clinically used antibiotics on phenotypic traits of Gram-negative species from the ESKAPEE group

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Résumé

L'utilisation massive de glyphosate et des herbicides à base de glyphosate (HBG), combinée à leur persistance dans l'environnement, soulève des questions quant à la résistance croisée avec des antibiotiques utilisés en médecine humaine et vétérinaire, étant donné que le glyphosate présente des propriétés antimicrobiennes. L'objectif de cette étude était d'évaluer l'impact d'un HBG en combinaison avec des antibiotiques courants sur des bactéries Gram-négatives du groupe ESKAPEE. Dix souches, incluant des souches de référence et des souches multirésistantes, ainsi que huit antibiotiques (céfotaxime, méropénème, aztréonam, ciprofloxacine, gentamicine, tigécycline, sulfaméthoxazole-triméthoprime et colistine) ont été testées en présence et en absence de HBG. En parallèle, l'effet du HBG en combinaison avec trois carbapénèmes (imipénem, ertapénem et méropénem) sur 14 souches résistantes aux carbapénèmes a été examiné pour évaluer son impact sur l'activité des carbapénémases. Les résultats ont montré que la plupart des combinaisons présentent des effets additifs ou indifférents (70 associations). Des effets antagonistes ont été observés avec la ciprofloxacine et la gentamicine chez la souche multirésistante d'*A. baumannii*, uniquement avec la ciprofloxacine chez les deux souches testées d'*E. coli*, et uniquement avec la gentamicine chez la souche de *K. pneumoniae* CIP 82.91. Cela suggère que le glyphosate pourrait interagir avec les pompes à efflux, limitant l'accumulation intracellulaire de ces antibiotiques et réduisant leur efficacité. En revanche, le HBG a réduit la CMI de la colistine chez huit souches, ce qui a conduit à étudier son effet sur la perméabilité de la membrane externe. Pour cela, les BGN ont été exposées à une combinaison de HBG et de vancomycine. Bien que la vancomycine soit un antibiotique efficace contre les bactéries à Gram positif (BGP), elle ne peut pas traverser la membrane externe des BGN en raison de son poids

moléculaire élevé. L'inhibition complète de la croissance de ces BGN testées après l'exposition à la combinaison de HBG et de vancomycine a validé l'hypothèse selon laquelle le HBG agit sur la perméabilité de la membrane externe. En plus, une synergie avec les carbapénèmes contre les souches productrices de métallo-β-lactamases (MBL) a été observée. Le HBG a également inhibé l'activité des MBL, en séquestrant les ions métalliques indispensables à l'activité de cet enzyme Cet effet serait dû à l'activité chélatrice du glyphosate. Ces résultats suggèrent que l'effet chélateur du glyphosate pourrait avoir des effets multiples sur les cellules bactériennes, potentiellement en augmentant la perméabilité de la membrane externe et en inhibant l'activité des métalloenzymes.

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The effect of combinations of a glyphosate-based herbicide with various clinically used antibiotics on phenotypic traits of Gram-negative species from the ESKAPEE group

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The emission of glyphosate and antibiotic residues from human activities threatens the diversity and functioning of the microbial community. This study examines the impact of a glyphosate-based herbicide (GBH) and common antibiotics on Gram-negative bacteria within the ESKAPEE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp. and *Escherichia coli*). Ten strains, including type and multidrug-resistant strains for each species were analysed and eight antibiotics (cefotaxime, meropenem, aztreonam, ciprofloxacin, gentamicin, tigecycline, sulfamethoxazole-trimethoprim, and colistin) were combined with the GBH. While most combinations yielded additive or indifferent effects in 70 associations, antagonistic effects were observed with ciprofloxacin and gentamicin in five strains. GBH notably decreased the minimum inhibitory concentration of colistin in eight strains and displayed synergistic activity with meropenem against metallo-β-lactamase (MBL)-producing strains. Investigation into the effect of GBH properties on outer membrane permeability involved exposing strains to a combination of this GBH and vancomycin. Results indicated that GBH rendered strains sensitive to vancomycin, which is typically ineffective against Gram-negative bacteria. Furthermore, we examined the impact of GBH in combination with three carbapenem agents on 14 strains exhibiting varying carbapenem-resistance mechanisms to assess its effect on carbapenemase activity. The GBH efficiently inhibited MBL activity, demonstrating similar effects to EDTA (ethylenediaminetetraacetic acid). Chelating effect of GBH may have multifaceted impacts on bacterial cells, potentially by increasing outer membrane permeability and inactivating metalloenzyme activity.

Keywords Antibiotics, Carbapenem, ESKAPEE group, Glyphosate-based herbicide, Gram-negative bacteria

Glyphosate is a broad-spectrum weed-killing active molecule and the world's most widely used herbicide[1]. Its annual use in recent years has been estimated to be between 600 thousand 750 thousand tonnes, with predictions that this could increase to 920 thousand tonnes by 2025[2]. Glyphosate-based herbicide (GBH) is commonly used to control weeds in agricultural fields and urban areas such as industrial sites, public parks, and infrastructures[3]. Glyphosate and the primary product of its degradation, aminomethylphosphonic acid (AMPA), have been detected in soil, water (including surface water, groundwater, and drinking water)[4], foodstuffs[5], milk, urine

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and faeces[6]. Glyphosate functions as a herbicide by targeting the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, crucial in the shikimate pathway responsible for synthesising aromatic amino acids in plants. This target is also found in microorganisms, imparting additional antimicrobial properties to glyphosate (Patent No.: US 7,771,736, 2010)[7,8]. Moreover, aside from its antimicrobial attributes, glyphosate, which was initially patented for its chelating properties (Patent No.: US 3,160,632, 1964), can form complexes with various metal ions such as Cu[9], Cd[10], Zn[11], and Mn[12] upon contact with soil and water. This impacts the availability of essential metals to plants and microorganisms, potentially altering their metabolic activity[13].

Consequently, glyphosate poses a potential threat to microbial biodiversity, as evidenced by previous studies examining the composition of species in the rhizosphere[14], zooplankton[15], as well as dysbiosis observed in the intestinal microbiota of bees[16], earthworms[17], and rats[18].

Microorganisms are pivotal in preserving biodiversity, undertaking essential physiological and metabolic functions crucial for the integrity of the environment and host organisms[19,20].

Moreover, the co-occurrence of other antimicrobial substances exacerbates the health and ecological risks posed by glyphosate[21].

Peri-urban regions serve as significant reservoirs of antibiotics, primarily sourced from industrial and municipal waste, along with agricultural activities such as animal feed supplementation, organic fertilisation employing cattle manure, and wastewater irrigation[22,23].

The amendment of organic fertilizers and manures to increase the productivity of agriculture is a common practice, but the presence of antibiotic residues potentially boosts antibiotic resistance in agricultural soils[24]. Several research studies have consistently found antibiotic residues and multidrug-resistant strains in agricultural soils. These include antibiotics from various clinically relevant classes such as tetracyclines, sulphonamides, ciprofloxacin, and carbapenems[22,23,25–27].

The use of GBHs and antibiotics together in farming and peri-urban areas poses a serious threat to the environment. Their accumulation can harm the diversity and functions of microbial communities, ultimately impacting agricultural productivity as well as human and animal health[28,29]. Recently, emphasis has been placed on the link between glyphosate exposure and the emerging antibiotic resistance in enteric and environmental opportunistic bacteria, particularly in bacterioplankton and soil communities[30,31], as well as *Pseudomonas aeruginosa*[32], *Escherichia coli,* and *Salmonella* spp. strains[33–35]. Furthermore, it was reported that glyphosate exposure increases cell membrane permeability, which can lead to increased horizontal transfer of multidrug-resistant plasmids[36,37]. The accumulation of glyphosate and antibiotics is a pressing issue that threatens environmental safety and global health. Despite this, only a few studies have examined their combined impact on opportunistic pathogenic microorganisms, particularly bacteria of the ESKAPEE group (*Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, *Enterobacter* spp., and *E. coli*). We focused on Gram-negative bacteria (GNB) from this group (*A. baumannii*, *P. aeruginosa,* and Enterobacteriaceae) that are highly resistant to several antibiotics and which have been classified as top priority (priority 1: critical) by the WHO, requiring new control agents or emergency measures to manage their spread[38]. Additionally, these Gram-negative opportunistic pathogen species are omnipresent in soil, water, and human/animal microbiota and are known to cause severe infections and outbreaks, often resulting in high mortality rates[39].

Our primary hypothesis is that exposure to GBHs could induce modifications in the antibiotic susceptibility levels of clinically significant bacterial strains, such as GNB in the ESKAPEE group. To investigate this hypothesis further, we examined the impact of the combination of a GBH and commonly used medical and veterinary antibiotics on GNB in the ESKAPEE group, including both susceptible and MDR strains. Subsequently, based on our observations, we investigated the effect of a GBH on outer membrane permeability and metallo-β-lactamase (MBL) activity.

Results

Minimum inhibitory concentration assay

All strains tested in this study and their antibiotic susceptibility profiles are described in Table 1 (details of antibiotics susceptibilities are available in Supplementary Table 1). The minimum inhibitory concentration (MIC) of the GBH was closely distributed among strains within the same species, but there was a clear difference between the various species tested (MIC values ranged from 2 g/L to 10 g/L, corresponding to 1.5 g/L to 7.5 g/L of glyphosate acid) (Supplementary Table S2). Most strains, including both type and MDR strains of *K. pneumoniae* and *E. coli* and the *E. cloacae* type strain, showed growth inhibition at a concentration of 4 g/L to 6 g/L of GBH (corresponding to 3 g/L to 4.5 g/L of glyphosate acid). The highest GBH-MICs (8 g/L to 10 g/L, equivalent to a glyphosate acid concentration of 6 g/L to 7.5 g/L) were observed in both *P. aeruginosa* strains and the MDR *E. cloacae* isolate. The lowest GBH-MIC (2 g/L) equivalent to a glyphosate acid concentration of 1.5 g/L was measured in both *A. baumannii* strains tested.

The MDR isolates showed higher MIC values for most antibiotics, except for colistin. Tigecycline was effective on all strains except for *P. aeruginosa*. It is worth mentioning that *E. cloacae-bla*$_{\text{OXA-48}}$ (Q9425) showed a low MIC value of 1 mg/L for meropenem, while gentamicin was effective against *K. pneumoniae-bla*$_{\text{KPC}}$ (Q9432) with an MIC of 0.25 mg/L (Supplementary Table S2).

The effect of the GBH in combination with clinically relevant antibiotics

The ten strains were tested with eight combinations of the GBH and antibiotics. Most associations showed either an additive (n = 35) effect or an indifferent effect (n = 35), with only five combinations showing antagonistic or synergistic effects (Table 2). The GBH had an antagonistic effect with ciprofloxacin and gentamicin on the MDR strain of *A. baumannii,* and only with ciprofloxacin on both the tested strains of *E. coli*. The strongest antagonistic effect was observed with *E. coli*, where the MIC of ciprofloxacin increased from 0.003 mg/L to 0.03 mg/L.

Stains and no	Origin (year/country)	Resistant antibiotic susceptibility testing phenotype	Used for checkerboard combinations assays	ARGs of interest	Reference	GenBank accession No
A. baumannii (CIP 70.10)	Type strain	ATM	Preliminary ATBs + GBH; Carbapenems + GBH	–	–	–
A. baumannii (Q9428)	Clinical isolate (2012/ Iran)	TIC, TIM, TPZ, ATM, CAZ, FEP, MER, IPM, RA, SXT, AK, CIP, GN	Preliminary ATBs + GBH; Carbapenems + GBH	bla_{OXA-23}	Hadjadj et al 2018[40]	NKXQ00000000
A. baumannii (Q9426)	Clinical isolate (2012/Liban)	TIC, TIM, TPZ, ATM, CAZ, FEP, MER, IPM, SXT, CIP	Carbapenems + GBH	bla_{NDM}*	Rafei et al 2014[41]	–
K. pneumoniae (CIP 82.91)	Type strain	AMX	Preliminary ATBs + GBH; Carbapenems + GBH	–	–	–
K. pneumoniae (Q9432)	Clinical isolate (2008/Israel)	AMX, AMC, FEP, TPZ, MEC, CRO, ETP, IPM, FF, NIT, SXT, AK, CIP	Preliminary ATBs + GBH; Carbapenems + GBH	bla_{KPC}	Lalaoui et al 2018[42]	–
K. pneumoniae (P1571)	Clinical isolate (2010/France)	AMX, AMC, FEP, TPZ, MEC, CRO, ETP, IPM, FF, NIT, SXT, AK, CIP, TET, GN	Carbapenems + GBH	bla_{NDM}*	Diene et al 2012[43]	HQ328085
K. pneumoniae (Q9430)	Clinical isolate (2018/France)	AMX, AMC, FEP, TPZ, MEC, CRO, ETP, SXT, AK, CIP, TET, GN	Carbapenems + GBH	bla_{OXA-48}	–	–
K. pneumoniae (Q9427)	Clinical isolate (2012/France)	AMX, AMC, FEP, TPZ, MEC, CRO, ETP, NIT, SXT, AK, CIP, TET, GN	Carbapenems + GBH	bla_{VIM}*	–	–
K. pneumoniae (Q9429)	Clinical isolate (2018/France)	AMX, AMC, FEP, TPZ, MEC, CRO, ETP, IPM, FF, NIT, SXT, CIP	Carbapenems + GBH	Membrane impermeability	–	–
E. coli (CIP 7624)	Type strain	–	Preliminary ATBs + GBH; Carbapenems + GBH	–	–	–
E. coli (Q1950)	Clinical isolate (2019/ Lebanon)	AMX, AMC, FEP, TPZ, MEC, CRO, ETP, IPM, SXT, AK, CIP, TET, GN	Preliminary ATBs + GBH; Carbapenems + GBH	bla_{NDM}*	Chamieh et al 2021[44]	JAFEVK010000000
E. coli (P1570)	Poultry isolate (2013/ Lebanon)	AMX, AMC, TPZ, ETP, SXT	Carbapenems + GBH	bla_{OXA-48}	Al Bayssari et al 2014[45]	KM390023
E. coli (Q9431)	Clinical isolate (2011/France)	AMX, AMC, MEC, NIT, TET	Carbapenems + GBH	bla_{GES}	–	–
P. aeruginosa (CIP 76.110)	Type strain	RA, SXT, MIN	Preliminary ATBs + GBH; Carbapenems + GBH	–	–	–
P. aeruginosa (Q5596)	Clinical isolate (2016/Pakistan)	TIC, TIM, TPZ, ATM, CAZ, FEP, MER, IPM, RA, SXT, AK, CIP, MIN, GN	Preliminary ATBs + GBH; Carbapenems + GBH	bla_{VIM}*	Hadjadj et al 2021[46]	–
P. aeruginosa (Q9424)	Clinical isolate (2016/Pakistan)	TIC, TIM, TPZ, ATM, CAZ, FEP, MER, IPM, RA, SXT, AK, CIP, MIN, GN	Carbapenems + GBH	Alterations of porin OprD	Hadjadj et al 2021[46]	–
E. cloacae (CIP 104,674)	Type strain	AMX, AMC, NIT, SXT	Preliminary ATBs + GBH; Carbapenems + GBH	–	–	–
E. cloacae (P7536)	Clinical isolate (2017/France)	AMX, AMC, FEP, TPZ, MEC, CRO, ETP, NIT, SXT, AK, CIP, TET, GN	Preliminary ATBs + GBH; Carbapenems + GBH	bla_{OXA-48}	Hadjadj et al 2022[47]	CP071788
E. cloacae (Q9425)	Clinical isolate (2016/Pakistan)	AMX, AMC, FEP, TPZ, MEC, CRO, ETP, SXT, GN	Carbapenems + GBH	bla_{NDM}*	Hadjadj et al 2021[46]	-

Table 1. Details of strains used in checkerboard assays. ARGs: antibiotic resistance genes, * Class B carbapenemase (MBL). Antibiotics tested on non-fermenting Gram-negative bacilli strain: TIC: Ticarcillin, TIM: Ticarcillin-clavulanic acid, TPZ: Piperacillin-tazobactam, ATM: Aztreonam, CAZ: Ceftazidime, FEP: Cefepime, MER: Meropenem, IPM: Imipenem, FF: Fosfomycin, RA: Rifampicin, SXT: Sulfamethoxazole-trimethoprim, AK: Amikacin, CIP: Ciprofloxacin, MIN: Minocycline, CS: Colistin, GN: Gentamicin. Antibiotics tested on enterobacteria strains: AMX: Amoxicillin, AMC: Amoxicillin-clavulanic acid, FEP: Cefepime, TPZ: Piperacillin-tazobactam, MEC: Mecillinam, CRO: Ceftriaxone, ETP: Ertapenem, IPM: Imipenem, FF: Fosfomycin, NIT: Nitrofurantoin, SXT: Sulfamethoxazole-trimethoprim, AK: Amikacin, CIP: Ciprofloxacin, TET: Tetracycline, CS: Colistin, GN: Gentamicin.

However, the GBH-colistin combinations had a synergistic effect against the *A. baumannii* strains tested, with a decrease in the MIC of colistin. For other strains, the effect was either indifferent or additive, but the addition of the GBH reduced the MIC of colistin at least three-fold. For example, the MIC of colistin for *E. coli* (CIP 7624) decreased from 1 mg/L to 0.06 mg/L, while for *E. coli-*bla_{NDM} (Q1950) and *P. aeruginosa* (CIP 76.110), it decreased from 1 mg/L to 0.125 mg/L and from 1 mg/L to 0.01 mg/L, respectively (Table S2 in the supplementary file). Similarly, the colistin MIC of *P. aeruginosa-*bla_{VIM} (Q5596) decreased from 2 mg/L to 0.007 mg/L, while for *E. cloacae* (CIP 104,674) and *E. cloacae-*bla_{OXA-48} (P7536), the colistin MIC decreased from 2 mg/L to 0.25 mg/L and

Antibiotics + GBH combinations	Cefotaxime + GBH	Meropenem + GBH	Aztreonam + GBH	Tigecycline + GBH	SXT + GBH	Gentamicin + GBH	Ciprofloxacin + GBH	Colistin + GBH
A. baumannii (CIP 70.10)	Indifference (FICI = 1)	Additive (FICI = 0.75)	Indifference (FICI = 1)	Additive (FICI = 0.75)	Indifference (FICI = 1)	Indifference (FICI = 1.12)	Indifference (FICI = 1.24)	**Synergy (FICI = 0.37)**
A. baumannii (Q9428)	Indifference (FICI = 1.25)	Additive (FICI = 0.75)	Indifference (FICI = 1.03)	Indifference (FICI = 1.25)	Indifference (FICI = 1.5)	**Antagonism (FICI = 2)**	**Antagonism (FICI = 2)**	**Synergy (FICI = 0.37)**
K. pneumoniae (CIP 82.91)	Indifference (FICI = 1.16)	Indifference (FICI = 1.36)	Additive (FICI = 0.9)	Additive (FICI = 0.91)	Additive (FICI = 0.91)	**Antagonism (FICI = 2.66)**	Indifference (FICI = 1.66)	Additive (FICI = 0.91)
K. pneumoniae (Q9432)	Additive (FICI = 0.67)	Additive (FICI = 0.75)	Additive (FICI = 0.72)	Additive (FICI = 0.91)	Additive (FICI = 0.91)	Indifference (FICI = 1.66)	Indifference (FICI = 1.66)	Indifference (FICI = 1.16)
E. coli (CIP 7624)	Additive (FICI = 0.99)	Indifference (FICI = 1.17)	Additive (FICI = 0.87)	Additive (FICI = 0.62)	Indifference (FICI = 1)	Indifference (FICI = 1.25)	**Antagonism (FICI = 10.75)**	Additive (FICI = 0.81)
*E. coli** (Q1950)	Additive (FICI = 0.62)	**Synergy (FICI = 0.3)**	Additive (FICI = 0.75)	Indifference (FICI = 1)	Indifference (FICI = 1)	Indifference (FICI = 1.16)	**Antagonism (FICI = 2.5)**	Additive (FICI = 0.62)
P. aeruginosa (CIP 76.110)	Indifference (FICI = 1.25)	Indifference (FICI = 1.12)	Indifference (FICI = 1.25)	Indifference (FICI = 1.12)	Indifference (FICI = 1.31)	Indifference (FICI = 1.24)	Indifference (FICI = 1.04)	Indifference (FICI = 1.01)
*P. aeruginosa** (Q5596)	**Synergy (FICI = 0.46)**	**Synergy (FICI = 0.21)**	Indifference (FICI = 1.3)	Indifference (FICI = 1.3)	Additive (FICI = 0.86)	Indifference (FICI = 1.3)	Indifference (FICI = 1.8)	Additive (FICI = 0.8)
E. cloacae (CIP 104,674)	Additive (FICI = 0.75)	Indifference (FICI = 1.1)	Additive (FICI = 0.56)	Additive (FICI = 0.69)	Additive (FICI = 0.67)	Additive (FICI = 0.78)	Additive (FICI = 0.92)	Additive (FICI = 0.78)
E. cloacae (P7536)	Indifference (FICI = 1)	Additive (FICI = 0.75)	Additive (FICI = 0.75)	Additive (FICI = 0.75)	Additive (FICI = 0.75)	Additive (FICI = 0.65)	Additive (FICI = 0.65)	Additive (FICI = 0.75)

Table 2. FICI values and outcome of a GBH in combination with antibiotics in the checkerboard assay on Gram-negative bacteria of ESKAPEE group. FICIs were calculated based on the concentration of glyphosate-isopropylamine salt in the tested GBH and its equivalence to glyphosate acid. Since the results were similar, only the data for the GBH are reported here. Synergistic results are highlighted in bold.

from 1 mg/L to 0.001 mg/L, respectively (Table S2 in supplementary file). The GBH-meropenem combinations also showed a synergistic effect on MBL producing strains. The addition of the GBH reduced the meropenem MICs from 16 mg/L to 0.06 mg/L for *E. coli-bla*$_{NDM}$ (Q1950), with a FICI of 0.3, and from 32 mg/L to 0.5 mg/L for *P. aeruginosa-bla*$_{VIM}$ (Q5596), with a FICI of 0.2 (Table 2).

Outer membrane destabilisation of GNBs by the GBH

In combination with the GBH, colistin MICs were reduced by a factor of at least three for eight of the ten strains tested. To investigate further, we assessed the permeabilising effect of GBH by evaluating its ability to render vancomycin active on GNB. Vancomycin is a glycopeptide antibiotic that acts on Gram-positive bacteria but cannot penetrate the outer membrane of GNBs due to its high molecular weight. The combination of a GBH and vancomycin on ten isolates increased bacterial growth inhibition from an average of 9% (2% to 19%) to an average of 97% (94% to 99%) (Fig. 1). These observations suggest that GBH increases outer membrane permeability, facilitating the action of antibiotics such as vancomycin and colistin.

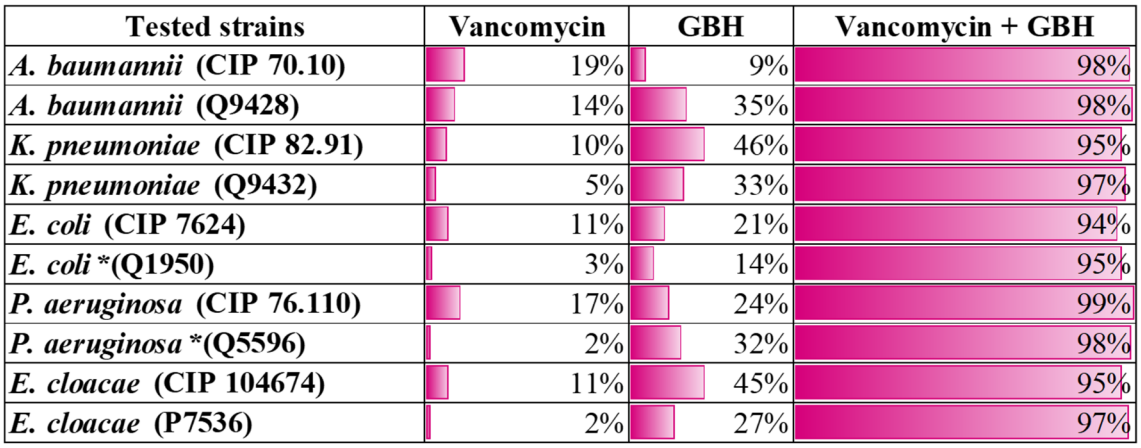

Figure 1. The percentage of bacterial growth inhibition in the presence of vancomycin alone, GBH tested alone, and a combination of vancomycin and the tested GBH. The concentration of vancomycin tested was 150 mg/L. The concentration of GBH tested was 0.1 mM (28 mg/L of glyphosate-isopropylamine salt in the tested GBH equivalent to 0.021 g/L of glyphosate acid). *Strain producing class B carbapenemase (MBL).

Investigation of GBH-carbapenem combinations

Our research uncovered significant synergistic effects of the combination of GBH and meropenem on MBL-producing strains. To delve deeper into these findings, we examined the effects of GBH combinations with three distinct carbapenem antibiotics on 19 strains including the five carbapenem-susceptible type strains (*K. pneumoniae, A. baumannii, P. aeruginosa, Enterobacter* spp. and *E. coli*), and 14 strains exhibiting varying carbapenem resistance mechanisms (bla_{KPC}, bla_{GES}, bla_{NDM}, bla_{VIM}, bla_{OXA-23}, bla_{OXA-48}, membrane permeability mutations, and porin-deficient OprD). The MIC values of a GBH and carbapenems for all tested strains are shown in Table S3 in the supplementary file.

All six of the MBL-producing strains we tested, namely *K. pneumoniae-*bla_{NDM} (P1571), *K. pneumoniae-*bla_{VIM} (Q9427), *A. baumannii-*bla_{NDM} (Q9426), *P. aeruginosa-*bla_{VIM} (Q5596), *E. cloacae-*bla_{NDM} (Q9425), and *E. coli-*bla_{NDM} (Q1950), showed synergistic effects when combined with the GBH and all three carbapenem agents (Table 3). However, only additive or indifferent effects were found with other isolates, including serine-carbapenemase-producing strains, oxacillinase-positive strains, porin-deficient OprD *P. aeruginosa*, *K. pneumoniae* with membrane impermeability, and type strains (Table 3).

Study of the effect of GBH on MBL-producing strains

The test aims to differentiate MBL-producing strains from other carbapenemases by studying the effect of EDTA on their enzymatic activity. EDTA inactivates metalloenzymes by binding to Zn^{2+} ions. We reversed the experiment to assess the chelating effect of a GBH, testing a collection of strains with known resistance mechanisms. The addition of GBH to bacterial suspensions produced an effect like that of EDTA for all strains tested (Fig. 2). An inhibition zone was observed around meropenem discs supplemented with GBH and EDTA for the six MBL-producing enterobacteria. In contrast, we observed no inhibition zone around the meropenem discs which were not supplemented (Fig. 3). Following the addition of $ZnSO_4$ in combination with GBH, we observed a restoration of MBL activity. The same result was obtained with a mixture of $ZnSO_4$ and EDTA.

We observed no effect of either EDTA or GBH on type strains, and on strains that do not present metallo-beta-lactamases, OprD-deficient *P. aeruginosa*, *K. pneumoniae* with membranous impermeability, class A carbapenemase-producing (bla_{KPC}, and bla_{GES}), and class D carbapenemase-producing (bla_{OXA-48}, bla_{OXA-23}) strains (Fig. 2). Based on these results, chelating properties of glyphosate are presumed as indicated by previous studies[48–50].

Discussion

The objective of our work was to study the impact of the combination of a GBH and antibiotics on GNB in the ESKAPEE group. In this study, we observed various effects of this GBH on combinations of bacteria/antibiotics. We found that the addition of the GBH notably alters phenotypic antibiotic sensitivity. At first, our research revealed a specific synergistic effect between GBH and carbapenems on the growth of MBL-producing strains. Unlike other carbapenemase classes (classes A, C, and D), which use a serine-linked acyl intermediate to catalyse β-lactam hydrolysis, class B MBLs require zinc for their activity[51], which can be neutralised by metal chelators

Tested strains	Imipenem + GBH	Ertapenem + GBH	Meropenem + GBH
A. baumannii (CIP 70.10)	Additive (FICI = 0.74)	Indifference (FICI = 1.42)	Additive (FICI = 0.75)
A. baumannii (Q9428)	Additive (FICI = 0.75)	Additive (FICI = 0.62)	Additive (FICI = 0.75)
*A. baumannii** (Q9426)	**Synergy (FICI = 0.31)**	**Synergy (FICI = 0.26)**	**Synergy (FICI = 0.28)**
K. pneumoniae (CIP 82.91)	Additive (FICI = 0.83)	Indifference (FICI = 1.66)	Indifference (FICI = 1.36)
K. pneumoniae (Q9432)	Indifference (FICI = 1.16)	Additive (FICI = 0.78)	Additive (FICI = 0.75)
*K. pneumoniae** (P1571)	**Synergy (FICI = 0.37)**	**Synergy (FICI = 0.26)**	**Synergy (FICI = 0.26)**
K. pneumoniae (Q9430)	Additive (FICI = 0.85)	Additive (FICI = 0.85)	Indifference (FICI = 1.1)
*K. pneumoniae** (Q9427)	**Synergy (FICI = 0.43)**	**Synergy (FICI = 0.40)**	**Synergy (FICI = 0.42)**
K. pneumoniae (Q9429)	Additive (FICI = 0.8)	Additive (FICI = 0.8)	Additive (FICI = 0.8)
E. coli (CIP 7624)	Additive (FICI = 0.58)	Additive (FICI = 0.92)	Indifference (FICI = 1.17)
*E. coli** (Q1950)	**Synergy (FICI = 0.36)**	**Synergy (FICI = 0.3)**	**Synergy (FICI = 0.3)**
E. coli (P1570)	Additive (FICI = 0.74)	Additive (FICI = 0.73)	Indifference (FICI = 1.23)
E. coli (Q9431)	Additive (FICI = 0.75)	Indifference (FICI = 1.2)	Additive (FICI = 0.92)
P. aeruginosa (CIP 76.110)	Indifference (FICI = 1)	Additive (FICI = 0.81)	Indifference (FICI = 1.12)
*P. aeruginosa** (Q5596)	**Synergy (FICI = 0.43)**	**Synergy (FICI = 0.41)**	**Synergy (FICI = 0.21)**
P. aeruginosa (Q9424)	Indifference (FICI = 1.16)	Additive (FICI = 0.91)	Additive (FICI = 0.91)
E. cloacae (CIP 104,674)	Indifference (FICI = 1.1)	Indifference (FICI = 1.1)	Indifference (FICI = 1.1)
E. cloacae (P7536)	Additive (FICI = 0.75)	Additive (FICI = 0.62)	Additive (FICI = 0.75)
*E. cloacae** (Q9425)	**Synergy (FICI = 0.46)**	**Synergy (FICI = 0.41)**	**Synergy (FICI = 0.41)**

Table 3. FICI values and outcome of a GBH in combination with carbapenems in the checkerboard assay on carbapenem-resistant strains. FICIs were calculated based on the concentration of glyphosate-isopropylamine salt in the tested GBH and its equivalence to glyphosate acid. Since the results were the same, only the data for the GBH are reported here. *: Strain producing class B carbapenemase (MBL). Synergistic results are highlighted in bold.

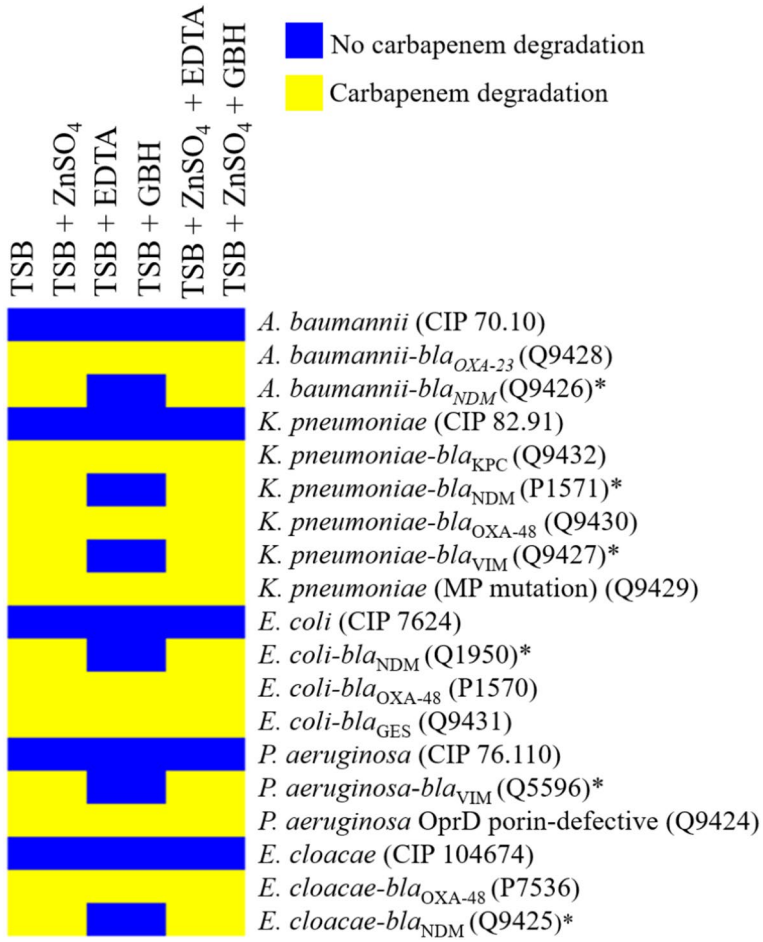

Figure 2. A heat map was generated to illustrate the degradation or non-degradation of carbapenems, determined by employing the modified carbapenem inactivation method combined with the addition of $ZnSO_4$ (zCIM), EDTA (eCIM), GBH (gCIM), $ZnSO_4$-EDTA, and $ZnSO_4$-GBH. This approach aimed to evaluate the influence of the tested GBH on strains producing MBLs. *Strain producing class B carbapenemase (MBL). MP mutation: membrane permeability mutation.

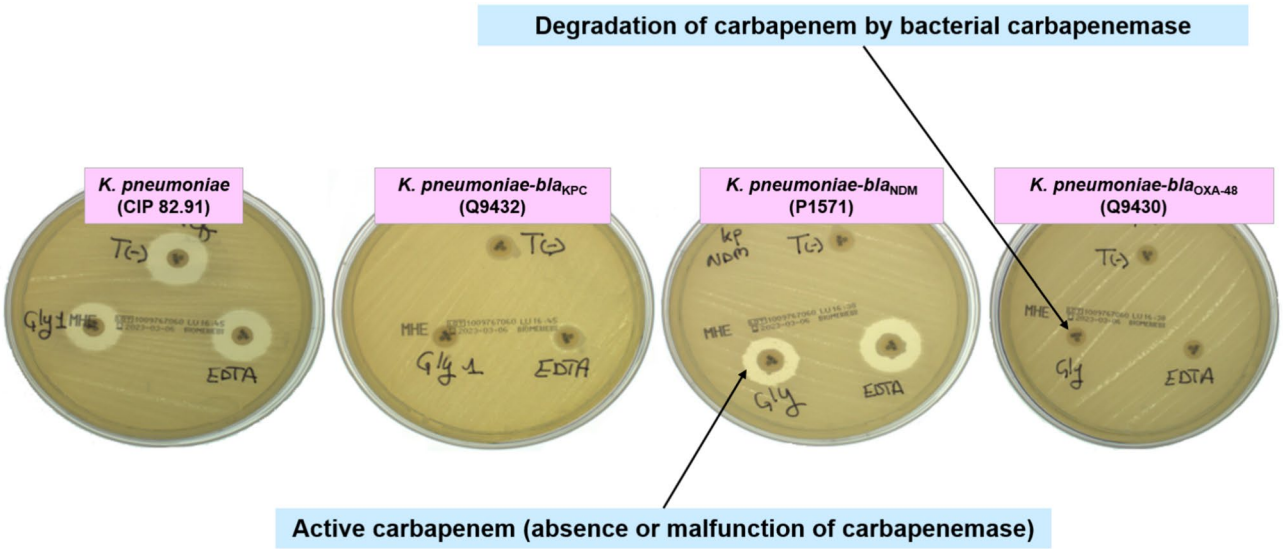

Figure 3. Photo of mCIM, eCIM, and gCIM test results. We present the results obtained on *K. pneumoniae* strains, including one antibiotic-sensitive type strains and three carbapenemase-producing strains of classes A, B, and D. T- is the name of the tube containing only TSB, while the symbols EDTA and Gly have been given to the other tubes supplemented with EDTA (eCIM) and the GBH (gCIM), respectively.

such as EDTA[52]. Research suggests that glyphosate's isopropylamine salt has EDTA-like chelating properties, allowing it to form stable complexes with divalent and trivalent metal ions and micronutrients like zinc[50]. Glyphosate acid acts as a tridentate zwitterionic chelating agent, possessing three functional groups: carboxylate, amine, and phosphonate, with both positive and negative charges[53]. Additionally, isopropylamine salt is also a chelator, thereby enhancing the chelating potential of the herbicide based on glyphosate isopropylamine salt[54].

We observed the reactivation of MBLs after the addition of $ZnSO_4$. Consequently, we conclude that the GBH is capable of sequestering metal ions in its environment and has a potential impact on various enzymatic activities reliant on zinc as a cofactor, including MBL activity[51,55]. Although this may seem like a positive development in the fight against MDR strains, it can seriously affect the health of plants, animals, and humans. The MBL superfamily is widespread and has been described in all life forms, including prokaryotic organisms such as bacteria and archaea, as well as eukaryotes, including humans[56]. This class of enzymes is heterogeneous and has been implicated in several physiological processes, such as the transport of molecules across membranes, communication through quorum sensing, and the hydrolysis of antibiotics (β-lactamase), anticancer drugs (cisplatin and mitomycin C), nucleic acids, ascorbic acid and xenobiotics (e.g. glyoxalase and lactonase enzymes)[57]. We have shown the effect of the GBH on beta-lactamases, but it would be interesting to evaluate its effect on other MBL-fold enzymes.

In our work, we also highlighted an effect on the outer membrane that could be linked to the chelating properties of GBH. The GBH addition rendered our strains susceptible to colistin and vancomycin. Vancomycin is naturally active only against Gram-positive bacteria. However, GNBs become susceptible to this antibiotic when the structure of their outer membrane is disrupted[58]. Studies have shown that adding chelating agents such as EDTA can potentiate the effect of colistin on MDR strains such as *Salmonella* spp[59]. and *K. pneumoniae*[60], leading to a decrease in their MICs to colistin and can make vancomycin effective against *E. coli* MDR strains[61,62]. These results align with our observations. We suggest that the chelating properties of GBH, which may sequester divalent cations Mg^{2+} or Ca^{2+}, thereby increase the permeability of the outer membrane and facilitate the entry of antibiotics[63]. Additionally, we suggest that the chelating effect of glyphosate-isopropylamine saltcould have multifaceted impacts on bacterial cells, potentially rendering the outer membrane more permeable and inactivating metalloenzymes.

In our study, we found that the association of GBH and gentamicin had antagonistic effects on the *A. baumannii-bla*$_{OXA-23}$ (Q9428) strain and the *K. pneumoniae* (CIP 82.91) strain. Similarly, the combination of GBH and ciprofloxacin had an antagonistic reaction on the *A. baumannii-bla*$_{OXA-23}$ (Q9428) strain and both *E. coli* strains tested. Other studies have also shown that being exposed to glyphosate increases the MICs of ciprofloxacin and aminoglycosides in *E. coli* and *Salmonella enterica* serovar Typhimurium strains[34,35]. The authors suggest that this could be an adaptive response leading to a cross-response to antibiotics by increasing membrane efflux[31]. However, it has been demonstrated that the targeted deletion of efflux pump genes may counteract the increased tolerance to kanamycin and ciprofloxacin in *E. coli* and *S. enterica* serotype Typhimurium, when exposed to GBH[35].

The main limitation of our study lies in the concentrations of GBH used in the preliminary tests. Our experimental approach aimed to explore the effects of combining a GBH and antibiotics through checkerboard tests, using separately determined MICs for each substance[64]. The GBH exhibited high MICs for our bacterial panel, however, subsequent experiments showed a comparable effect at lower GBH concentrations, particularly with the GBH-supplemented carbapenem inactivation method (gCIM) and the GBH/vancomycin combination tests. The concentration of GBH tested in these experiments was 0.1 mM, (28 mg/L equivalent to 0.021 g/L of glyphosate acid), falling within the acceptable limits of regulated glyphosate for short-term freshwater exposure in the United States (49.9 mg/L) and Canada (27 mg/L)[31]. It is worth mentioning that we evaluated a GBH formulation containing quaternary ammonium compounds alongside glyphosate isopropylamine salt. We opted for a GBH with quaternary ammoniums due to their reduced toxicity and widespread use as alternatives to GBHs containing polyethoxylated amines (POEAs). While regulatory authorities consider glyphosate as the active ingredient in GBHs, with other chemical compounds presumed to be inert[65]. We recognise that quaternary ammoniums also possess antibiotic properties, potentially heightening the risks associated with microbial exposure to GBH. In our study, we did not test glyphosate salt alone; rather, we analysed it as part of the complete herbicide formulation, simulating environmental contamination. The impact of the chelating effect of glyphosate-isopropylamine salt has already been demonstrated by altering plant[66,67] and algae[54] growth and vegetal enzymes[50]. This chelating activity could varies depending to the tested formulation[50], as it has been demonstrated for the combination effect of different GBHs formulation and imipenem on *P. aeruginosa*[32].

The simultaneous presence of herbicides and antibiotics is frequent in agricultural, urban and aquatic environments receiving effluents or leachates from agroecosystems, livestock farms, and clinical settings[35,68]. The relationship between GBH and antibiotics, and how they impact opportunistic pathogenic and MDR Gram-negative bacteria has been studied only sporadically, even though these bacteria are widespread in areas of human activity and are frequently linked to severe infections. The presence of a pesticide in diverse environments (such as soil, water, and wastewater), where antibiotic concentrations are typically low, may elevate these levels to a degree that inhibits sensitive species and selects tolerant strains[35].

These findings raise concerns about the potential risks that GBHs could cause to microbial diversity and global health. In addition to its antimicrobial properties, the chelation capacity of glyphosate, even at low concentrations, enables it to bind to macro- and micronutrients, inhibitors or activators of numerous critical physiological processes in plants, mammals, and microorganisms[69]. Further investigation is crucial to understand the effects of other glyphosate formulations on microbial antibiotics sensitivity.

Materials and methods

Collection and susceptibility of bacterial strains

We analysed Gram-negative ESKAPEE pathogenic strains, encompassing *K. pneumoniae, A. baumannii, P. aeruginosa, Enterobacter spp.*, and *E. coli*. For preliminary combination assays of ATBs with the GBH, we selected ten strains (Table 1), including five type strains and five MDR strains: *K. pneumoniae* (CIP 82.91 and Q9432), *A. baumannii* (CIP 70.10 and Q9428) *P. aeruginosa* (CIP 76.110 and Q5596), *Enterobacter* spp. (CIP 104,674 and P7536), and *E. coli* (CIP 7624 and Q1950). The type strains were obtained from the Institut Pasteur collection (Paris, France), while the MDR strains were isolated and characterised at the Institut Hospitalier Universitaire Méditerranée Infection (IHU-MI) (Marseille, France). We classified MDR strains according to the definition established by Magiorakos et al., indicating acquired non-susceptibility to at least one agent from three or more classes of antibiotics[70]. In a subsequent step, to explore the impact of carbapenems and GBH combinations, we expanded our strain collection to include nine carbapenem-resistant strains, each exhibiting various mechanisms of resistance (Table 1). Strain identification was performed using matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik, Bremen, Germany), as described previously[71]. Antibiotic susceptibility of the growing bacteria was determined using the disk diffusion method following guidelines from the European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2022). Two different panels of 16 antibiotics were tested on enterobacteria and non-fermenting GNB (see Table 1 legend). Standard qPCR and PCR assays were conducted to confirm the presence of carbapenemase genes (bla_{NDM}, bla_{VIM}, bla_{KPC}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-48}, and bla_{OXA-58})[72].

Chemicals and stock solutions used

We used cation adjusted Mueller–Hinton broth media (CAMHB, Merck KGaA, Darmstadt, Germany) and Trypticase soy agar (TSA, bioMérieux, Marcy-l'Étoile, France) medium for bacterial culture. We chose to investigate antibiotics used in human and veterinary medicine, notably ciprofloxacin, gentamicin, tigecycline, sulfamethoxazole + trimethoprim, and colistin (Sigma Aldrich, Saint-Quentin-Fallavier, France). Additionally, we tested antibiotics exclusively used in human medicine, such as cefotaxime, meropenem, imipenem, ertapenem and aztreonam (Sigma Aldrich, Saint-Quentin-Fallavier, France). For each antibiotic, we prepared a 1 g/L stock solution in deionised water using either a lyophilised or solution formulation. Additionally, we used a commercial GBH (Radikal, Fitogal, Spain), which is a soluble concentrated solution formulated with 360 g/L of glyphosate-isopropylamine salt, corresponding to 270 g/L of glyphosate acid. This formulation also includes a quaternary ammonium as a coformulant, but the specific type and concentration are not disclosed by the manufacturer (Expiration date of approval: 30/04/2028). This solution was diluted to a concentration of 20 mg/mL in a sterile CAMHB medium.

Minimum inhibitory concentration assays

MICs for antibiotics and the GBH were determined using the broth microdilution method. The sterile CAMHB medium was used as a negative control, while the *E. coli* type strain, with known antibiotic sensibility, served as a positive control. The final concentration of GBH solution in the microtiter plates ranged from 0.0625 g/L to 32 g/L for all strains. Antibiotic concentrations tested ranged from 0.5 mg/L to 32 mg/L for susceptible strains, and from 0.5 mg/L to 256 mg/L for MDR strains. Antibiotic sensitivity was determined using 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Aldrich®, St Louis, Missouri, USA). A 0.1% (w/v) TTC solution was prepared and added after 22 h of incubation at 37 °C, followed by an additional two hours of incubation, to determine the MIC[73]. The experiment was repeated in triplicates and performed independently.

Checkerboard assay

The antibacterial effects of the combination between GBH and the tested antibiotic were assessed using the checkerboard test, as previously described by Sopirala et al.[74]. Serial dilutions of each antibiotic and a GBH at decreasing concentrations were prepared in CAMHB ranging from 4 × MIC to 1/128 × MIC. Each checkerboard well was streaked with 45 µL of each agent and 10 µL of a bacterial suspension at 10^5 CFU/mL. Additional rows were used to determine the MIC of each antimicrobial compound alone by adding 90 µL of each agent. For each drug combination, the Fractional Inhibitory Concentration (FIC) and the Fractional Inhibitory Concentration Index (FICI) were calculated according to the equation $FICI = FIC_A + FIC_G$ = (MIC of the tested antibiotic in combination with the GBH/MIC of antibiotic alone) + (MIC of GBH in combination with antibiotic /MIC of GBH alone). The interaction was defined as synergy (FICI ≤ 0.5), additive (FICI > 0.5 and < 1), indifferent (FICI > 1 and < 2), and antagonistic (FICI > 2)[75,76].

Screening of a GBH effect on the outer membrane

We conducted an experiment to study the impact of GBH on enhancing the activity of colistin and its effect on the stability of the outer membrane of GNB. To do so, we tested the sensitivity of ten strains, including five type strains and five MDRs (used in the preliminary tests and previously cited), to the combination of GBH and vancomycin, which is typically only active against Gram-positive bacteria. We followed the methodology described by Muheim et al., with a few modifications[77]. We prepared overnight bacterial cultures and diluted them to an optical density of $OD_{600} = 0.01$. Subsequently, we added either 150 mg/L of vancomycin, 0.1 mM of the GBH (28 mg/L of glyphosate-isopropylamine salt in the tested GBH equivalent to 21 mg/L of glyphosate acid), or a combination of both vancomycin and GBH (150 mg/L and 0.1 mM, respectively). Next, 100 µL of each mixture was added to the wells of microtiter plates. These plates were then incubated for five hours at 37 °C without shaking, and the optical density at OD_{600} was measured using a spectrophotometer (Multiskan Spectrum, ThermoFisher Scientific, Waltham, MA, USA). Three growth controls were included: bacterial suspension alone,

bacterial suspension with 150 mg/L of vancomycin, and bacterial suspension with 0.1 mM a GBH. A sterility control consisting of sterile growth medium was also included. The bacterial growth inhibition rate was determined using the following equation, which correlates with the absorbance (A) measured in the wells: bacterial growth inhibition (%) = $[1 - (A_{sample} - A_{negative\ control} / A_{positive\ control} - A_{negative\ control})] \times 100$.

Investigation of the association of the GBH and carbapenem

After the initial results, we aimed to investigate how GBH and carbapenem combinations affect carbapenem-resistant strains. We selected 19 Gram-negative strains from the ESKAPEE group, which included the five type strains mentioned above, and 14 strains exhibiting varying carbapenem resistance mechanisms (*A. baumannii-bla*$_{OXA-23}$ (Q9428), *A. baumannii-bla*$_{NDM}$ (Q9426), *K. pneumoniae-bla*$_{OXA-48}$ (Q9430), *K. pneumoniae-bla*$_{NDM}$ (P1571), *K. pneumoniae- bla*$_{VIM}$ (Q9427), *K. pneumoniae-bla*$_{KPC}$ (Q9432), *E. coli-bla*$_{OXA-48}$ (P1570), *E. coli-bla*$_{NDM}$ (Q1950), *E. coli-bla*$_{GES}$ (Q9431), *P. aeruginosa- bla*$_{VIM}$ (Q5596), *E. cloacae-bla*$_{OXA-48}$ (P7536), *E. cloacae-bla*$_{NDM}$ (Q9425), OprD-deficient *P. aeruginosa* (Q9424), and one with membrane permeability mutations, *K. pneumoniae* (Q9429) (Table 1). We tested three different carbapenem antibiotics (imipenem, ertapenem, and meropenem) and the GBH, individually and in combination, using the experimental procedure reported above.

Study of the effect of a GBH on MBL-producing strains

To explore the potential synergistic impact of carbapenem agents and GBH on MBL strains, the modified carbapenem inactivation method combined with the EDTA-carbapenem inactivation method (eCIM) developed by the Clinical and Laboratory Standards Institute (CLSI) was used, with a few modifications. The EDTA was used in this assay to differentiate MBL from other carbapenemases, specifically class A and D. Additionally, we tested GBH to confirm whether it exhibits similar ion chelating properties. Fourteen strains which were resistant to carbapenems by various means were tested (Table 1). The test was conducted following the description published by Sfeir et al.[78]. Briefly, a colony of the tested strain was resuspended in three tubes with 2 mL of tryptic soy broth (TSB, bioMérieux) from the overnight culture. The first tube contained only TSB, while the second tube was supplemented with EDTA (ThermoFisher Scientific, Waltham, Massachusetts, USA) to a final concentration of 0.1 mM and used as a positive control to validate the experiment. The third tube had an additional 0.1 mM of GBH. A meropenem disc (10 µL) was placed in all tubes. After four hours of incubation at 35 °C ± 15 min, the discs were placed on Mueller–Hinton (BD) agar plates inoculated with a 0.5 McFarland suspension of a carbapenem-susceptible type strain (*E. coli* CIP 7624). In a second step, we experimented to determine whether the GBH binds to zinc ions in the active site of MBL enzymes or chelates free zinc ions. To do so, we combined a GBH with the zinc-supplemented carbapenem inactivation method (zCIM)[79]. We added a meropenem disc to a bacterial suspension grown in TSB supplemented with $ZnSO_4$ (final concentration 1.5 mM) and the GBH (final concentration 0.1 mM). We prepared a bacterial suspension containing $ZnSO_4$ alone (final concentration 1.5 mM) and the GBH alone.

We used previously established thresholds: an inhibition zone ≤ 15 mm indicates carbapenem degradation, while an inhibition zone > 15 mm indicates the absence of carbapenem degradation to interpret the results. We used type strains that are incapable of degrading meropenem, along with strains producing class A and D carbapenemases that would not be affected by EDTA, as negative controls. The addition of EDTA, a known agent for chelating MBLs, served as a positive control in our experiment.

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Author contributions

L.H., J.M.R. and S.A.B.: designed and conceived the study. H.Z., A.H., L.H.: performed the experiments and interpreted the data. H.Z., A.H., L.H. and S.A.B. drafted the manuscript. H.Z., L.H. J.M.R., and S.A.B.: made critical corrections and revisions. All authors read and approved the final version of the manuscript.

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Competing interests

The authors declare no competing interests.

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Conclusions

L’objectif principal de cette thèse était de résumer les connaissances actuelles sur les mécanismes de tolérance au glyphosate chez les microorganismes dans la littérature, de décrire les mécanismes de tolérance au glyphosate chez les bactéries à Gram négatif (BGN), et d’évaluer le rôle du glyphosate dans la résistance aux antibiotiques.

L'étude des mécanismes de tolérance au glyphosate a révélé que, bien que plusieurs mécanismes soient identifiés et documentés dans la littérature, il subsiste des lacunes importantes concernant des mécanismes encore mal décrits. Les bactéries ont développé des stratégies d'adaptation face à la présence de cet herbicide qui partagent des similarités avec les mécanismes de résistance aux antibiotiques. Ces mécanismes incluent des modifications de la perméabilité de la membrane cellulaire ou l'activation de systèmes d'efflux (56), ainsi que la modification de la cible enzymatique. Ainsi, l’altération de la 5-énolpyruvylshikimate-3-phosphate synthase (EPSPS) peut induire une réduction de son affinité à cet herbicide. En revanche, la modification de l'herbicide lui-même n'est pas un mécanisme courant dans le cas du glyphosate, bien que certaines espèces de microorganismes soient capables de le dégrader.

Les mécanismes identifiés à ce jour sont principalement d'origine chromosomique, reflétant une adaptation évolutive au stress imposé par le glyphosate. Cependant, certaines études suggèrent la présence de mécanismes extra-chromosomiques. Ainsi, l'opéron *phn*, normalement impliqué dans la dégradation du glyphosate et localisé au niveau chromosomique, a également été trouvé sur des plasmides (57,58). A notre connaissance, le gène plasmidique *aroA* a déjà été rapporté dans la littérature, mais nos travaux caractérisent pour la première fois ce gène et son environnement génétique (59). Nous avons montré que cette enzyme possédait bien une activité de la 5-

énolpyruvylshikimate-3-phosphate synthase, et qu'elle présentait une affinité diminuée au glyphosate comparativement aux enzymes de classe I. Cependant, nous n'avons pas mis en évidence de modification de la CMI au glyphosate des bactéries porteuses de ce gène comparativement aux autres. Cette voie étant essentielle à la survie des bactéries, ce gène pourrait permettre à la bactérie de survivre plus facilement dans ce milieu, sans être tolérante à de plus fortes concentrations au glyphosate.

Au sein du transposon, le gène *aroA* est associé à un gène *MFS-like*, codant pour une pompe d'efflux connue pour son rôle dans la résistance multidrogue (60). Nos résultats suggèrent un rôle potentiel de cette pompe dans la tolérance au paraquat, un herbicide également largement utilisé dans la région dans laquelle les prélèvements ont été effectués. Il faudrait cependant insérer le gène seul dans une souche d'*E. coli* pour confirmer son rôle dans la résistance au paraquat.

La présence d'un tel transposon chez des bactéries clinique et d'origine alimentaires est intéressante car elle souligne le risque d'une diffusion à large échelle dans des zones polluées. Nous avons isolé ce plasmide de souche cliniques et alimentaires, suggérant un passage de ces bactéries résistantes dans le tube digestif humain. Si le risque direct lié à la tolérance au glyphosate et/ou au paraquat semble faible, nous avons remarqué que ce transposon était situé sur des plasmides de type IncF. Ces plasmides sont souvent retrouvés chez des bactéries à Gram négatif (61), et sont rapportés dans la littérature comme étant des éléments importants dans la dissémination de la résistance aux antibiotiques (61,62). Le principal risque dans ce cas repose sur la co-sélection de gènes de résistance aux antibiotiques porté par ce même plasmide, sous la pression de cet herbicide. Le terme « co-sélection » désigne le phénomène par lequel l'utilisation d'un agent, favorise la sélection simultanée de gènes de résistance à d'autres agents, même s'ils ne sont pas directement ciblés (63). Les prélèvements à partir desquelles sont issues ces souches

bactériennes datent de 2018. Il serait intéressant de savoir depuis quand ce gène a émergé, en le testant sur une collection de souches environnementales et cliniques de la région antérieur. De plus, l'utilisation du paraquat a été interdite en 2020 (64). Il serait intéressant de réaliser de nouveaux prélèvements dans la région pour évaluer le niveau de dissémination de ce plasmide, et de réaliser une enquête des pratiques de désherbage, pour évaluer le niveau d'utilisation de ces herbicides malgré l'interdiction. Cela nous permettra également de voir si le plasmide porte d'autres gènes de résistance, et si d'autres plasmides sont maintenant porteurs de ce transposon.

Les résultats des tests enzymatiques et des CMI des souches porteuses du gène *aroA* ont mis en lumière la problématique de l'utilisation des termes « tolérance » ou « résistance » au glyphosate en fonction de l'utilisation de méthodes d'évaluation *in vitro* ou *in silico.* Durant nos travaux, nous avons noté que peu d'études ont examiné la tolérance des microorganismes en déterminant leur CMI au glyphosate. Les méthodes de détermination de la CMI ne sont pas harmonisées et plusieurs formulations à base de glyphosate ont été utilisées, certaines étant plus toxiques que d'autres (65). De plus, diverses méthodes ont été employées pour déterminer la CMI, mais la méthode en milieu liquide est généralement considérée comme la plus efficace, car le glyphosate se dissout difficilement et la diffusion dans les géloses n'est pas toujours homogène (66).

Ces observations faites, nous avons réalisé une étude comparative de ces deux méthodes d'évaluation sur 60 souches bactériennes. Les résultats préliminaires de l'analyse combinée des deux approches *in silico* et *in vitro* ont montré l'absence de corrélation entre la tolérance au glyphosate mesurée *in vitro* par la détermination des CMI et la classification *in silico* des enzymes EPSPS. Nous avons choisi d'utiliser le milieu liquide avec deux formulations de glyphosate (IPA et HBG) afin de tester une formulation à base de glyphosate tout en ayant l'IPA comme contrôle pour nous comparer aux résultats ici de la littérature. Il aurait été intéressant de tester le Roundup®,

qui est l'HBG le plus utilisé, mais nous n'avons pas réussi à nous en procurer. Suite à cette étude, nous proposons d'utiliser le terme « d'affinité au glyphosate » lorsqu'il s'agit d'études concernant l'affinité enzymatique du glyphosate pour sa cible, et de « tolérance au glyphosate » lorsque l'on étudie la tolérance d'une bactérie au glyphosate.

Enfin, nous n'avons pas trouvé de lien entre la résistance aux antibiotiques et la tolérance au glyphosate, mais notre travail présentait de nombreuses limitations, notamment en ce qui concerne la distinction des groupes de patients exposés et non exposés. Des travaux réalisés dans notre équipe ont montré que l'association de certains antibiotiques au glyphosate avaient un impact sur la résistance à certains antibiotiques (67). L'ensemble de ces résultats suggèrent un lien entre la résistance aux antibiotiques et l'utilisation massive d'herbicides dans l'environnement. Il est important de poursuivre l'étude du lien entre ces phénomènes pour mieux comprendre l'impact de ces composés dans la santé humaine et animale.

Perspectives

Les résultats de cette thèse ouvrent plusieurs pistes pour des recherches futures dont certaines ont déjà été mentionnées. Il nous parait important de continuer à caractériser le gène *aroA* de classe II afin de mieux comprendre son rôle. La réalisation de prélèvements humains et environnementaux dans la même zone géographique, cinq ans après les premiers échantillons, permettront de suivre l'évolution de ce gène et d'évaluer s'il est toujours présent sur des transposons ou des chromosomes et dans quelle proportion. Il serait également pertinent d'étendre cette analyse pour identifier l'impact de la dissémination de ce gène dans l'environnement et chez l'Homme et l'animal. Une autre piste intéressante serait d'étudier l'effet du glyphosate sur le microbiote digestif des personnes exposées et non exposées, le glyphosate ayant déjà montré un rôle de perturbateur de la flore microbienne chez l'abeille et le rat. Il serait également pertinent d'examiner la présence du gène *aroA* de classe II dans des échantillons de patients n'ayant jamais été en contact avec des pesticides, afin de voir si ce gène plasmidique est présent, suggérant un autre rôle de ce gène. En parallèle, il serait intéressant d'étudier l'effet des combinaisons d'herbicides, d'antiseptiques et d'autres biocides sur les bactéries, notamment en menant des études *in vitro* et *in silico* pour comprendre le rôle des autres composés chimiques retrouvés dans l'environnement sur l'émergence de la résistance aux antibiotiques. Ces travaux pourraient nous permettre de promouvoir l'utilisation de pratiques agricoles alternatives, telles que l'agriculture biologique et l'utilisation de biopesticides, afin de réduire la dépendance aux produits chimiques synthétiques et minimiser leur impact sur l'environnement et la santé humaine. Ces recherches doivent être menées dans une optique « One Health », reconnaissant l'interconnexion entre la santé humaine, animale et environnementale dans la lutte contre la résistance aux antibiotiques.

Travaux Annexes

Avant-propos

Au cours de mes trois années de thèse, j'ai eu l'opportunité de collaborer à plusieurs projets de recherche diversifiés qui ont considérablement élargi mes compétences et enrichi mon parcours scientifique. Ces travaux ont exploré des domaines variés mais interconnectés, illustrant la portée et la profondeur de l'application des techniques de métagénomique et de génomique dans la recherche microbiologique.

Parmi ces projets, une étude s'est concentrée sur la caractérisation des communautés fongiques du sol, en utilisant une approche combinée de culturomique et de métabarcodage ITS1/ITS2 pour analyser les environnements pollués par les hydrocarbures aromatiques polycycliques (HAP). Cette étude a permis d'obtenir 165 isolats fongiques à partir de 14 échantillons de sol, identifiés à différents niveaux taxonomiques, révélant une prévalence marquée du phylum Ascomycota. L'analyse a montré que les communautés fongiques des sols pollués par les HAP étaient distinctes de celles des sols non pollués, avec des genres spécifiques associés à chaque type d'échantillon. En intégrant les approches de culturomique et de métagénomique, cette recherche a fourni une caractérisation précise et complète de la structure des communautés fongiques du sol, offrant ainsi une meilleure compréhension de l'écologie des champignons dans des environnements contaminés. Ces expériences ont illustré la combination de l'application approfondie des techniques de génomique et ainsi de la culturomique dans l'exploration de la diversité microbiologique et ont renforcé la pertinence des méthodes intégrées pour la recherche environnementale (**Article 6**).

Une autre étude a porté sur l'analyse du microbiote intestinal chez des patients atteints de cancer colorectal (CCR), l'un des cancers les plus meurtriers au niveau mondial, à différents stades de traitement. Nous avons analysés les échantillons fécaux de dix patients avant, pendant et après les traitements de chimiothérapie et/ou radiothérapie, en utilisant la technique métagénomique

Illumina pour séquencer la région V3-V4 du gène 16S rRNA. Les analyses ont permis de décrire la diversité et la composition du microbiote intestinal tout au long du traitement. Les résultats ont montré que les phyla les plus abondants étaient les Firmicutes, Bacteroidetes et Proteobacteria, sans déviation significative dans la composition du microbiote entre les différentes périodes de traitement. Cette étude fournit une vue détaillée de la composition du microbiote intestinal chez les patients atteints de cancer colorectal tout au long du parcours de traitement, offrant ainsi des insights précieux sur l'évolution de la communauté microbienne dans ce contexte clinique (**Article 7**).

Étant particulièrement intéressée par la résistance aux antibiotiques et les éléments génétiques mobiles tels que les transposons, j'ai travaillé sur une étude explorant les mécanismes de résistance aux carbapénèmes chez *Klebsiella pneumoniae* en Polynésie française. Cette recherche s'est focalisée sur la caractérisation génomique des souches résistantes et l'analyse des éléments génétiques mobiles impliqués dans leur propagation, afin de comprendre comment ces éléments contribuent à la diffusion de la résistance et d'identifier des stratégies pour prévenir ce phénomène. (**Article 8).**

Article 6

Integrating culturomics and metagenomics to characterise fungal communities in PAH-contaminated soils

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(Soumis dans Applied Soil Ecology)

Integrating culturomics and metagenomics to characterise fungal communities in PAH-contaminated soils

Short title: Fungal culturomics and metagenomics of PAH-contaminated soils

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Abstract

Fungi play a pivotal role in soil ecology and have the potential to be utilized as bioremediation agents. However, there is still a paucity of knowledge regarding the diversity and structure of soil fungal communities. In this study, we present a novel approach to environmental mycobiome characterisation, combining culturomics and ITS1/ITS2 metabarcoding, to investigate the mycobiome of soil environments exhibiting profiles of PAH pollution. A total of 165 fungal isolates were obtained from 14 soil samples through the culturomics approach. These isolates were identified as belonging to 51 fungal genera, 31 families, 18 orders, 9 classes and 3 phyla. In addition, five distinct culture media were employed in the culturomics approach. Of these, FastFung emerged as the most effective medium, yielding the highest number of fungal isolates (78/165, 47.3%). Using high-throughput ITS1/2 amplicon-based sequencing, a total of 595 assigned operational taxonomic units (OTUs) and 1062 OTUs were recovered from the ITS1 and ITS2 regions, respectively. Ascomycota was the most prevalent fungal phylum across all three datasets, accounting for 99.4% and 88.6% of OTUs in the ITS1 and ITS2 datasets, respectively, and 93.3% of species isolated through culturomics. The fungal community structure of PAH-polluted soils was found to be distinct and dissimilar from that of unpolluted soil ecosystems. The LEfSe analysis demonstrated that *Coniosporium*, *Ovatospora*, and *Phialemonium* were associated with unpolluted

samples, whereas *Gliomastix* and *Emericellopsis* were associated with PAH contamination. The integration of culturomics and metagenomics, based on ITS1/2 amplicons, provides comprehensive and accurate characterization of the soil fungal community structure, which facilitates a deeper insight into the ecology of soil fungi.

Keyword: Mycobiota; soil microbiology, soil pollutants; environmental restoration and remediation; population genomics; mycology.

1. Introduction

Soil contamination by persistent organic pollutants (POPs) has emerged as a major environmental problem in recent years. Their widespread occurrence in the soil environment can be attributed to various anthropogenic activities, including oil spills, discharges of petroleum products and untreated sewage sludge, mismanagement of industrial, nuclear and biomedical waste, and the extensive use of fertilisers, herbicides and pesticides in agriculture [1]. Polycyclic aromatic hydrocarbons (PAHs) are among the POPs of increasing concern [2]. PAHs are ubiquitous environmental pollutants formed by the fusion of two or more aromatic benzene rings in linear, angular or cluster arrangements. They are formed primarily by the incomplete combustion of organic matter, namely wood, coal, tar, municipal solid waste and petroleum products [3]. This widespread environmental burden not only poses a significant threat to human health due to their known or potential toxicity and risk of human exposure [4], but also alters the microbial community structure, the study of which may provide valuable insights into the magnitude of the environmental impact [4].

Fungi are key players in the soil environment, constituting an important fraction of the soil biomass and participating, through their mycelial networks, in vital processes contributing to nutrient and organic matter cycling, biotic interactions and nutrient channelling [5]. Despite their crucial role in soil dynamics as nutrient cycling agents and organic matter decomposers, our knowledge of soil fungal diversity and community structure remains relatively scarce and fragmentary [6]. The study and cultivation of soil fungal communities, now known as mycobiomes, dates back to the 1910s with the pioneering work of C.L. Waksman [7]. However, the technological advances available at that time in terms of culture media and identification techniques were fallible and flawed. Since then, groundbreaking advances in next-generation

sequencing (NGS) and the integration of metagenomic approaches into soil mycobiome studies have revolutionised our understanding of fungal communities in soil ecosystems [6], [8]–[11]. Indeed, a recent study has shown that soil fungal communities worldwide are dominated by a few Ascomycota taxa [10]. Despite its revolutionary contributions, one of the major limitations of metagenomics is the relatively high proportion of unassigned sequences generated by this approach [12]. In addition, most taxonomic analyses are limited to higher taxa levels due to limitations in accurately identifying and assigning OTUs at the genus and species level. Furthermore, culture-dependent approaches were abruptly marginalised as attention shifted to the more sophisticated and less laborious metagenomic approaches. Notwithstanding the contribution of the latter to our understanding of the soil mycobiome, culturing the soil microbiome is of great importance to further understand and characterise active fungal communities in soil ecosystems. While the exact number of fungal species isolated from soil environments remains elusive, estimates suggest the presence of approximately 55,600 fungal species [13]. In addition, a comprehensive repertoire of soil-dwelling fungal species is still not available, especially in soils contaminated by PAHs. Due to their unique capabilities and metabolic versatility, fungi have emerged as valuable bioremediation agents capable of addressing numerous aspects of environmental contamination. [14]–[17]. Addressing the issue of fungal diversity in polluted soil matrices using only metabarcoding-based approaches allows at most the presence of some fungal species or genera to be associated with a given pollutant, without the possibility of assessing their degradation capabilities [18].

In this context, pure culture of fungi remains an indispensable tool for the study of the mycobiome. The emergence of culturomics in the last decade has significantly contributed to unravelling gut microbial community structure [19], [20]. Therefore, the aim of this study was to

apply this pioneering approach in environmental sciences alongside the established ITS1/ITS2 metabarcoding technique to reveal and characterise the fungal community structure within PAH-contaminated soil samples. Using advanced identification technologies, it should provide deeper and more accurate characterisation of the mycobiome at lower taxonomic levels, paving the way for eventual use of specific fungi as potential bioremediation agents.

2. Materials and Methods

2.1. The samples. The study was conducted on 19 soil samples, collected from various geographical locations in the Southern Region of France, and stored at 4°C until further use. These samples comprised six PAH-polluted samples collected from Port-Saint-Louis-du-Rhône (SS1, SS7, SS8, SS9, SS10, SS11), one sample collected from France's high-speed rail network (SNCF) (SS2), four samples displaying different pollution and pretreatment profiles were acquired from the Green PRAXIS Société at Gare de Corligrey, of which, two were unpolluted (SS3 and SS5), and the remaining two were PAH-polluted (SS4 and SS6). Five more samples displaying various levels and profiles of PAH pollution were obtained from BioCentre (SS12-SS16), and finally three unpolluted samples, one from a topsoil, one from Jardin la Verdière and one from the commune of Pennes-Mirabeau were considered for this study. Of these 19 samples, 14 were subjected to the culturomics approach, while all 19 were analysed using high-throughput amplicon-based sequencing. The description of the samples is given in Supplementary Table 1.

Supplementary Table 1. Description of the samples (Sample ID, Pollution Status, and Source).

Sample ID	Description
SS1	Port-Saint-Louis-du-Rhône 105, PAH-Polluted (ug/g), 29/11/2020
SS2	SNCF, high PAH-Pollution (10 ug/g), 22/08/2017
SS3	Green Praxis, Gare de Corligrey, unanthropized soil far away from human activity, non-polluted, 04/2022
SS4	Green Praxis, Gare de Corligrey, > 20m from the tracks, PAH-polluted, 04/2022
SS5	Green Praxis, Gare de Corligrey, unanthropized soil far away from human activity, non-polluted, sieved and lyophilized, 04/2022
SS6	Green Praxis, Gare de Corligrey, > 20m from the tracks, PAH-polluted, sieved and lyophilized, 04/2022
SS7	S5, Unsorted Soils, HAPSIS PSL 11/03/20
SS8	S9, PSL-HAPSIS 11/03/2020
SS9	S1, Friche, PSL-HAPSIS 05/03/2020
SS10	S3, PSL-HAPSIS 05/03/2020
SS11	S2, Unsorted Soils, PSL-HAPSIS, 11/03/2020
SS12	BioCentre Co-Pollution HAP (50-200 PPM), 06/23
SS13	BioCentre Co-Pollution Sb, 06/23
SS14	BioCentre PAH + PCB
SS15	BioCentre Co-Pollution HCT-HAP
SS16	BioCentre HAP Soluble
SS17C	Topsoil, ref 500 um, non-polluted
SS18C	Jardin la Verdière, 83560 500 um, non-polluted
SS19C	Soil from Pennes-Mirabeau commune, non-polluted

2.2. Culturomics. We employed the culturomics approach initially developed by Lagier *et al.* [21] for the study of the human gut microbiome. In this study, five different culture media were used for the study of the soil mycobiome; namely, Sabouraud Dextrose Agar (SDA) supplemented with Gentamicin and Chloramphenicol (GC) (Oxoid, Dardilly, France), ChromAgar (Becton Dickinson, Le Pont de Claix, France), Potato Dextrose Agar (PDA) supplemented with Chloramphenicol (CHL) (Clinisciences, Nanterre, France), modified Malt Extract Agar (MEA) + CHL, and FastFung [22]. Briefly, five grams of soil from each sample was diluted with 15 mL of Dulbecco's phosphate-buffered saline (DPBS). The resulting suspensions were then diluted to the $1/10^{th}$, $1/100^{th}$, $1/1000^{th}$ and $1/10000^{th}$. A total volume of 300 µl of each dilution was then plated onto the five aforementioned culture media and incubated at 25°C for seven days. Growth was monitored every 24 hours. Colonies with unique morphological features were subcultured under the same culture conditions for purification and subsequent identification.

2.3. Fungal colonies identification. Fungal colonies were identified using matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-TOF MS). Fungal colonies were subjected to a standard protein extraction protocol prior to identification [23]. Briefly, fungal colonies were incubated in 1 mL of 70% ethanol then centrifuged at 13000 rpm for 10 minutes. The supernatant was discarded, and the pellet was then treated with 10 µl of formic acid (70%) for 5 minutes followed by a treatment with acetonitrile (100%) for another 5 minutes. The mixture was then centrifuged at 13000 rpm for 5 minutes. Next, one µl of the resulting supernatant was deposited on the MSP96 target and allowed to dry at room temperature before adding 1 µl of a-cyano-4-hydroxycinnamic acid (HCCA) matrix on each spot and allowed to dry. *Candida krusei* was used as a positive control and the HCCA matrix as a negative control. Analysis was

performed on a Microflex LT™ mass spectrometer and the MALDI Biotyper™ system (Bruker Daltonics GmbH, Bremen, Germany). Spectra were acquired with the Flex Control™ v. 2.4 software (Bruker Daltonics). Both the MBT BDAL Library v11 (Bruker Daltonics) and in house reference spectra databases were used, as described in [24]. The MALDI-TOF MS-based identification of a strain was considered adequate if the identification Biotyper log(score) value (LSV) was less than 1.98.

2.4. Molecular identification. Fungal colonies that remained unidentified or that displayed a LSV < 1.98 were subjected to molecular identification. A preliminary lysis step was performed to ensure degradation of the cell wall and release of the cell contents. Fungal colonies were added to Lysing Matrix E tubes (MP Biomedicals, USA) containing ceramic and glass beads to which a volume of 700 µl of buffer G2 was added. Lysis was performed using a FastPrep-24 5G (MP Biomedicals, USA) benchtop bead beating system. After lysis, samples were centrifuged and 200 µl of supernatant was collected for DNA extraction. Total DNA was extracted using a BioRobot EZ1 (Qiagen, Les Ulis, France) with the commercial EZ1 DNA Tissue Kit (Qiagen, France) according to the manufacturer's instructions. Two genomic barcode regions were amplified and sequenced, including rRNA internal transcribed spacers (ITS) 1 and 2 and a fragment of the β-tubulin gene (β-tub2). For accurate identification of specific fungal genera, several additional genomic regions were also amplified, including the D1/D2 domain of the large ribosomal subunit (LSU), the partial translation elongation factor 1-alpha (TEF-1-α) gene or the partial RNA polymerase II subunit 2 (RPB2) gene (Supplementary Table 2). For PCR, 5 µl of extracted DNA was added to 20 µl of PCR mix. The mix consisted of 12.5 µl Ampli Taq Gold™ 360 Master Mix (Applied Biosystems), 6 µl sterile DNase/RNase-free water and 0.75 µl

154 forward/reverse primers. PCRs were performed as follows: an initial 15 minutes denaturation
155 step at 95°C for 15 minutes, followed by 39 cycles of denaturation at 95°C for 1 minute,
156 annealing at 56°C for 30 seconds and extension at 72°C for 1 minute, followed by a final
157 extension step at 72°C for 5 minutes. DNA sequencing was performed using the 3500 Genetic
158 Analyser (Applied Biosystems, Inc, Villebon-sur-Yvette, France). Raw sequence reads were then
159 assembled and corrected using ChromasPro (Technelysium Pty Ltd, South Brisbane, Australia)
160 version 1.34. Sequence similarity searches were performed using the Basic Local Alignment
161 Search Tool (BLAST)n against both the rRNA/ITS and the standard GenBank nucleotide
162 databases at the National Center for Biotechnology Information (NCBI).

Table 1. Overview of the ITS1/ITS2 amplicon-based Illumina sequencing data.

	Raw reads (Comnined)[1]			Clean reads[2]			OTUs[3]	
	Total	Minimum	Maximum	Reads after quality filtering and denoising	Merged reads	Non-chimeric reads	Total	The number of assigned OTUs
ITS1	2,394,642	86,375	249,578	926,894	584,164	432,362	1040	595
ITS2				1,888,227	1,338,600	1,154,095	2195	1062

1, 2: The number of reads before and after DADA2 denoising and filtering based on 19 soil samples (14 PAH-polluted samples and 5 non-polluted samples).
3: The number of recovered Operational Taxonomic Units (OTUs) after clustering, and the number of OTUs that were assigned at the phylum level using UNITE database.

Table 2. Taxonomic distribution of fungal isolates identified through culturomics along with the ASVs recovered with ITS1/ITS2 amplicon-based metagenomics at each taxon level and according to reads counts chosen as cutoff.

	ITS1			ITS2			Culturomics
	Total (>1 read)	Total (>10 reads)	Total (>100 reads)	Total (>1 read)	Total (>10 reads)	Total (>100 reads)	Total
Phylum	8	5	4	12	11	7	3
Class	23	19	14	45	37	25	9
Order	63	53	35	116	96	61	18
Family	164	143	92	282	229	142	31
Genus	394	341	183	650	530	303	51
Species	563	486	244	1,062	861	439	165

Supplementary Table 2. Sets of primers employed for the amplification of the ITS, B-tub2, TEF1, LSU (28S), and RPB2 genetic regions.

Primers	Sequences	Targeted Regions
ITS1	TCCGTAGGTGAACCTGCGG	18S-5.8S
ITS2	GCTGCGTTCTTCATCGATGC	18S-5.8S
ITS3	GCATCGATGAAGAACGCAGC	5.8S-28S
ITS4	TCCTCCGCTTATTGATATGC	5.8S-28S
ITS1	TCCGTAGGTGAACCTGCGG	18S-5.8S, 5.8S-28S
ITS4	TCCTCCGCTTATTGATATGC	18S-5.8S, 5.8S-28S
Bt-2a	CATCGAGAAGTTCGAGAAGG	B-tub2
Bt-2b	TACTTGAAGGAACCCTTACC	B-tub2
EF1-728F	GGTAACCAAATCGGTGCTGCTTTC	TEF1
EF1-986R	ACCCTCAGTGTAGTGACCCTTGGC	TEF1
D1	AACTTAAGCATATCAATAAGCGGAGGA	28S
D2	GGTCCGTGTTTCAAGACGG	28S
fRPB2-5F	GAYGAYMGWGATCAYTTYGG	RNA polymerase II gene
fRPB2-7cr	CCCATRGCTTGTYYRCCCAT	RNA polymerase II gene
EF1 (*Trichoderma*)	ATGGGTAAGGARGACAAGAC	TEF1
EF2 (*Trichoderma*)	GGARGTACCAGTSATCATGTT	TEF1

2.5. ITS1 and ITS2 Metabarcoding

2.5.1. DNA extraction, PCR amplifications, and ITS Metabarcoding. Total DNA was extracted from 500 mg soil samples using FastDNA® Spin kit for soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. We used both ITS1 and ITS2 barcodes and two hybridization temperature in triplicates to maximize the fungal reads diversity. All the amplification procedures and the steps of the Illumina MiSeq sequencing (Illumina, Inc., CA, USA) until the production of the reads have been previously described [25]. The ITS1 and ITS2 barcodes were amplified using the ITS1-F (5'- CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'- GCTGCGTTCTTCATCGATGC-3') or the ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS3 (5'- GCATCGATGAAGAACGCAGC-3') primers, respectively, with Illumina sequencing adapters. PCR amplification was performed for each sample as follows: an initial denaturation step at 95°C for 10 min; 40 cycles of denaturation at 95°C for 5s, annealing, at 52C° or 55°C, for 30s, and extension at 72°C for 1 min; and a final extension step at 72°C for 5 min. Each PCR contained 12.5 µl of Ampli Taq Gold^{TM} 360 Master Mix, 6 µl sterile DNase/RNase-free water, 0.75 µl of each primer and 5 µl template DNA for a total reaction volume of 25 µl. PCR reactions were performed in triplicate, separately for each set of primers and for each annealing temperature (52 and 55°C). Amplicons obtained from the replicates of each sample for the two annealing temperatures and the two sets of primers were then pooled.

Next, dual-index barcodes were ligated to the amplicons. The amplified and barcoded library was then purified using AMPure beads (Beckman Coulter Inc., Fullerton, CA, USA). Our library, along with several other multiplexed libraries were then pooled together. The pooled library concentration was quantified using a high-sensitivity Qubit assay (Life technologies, Carlsbad, CA, USA) to ensure sequencing efficiency. The pool was then diluted to a final

concentration of 8pM, prior to loading the library for metabarcoding onto the MiSeq™ sequencing platform. The sequencing run employed a single 39-hour cycle for automated cluster generation and paired-end reads sequencing with dual index reads. Raw data underwent quality filtering based on defined read quality scores. Finally, the filtered reads were configured into fastq files for forward (R1) and reverse (R2) sequences.

2.5.2. Sequence analysis, visualization and statistical analyses. Sequence analysis was performed using the QIIME2 interface (Quantitative Insights into Microbial Ecology 2, version 2019.2) and its plug-ins [26]. Demultiplexing was performed using the Cutadapt plugin *(qiime cutadapt demux-paired)*. Noise reduction and quality control were performed using the DADA2 (deficiency of adenosine deaminase type 2) plug-in *(qiime dada2 denoise-paired)* [27]. Reads were trimmed and truncated to avoid low-quality scores (>235 bp for forward and >142 bp for reverse reads). Clustering was performed using the VSEARCH algorithm *(qiime vsearch cluster-features-closed-reference)*, then Taxonomic assignment was conducted with UNITE dynamic database version 2019.2, with 97-99% similarity *(qiime feature-classifier classify-sklearn)* [28]. Data generated using QIIME2 (OTUs table, Metadata file, and Taxonomy table) was then uploaded and processed using marker data profiling in MicrobiomeAnalyst version 2.0 [29]. Data was rarefied to the minimum library size for visual exploration of community profiling (alpha diversity, core mycobiome), and clustering analysis (interactive heatmap). Approximate maximum-likelihood phylogenetic trees [29] were constructed with FastTree in MEGA 11[30], [31]. The trees were then uploaded and visualized with Interactive Tree Of Life (iTOL) version 6.7.4 [32]. Bar plots and dot plots of fungal isolates and OTUs at each taxon level distribution were constructed using GraphPad Prism version 9.1.0 for windows (GraphPad Software, Boston,

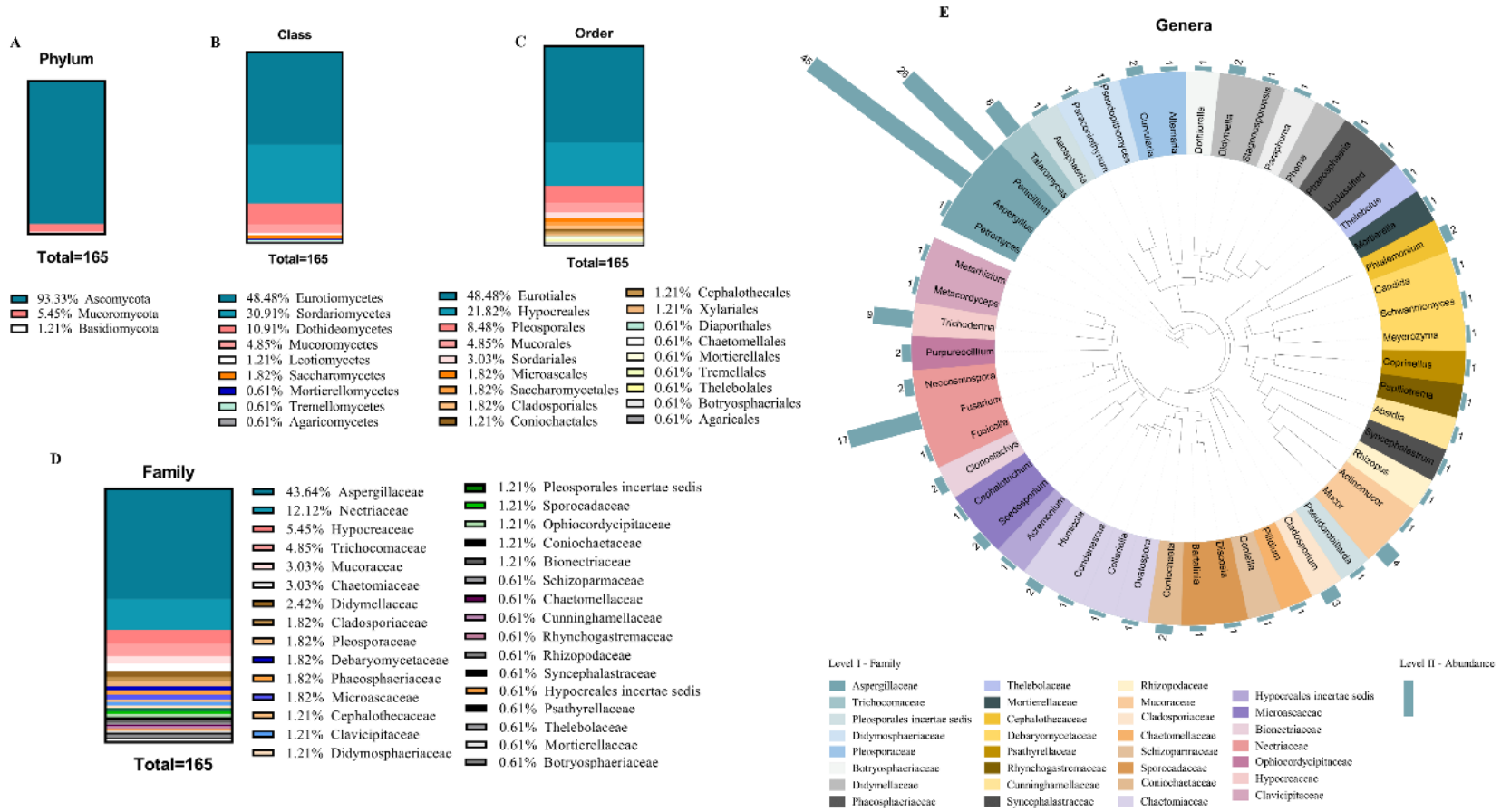
Massachusetts USA, www.graphpad.com). Venn Diagrams showing the comparison between different pollution profiles and fungal taxa recovered from different approaches were constructed using InteractiVenn [33]. Differential abundance features were visualised using heatmaps and extended error bars using the Statistical Analysis of Metagenomic Profiles (STAMP) Version 2.1.3 graphical software, with Welch's t-test for statistical support [34].

3. Results

3.1.The fungal culturomics approach

3.1.1. Fungal identification and diversity

All 14 soil samples analysed here yielded positive fungal cultures. A total of 742 fungal colonies were purified from the initial mixed fungal colonies, subcultured for 5-7 days and identified by, either or both, MALDI-TOF MS and Sanger sequencing. One hundred and sixty-five different fungal species were identified using this comprehensive approach, belonging to 51 genera, 31 families, 18 orders, 9 classes and 3 phyla (Figure 1).



243

244 **Figure 1.** Relative abundance of fungi isolated through the culturomics approach at each taxon level (A Phylum, B Class,
245 C Order, D Family, and E Genus). These graphs (A,B,C and D) were constructed using GraphPad Prism version 9.1.0 (GraphPad
246 Software). (E), Phylogenetic distributions of the 51 fungal genera isolated through the Culturomics approach. Colored ranges
247 identify fungal families within the tree. The phylogenetic tree was generated with the maximum-likelihood method by
248 FastTree and uploaded to iTOL (Interactive Tree Of Life). Colored bars represent the relative abundance of each genera.

Of the 165 fungal species isolated, 65 (39.4%) were successfully identified by MALDI-TOF mass spectrometry with LSV ≥1.95 (mean LSV = 2.21) and 100 (60.6%) by multilocus Sanger sequencing. (Supplementary Table 3). In addition, 39 of the 65 fungal species identified by MALDI-TOF mass spectrometry were also sequenced for confirmation, so 139 (84.2%) of the 165 isolated species underwent Sanger sequencing, either for identification or confirmation. The mass spectra of 56 isolates identified in this study, whose spectral fingerprints were not included in the reference database prior to this study, were added to our in-house reference mass spectra database allowing future identification of these environmental isolates.

Among the 165 fungal species isolated in this study, the majority (93.3%, 154 isolates) belonged to the Ascomycota phylum (Figure 2), while the remaining minority were distributed among Mucoromycota (5.5%, 9 isolates) and Basidiomycota (1.2%, 2 isolates). Thirty-one different fungal families were identified from the 165 isolates recovered, with Aspergillaceae being the most abundant (43.6%, 72 isolates), followed by Nectriaceae (12.1%, 20 isolates), Hypocreaceae (5.5%, 9 isolates) and Trichocomaceae (4.8%, 8 isolates). At the genus level, analysis of the 165 isolates showed that the soil fungal community was dominated by *Aspergillus* (27.3%, 45 isolates), *Penicillium* (15.8%, 26 isolates) and *Fusarium* (10.3%, 17 isolates), with *Trichoderma* (5.5%, 9 isolates) and *Talaromyces* (4.8%, 8 isolates) being relatively rare (Figure 2).

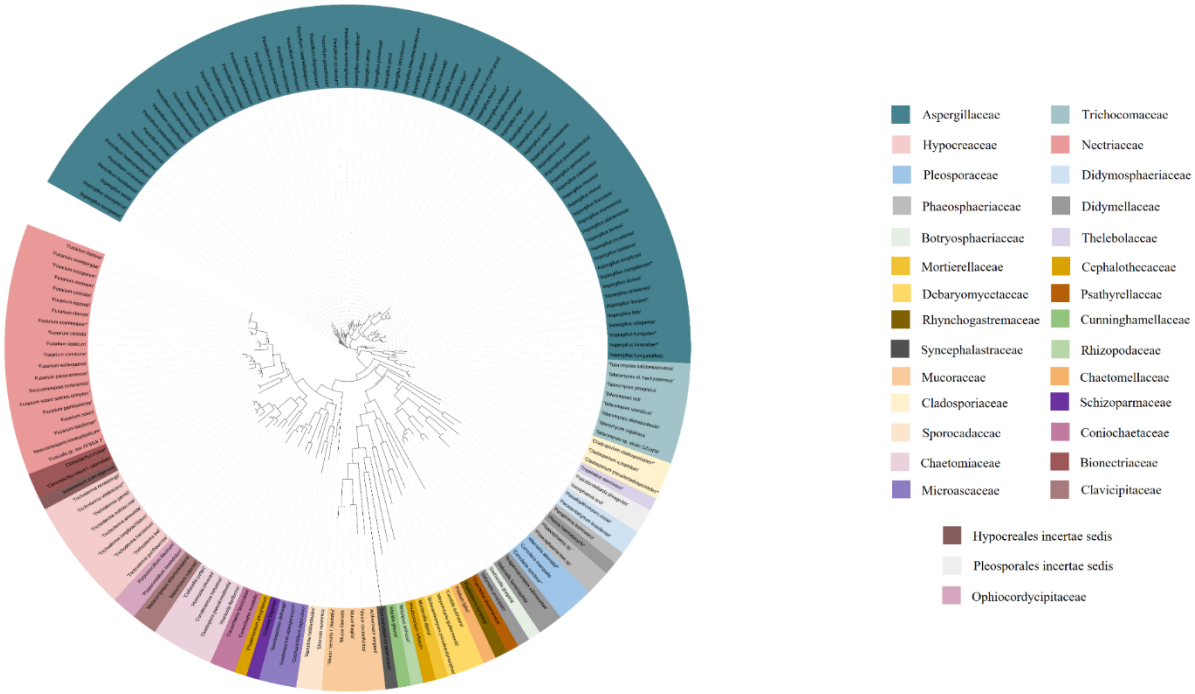


Figure 2. Phylogenetic distributions of the 165 fungal species isolated through the Culturomics approach. Colored ranges identify fungal families within the tree. The phylogenetic tree was generated with the maximum-likelihood method by FastTree and uploaded to iTOL (Interactive Tree Of Life).

Fungal species richness in PAH-polluted soils ranged from 8 to 30 per sample ($\bar{x}$ = 18.2). In contrast, the unpolluted soils exhibited a significantly higher fungal richness ranging from 17 to 34 species per sample ($\bar{x}$ = 25.8). *Purpureocillium lilacinum* was the most prevalent isolate, recovered from 9 samples, followed by *Fusarium oxysporum* and *Penicillium sizovae*, recovered from 8 samples each. *Mortierella alpina* was also isolated from 7 of the 14 samples, while *Aspergillus wentii* and *Aspergillus tubingensis* were identified in six samples. Furthermore, *Aspergillus niger*, *Aspergillus flavus* and *Fusarium solani* were isolated from 5 samples.

3.1.2. Polluted vs. Unpolluted Samples

Nine of the 14 soil samples analysed exhibited a profile indicative of PAH pollution, while the remaining five did not. Culturomics analyses revealed that the two sample groups displayed contrasting fungal communities, which were associated with contrasting pollution profiles. This dissimilarity was observed at both the species and genus levels. Only 24 (14.5%) of the 165 identified species, and 11 (21.6%) of the 51 identified genera, were common to both groups. Furthermore, additional analysis indicated that unpolluted soils exhibited a markedly higher diversity in *Penicillium* species compared to PAH-polluted soils, despite a smaller sample size (Figure 3A-C).

3.1.3. Culture media contribution to this approach

In this study, five distinct culture media were selected for the fungal culturomics approach. All isolates were incubated at 25°C under aerobic conditions. The culturomics approach yielded 165 distinct fungal species, with 17 species recovered with all five culture media. Of the five culture media employed in this study, FastFung emerged as the most effective medium yielding the highest number of fungal isolates 78/165 (47.3%), followed by Potato Dextrose Agar with 66/165 isolates (40%) and Sabouraud Dextrose Agar GC2 with 65/165

296 isolates (39.4%). Finally, ChromAgar and Malt Extract Agar permitted the isolation of 57/165
297 (34.5%) and 54/165 (32.7%) distinct fungal isolates, respectively (Figure 3D).
298 Furthermore, the analysis of the contribution of each culture medium revealed that FastFung not
299 only supported the growth of the greatest overall number of fungal isolates, but also yielded the
300 highest number of exclusive fungal isolates (32/98, 32.7%). It is noteworthy that these 32 fungal
301 isolates were recovered exclusively on FastFung medium, thereby demonstrating its superiority
302 in isolating fungal isolates that were not recoverable on other tested culture media (N=98).

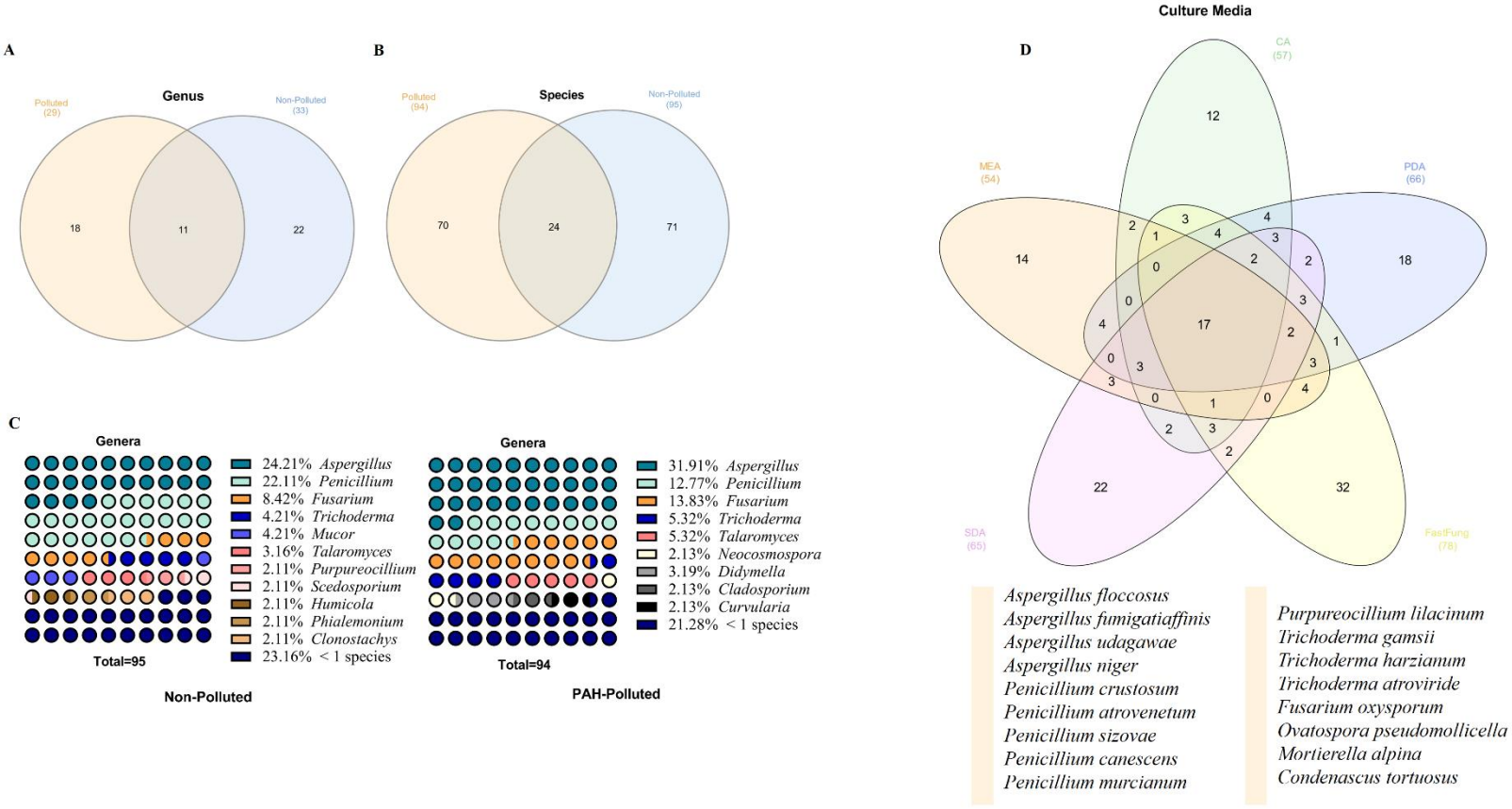


Figure 3. Variations in fungal community structure between PAH-polluted and non-polluted soils, visualized by Venn diagrams and parts of a whole dot plots (A-C).Venn Diagrams (A,B) depict the unique and shared fungal taxa at the genus and species levels between the two groups of analysed soils. (C) Parts of a whole dar plots representing the distribution of isolated fungi at the genus level, both in PAH-polluted and non-polluted soils. Dot plots were constructed using GraphPad Prism version 9.1.0 (GraphPad Software). The contribution of each culture medium used in the culturomics approach was depicted in a Venn diagram (D)., revealing the unique and shared fungal species isolated using each of these media.Venn diagrams (A, B and D) were constructed using InteractiVenn - https://www.interactivenn.net/

3.2. Fungal community composition using ITS1 and ITS2 Amplicon Sequencing

3.2.1. Amplicon sequencing analysis and processing

In this study, high-throughput sequencing technology was employed to assess the fungal diversity within 19 soil samples displaying different pollution profiles. The sequencing of both the ITS1 and ITS2 regions yielded a total of 2,394,642 raw reads, with a range of 86,375 to 249,578 reads per sample. Following denoising with DADA2, which filters low-quality sequences and chimeras, 1,586,457 high-quality reads were retained. The high-quality reads were subsequently clustered into Amplicon Sequence Variants (OTUs) with a 97% similarity, resulting in 3,235 unique fungal signatures. Of these, 1,657 (51.2%) were assigned at the phylum level, whereas 1,578 remained unassigned (48.8%) (Table 1). Among the taxonomically classified OTUs (>1 read), ITS1 sequencing revealed 595 OTUs, translating to 563 distinct species, 394 genera, 164 families, 63 orders, 23 classes, and 8 phyla. In contrast, ITS2 sequencing identified 1,062 OTUs corresponding to 1,062 distinct species, 650 genera, 282 families, 116 orders, 45 classes, and 12 phyla (Table 2).

3.2.2. Fungal community structure and diversity

The ITS1 barcode region analysis revealed that Ascomycota was the dominant phylum within the soil mycobiome, accounting for 99.4% of the recovered fungal reads. Basidiomycota represented a minor component, comprising only 0.4% of the total reads. At the class level, Dothideomycetes was the most abundant fungal class, comprising 37.4% of the reads, followed by Sordariomycetes (28.8%) and Eurotiomycetes (22.3%). The order Pleosporales emerged as the most prevalent within the fungal community, contributing to 29.7% of the total reads. This was followed by Eurotiales, Hypocreales and Glomerellales, which contributed 17%, 16.7% and 6.4%, respectively (data not shown). Analysis of the ITS2 region yielded similar results, with Ascomycota again dominating the soil mycobiome (88.6% of reads). However, analysis of the ITS2 region revealed a higher abundance of Mortierellomycota (5.9%) compared to ITS1 (0.03%). Additionally, Basidiomycota represented a slightly greater proportion in the ITS2 analysis (3.7% vs. 0.4% with ITS1). At the class level, Sordariomycetes emerged as the most prevalent class (35.3%), followed by Dothideomycetes (23.9%), which contrasted with its dominance in the ITS1 analysis (37.4%). Eurotiomycetes and Mortierellomycetes were also identified as notable classes using ITS2 sequencing, contributing to 20.1% and 5.9% of total reads, respectively. The order Pleosporales remained dominant, contributing to 18.8% of total reads. However, ITS2 sequencing identified Hypocreales (18%), Eurotiales (15.3%) and Sordariales (7.3%) at abundances comparable to Pleosporales, suggesting a more even distribution within this taxonomic level. As shown in Fig. 4D, the ITS2 analysis identified Aspergillaceae as the predominant fungal family within soil samples, accounting for 15.2% of the total sequence reads. This was followed by Nectriaceae (10.8%) and Mortierellaceae (5.7%). These three fungal families were the most prominent in the soil samples. To provide a visual

representation of the taxonomic distribution of the most dominant fungal genera within the soil samples, a phylogenetic tree encompassing the top 100 most abundant fungal genera was constructed. These genera collectively accounted for approximately 90% of the total read counts, spanning across eleven fungal classes: The most prevalent fungal classes were Sordariomycetes (38%), Dothideomycetes (26%), Eurotiomycetes (11%), Leotiomycetes (7%), Class Incertae sedis (an unclassified class) (7%), and Mortier The remaining 1% of the fungal kingdom is represented by the phyla Ellomycetes (3%), Agaricomycetes (3%), Tremellomycetes (2%), Archaeorhizomycetes (1%), Orbiliomycetes (1%) and Geoglossomycetes (1%) (Figure 4E).

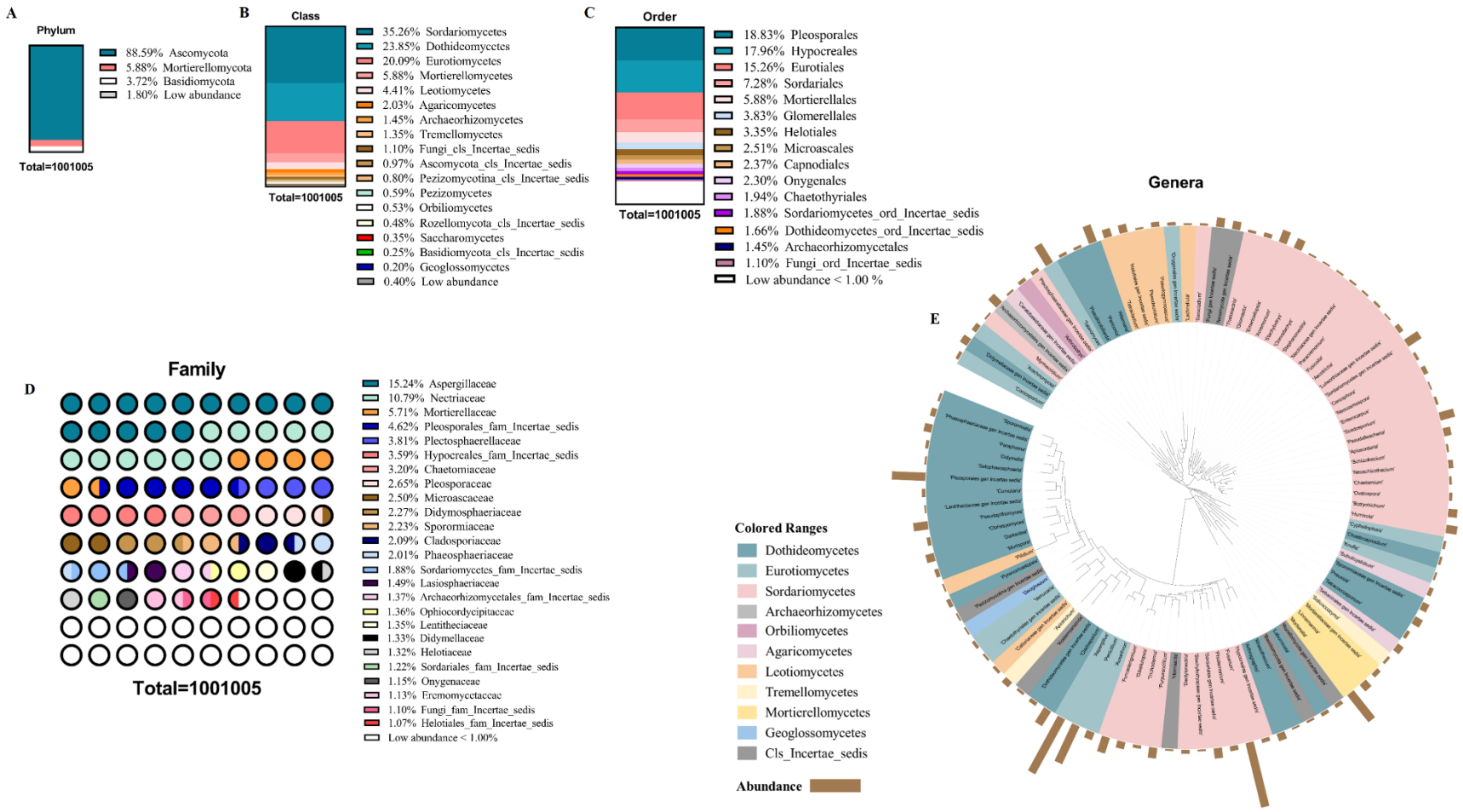
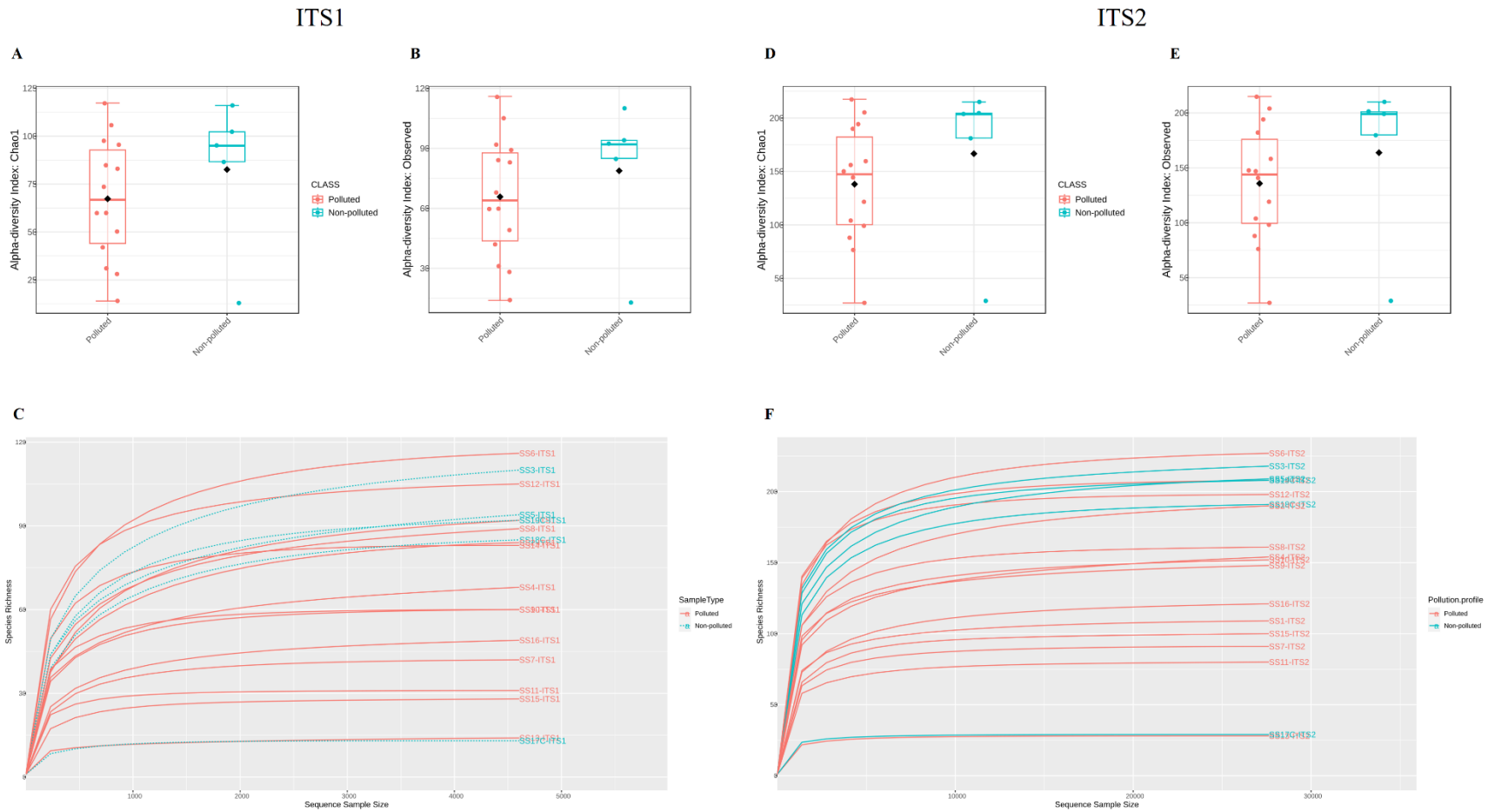


Figure 4. Relative abundance of fungal (A) Phylum, (B) Class, (C) Order, (D) Family and (E) Genus recovered through the ITS2 metabarcoding approach within 19 soil samples displaying various pollution profiles. (E) Phylogenetic distributions of the top 100 fungal genera accounting for ≈ 90% of total OTUs read counts. Colored ranges identify fungal classes within the tree. Colored bars represent the relative abundance of each genera. The phylogenetic tree was generated with the maximum-likelihood method by FastTree and uploaded to iTOL (Interactive Tree Of Life). Bar plots (A-C) and dot plots were constructed using GraphPad Prism version 9.1.0 (GraphPad Software).

3.2.3. PAH impact on soil fungal communities

The high-throughput sequencing of the ITS2 region was employed to assess and compare the composition of fungal communities between PAH-polluted and unpolluted soil samples. A total of 718,459 reads were recovered from the polluted group, which were assigned to 966 operational taxonomic units (OTUs) with an average number of 51,318.5 reads per sample. In contrast, the unpolluted group yielded a lower total read count (282,546), translating into 630 assigned OTUs with an average number of 56,509.2 reads per sample. Notably, despite the considerable disparity in total read counts, both groups exhibited a comparable average number of reads per sample. Alpha diversity indices, which permit an estimation of species richness within a sample, were calculated for both the PAH-polluted and unpolluted soil communities. Both the observed species richness and the Chao1 index exhibited a tendency to be higher in soil samples with no pollution profile compared to the PAH-polluted ones (Supplementary Figure 1).

Ascomycota was the dominant phylum in both PAH-polluted and unpolluted soils, accounting for 88% and 90.7% of total reads, respectively. The second most abundant phylum in PAH-polluted soils was Mortierellomycota (7.8%), followed by Basidiomycota (2.5%). Conversely, the proportion of Basidiomycota (6.8%) was greater than that of Mortierellomycota (0.9%) in unpolluted soils. The analysis at the class level revealed that Sordariomycetes and Dothideomycetes were the most dominant classes across both PAH-polluted and unpolluted soils. The classes exhibited comparable relative abundances in both soil profiles, with Sordariomycetes representing 34.1% and 38.3% and Dothideomycetes representing 23.8% and 24%. Eurotiomycetes also exhibited consistent abundance in both soil profiles, with a mean relative abundance of 21.6% and 16.9% in polluted and unpolluted soils, respectively.



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387 **Supplementary Figure 1.** Alpha diversity metrics estimates of the fungal communities in both soil groups (A, D Chao1 from ITS1

388 and ITS2 datasets, respectively; B, E Observed Richness from ITS1 and ITS2 datasets, respectively). Rarefaction curves for each soil

389 sample in both soil groups (C through the ITS1 dataset, F through the ITS2 dataset).

It is noteworthy that PAH contamination appeared to select for Mortierellomycetes (7.8% in polluted soils) with respect to the prevalence of Leotiomycetes in unpolluted soils (5.6%).

To identify taxa that are differentially abundant between PAH-polluted and unpolluted soils, we employed differential abundance analysis (Welch's t-test) at lower taxonomic levels (order, family, genus, and species). Furthermore, LEfSe (Linear Discriminant Analysis Effect Size) was employed to identify biomarker taxa. The differential abundance analysis revealed a significant enrichment of the order Mortierellales exclusively in soils with a PAH-pollution profile. This trend was also observed at lower taxonomic levels, with the family Mortierellaceae, and consequently at the genus level with Mortierella and Linnemania, which exhibited significant abundance increases in PAH-polluted soils (Fig. 5A). While differential abundance analysis revealed taxa displaying significant abundance shifts, LEfSe identified those that were particularly influential in differentiating fungal communities between PAH-polluted and unpolluted soils. At the order level, the Orbiliales and an unclassified Basidiomycota order were found to be enriched in unpolluted soils, while the Lulworthiales were associated with PAH-polluted soils (not shown). This trend was also observed at the family level. The Cephalothecaceae, Orbiliaceae, and an unclassified Basidiomycota family were enriched in unpolluted soils, while the Lulworthiaceae and an unclassified Hypocreales family were enriched in polluted soils (LDA score > 2) (Fig. 5B). Similarly, LEfSe identified differentially abundant genera with biomarker implications. The genera *Phialemonium*, *Ovatospora*, *Coniosporium*, and an unclassified Basidiomycota genus were found to be enriched in unpolluted soils, while the genera *Emericellopsis*, *Gliomastix*, and two unclassified genera belonging to the families Lulworthiaceae and Sporormiaceae were found to be enriched in PAH-polluted soils. The LEfSe analysis was also extended to the species level, where OTUs were identified that were associated

413 with either PAH-polluted or unpolluted soils. It is noteworthy that *Phialemonium inflatum*,
414 *Arthrobotrys amerosporus, Ovatospora mollicella,* and *Humicola repens* were enriched in
415 unpolluted soils, while *Aspergillus alabamensis* and *Gliomastix roseogrisea* were enriched in
416 PAH-polluted soils (LDA score > 2) (Fig. 5B). The Venn diagrams displayed the unique and
417 shared taxa at the family, genus, and species levels between the two soil groups. Soils
418 contaminated with PAHs and uncontaminated soils shared a total of 175 families, 325 genera and
419 443 species.

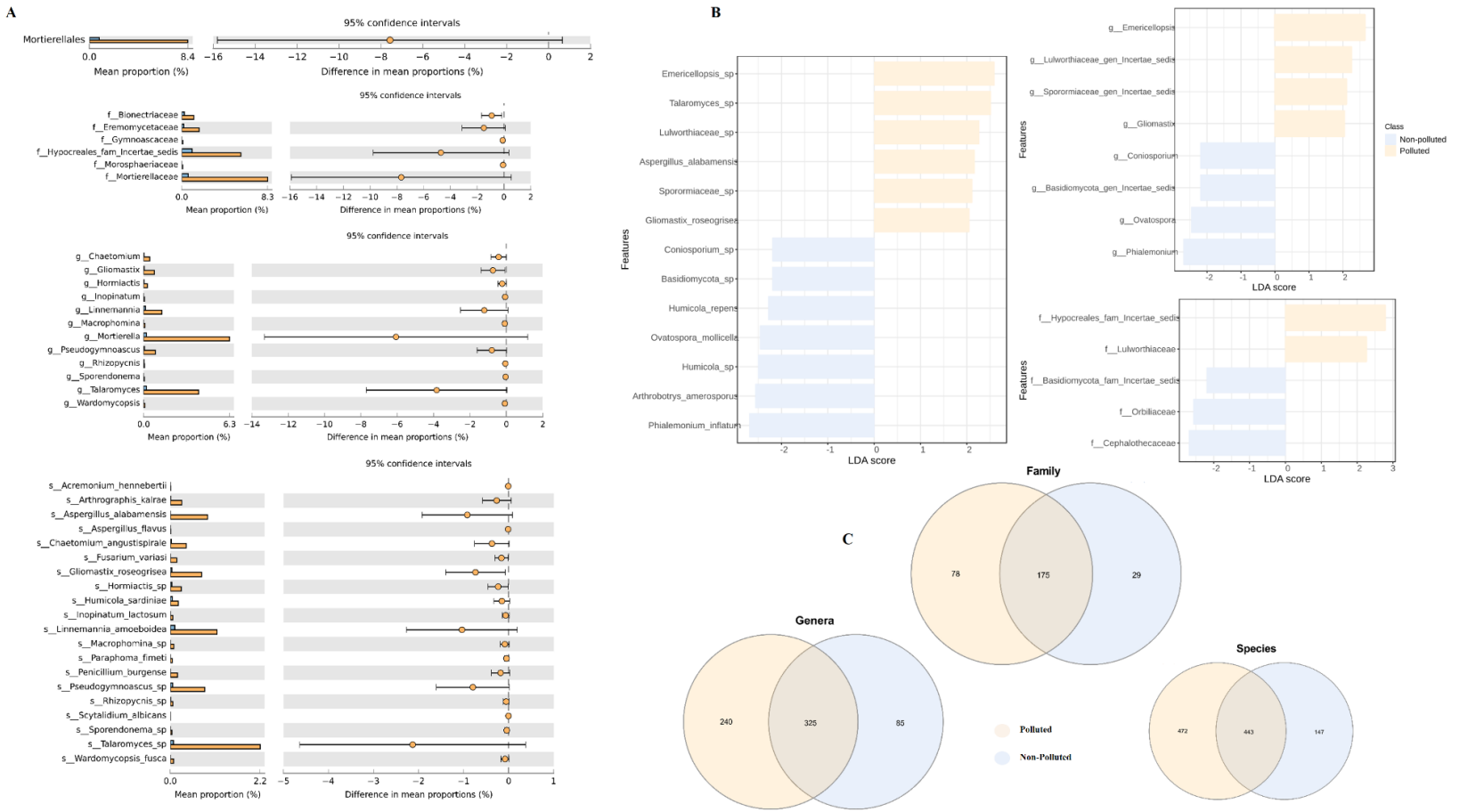

Figure 5. Differential abundance analysis (Welch's t-test, $p<0.05$) displayed fungal taxa exhibiting significant differences in abundance between the two soil groups at the order, family, genus and species level (A). LEfSe analysis (B) identified biomarker fungal taxa exhibiting both differential abundance and biological relevance in differentiating between the two soil groups ((LDA significance threshold > 2.0). Venn diagrams depicting the proportion of exclusive and shared fungal features at different taxa (family, genus and species) between the two soil groups.

However, they displayed remarkable dissimilarities, as evidenced by the unique taxa recovered from each group (Fig. 5C).

3.3. Soil Mycobiome through a holistic approach

Unique and shared classes, orders, families and genera across all 14 soil samples using a combined approach of culturomics, ITS1 and ITS2 metabarcoding were presented in Venn diagrams (Fig. 6A). Notably, the analysed soil samples exhibited a core fungal community comprising 8 classes, 12 orders, 19 families and 27 genera. Within the common classes, Sordariomycetes, Dothideomycetes and Eurotiomycetes were the most abundant. Similarly, within the shared orders, Eurotiales, Hypocreales and Pleosporales were the most abundant, regardless of the analysis method used. Interestingly, when analysed at the common family level, Aspergillaceae, followed by Nectriaceae, were consistently the most dominant in all three approaches. This trend was also observed at the genus level, with Aspergillus, Penicillium and Fusarium identified as the three most common taxa across all three analysis approaches. At the genus level, core fungal communities were defined as the common fungal genera recovered from soil samples analysed using all three approaches. The 27 core fungal genera were plotted in the phylogenetic tree below (Fig. 6B). These 27 genera accounted for a remarkable 81% of the species isolated using the culture approach. Furthermore, these same genera also contributed significantly to the fungal communities detected by metabarcoding, representing approximately 49% and 39% of the total reads recovered from ITS1 and ITS2 amplicon-based sequencing, respectively. These results highlight the importance and prevalence of these 27 core genera within soil fungal communities.

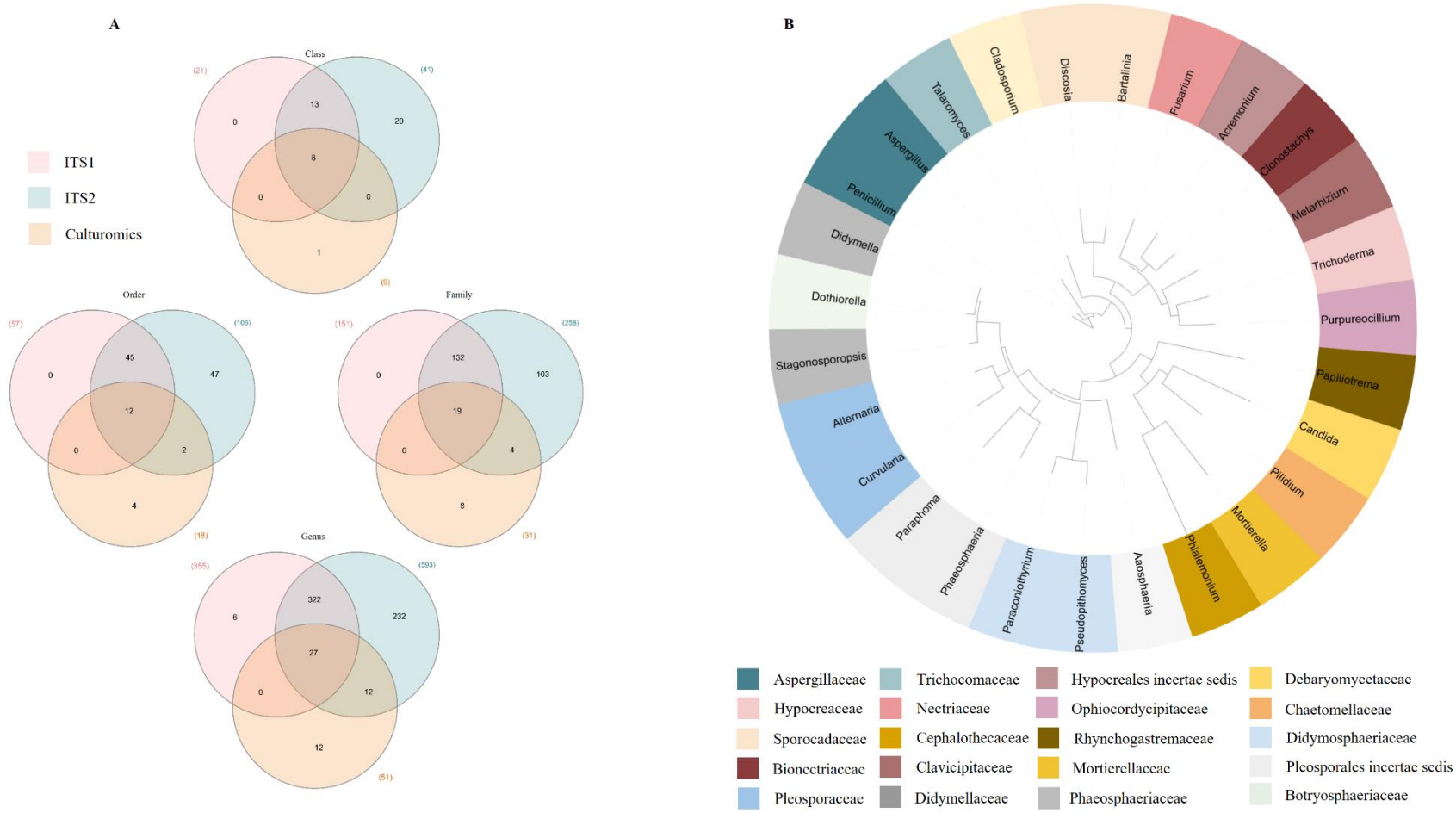

Figure 6. A Venn diagrams depicting the proportion of exclusive and shared fungal features at different taxon levels (Class, order, family, and genus) between the different approaches employed in this study (Culturomics, ITS1, ITS2). B Phylogenetic distribution of the core fungal mycobiome within soil samples analyzed in this study. Displayed are the 27 shared fungal genera recovered from analyzed soil samples using all three approaches. Colored ranges represent fungal families within the tree. The phylogenetic tree was generated with the maximum-likelihood method by FastTree and uploaded to iTOL (Interactive Tree Of Life).

4. Discussion

Fungi, one of the most taxonomically diverse eukaryotic kingdoms, are fundamental drivers of nutrient cycling and decomposition in terrestrial ecosystems [6]. In this study, we aimed to compare the fungal community structure of PAH-polluted and unpolluted soils collected across southern France. In particular, for the first time in this context, we have used a combined approach using culturomics alongside ITS1/ITS2 amplicon-based metagenomics to comprehensively characterise the soil mycobiome. This dual approach, using complementary techniques, aims to overcome the limitations of each of these methods alone.

Using the culturomic approach, a total of 165 species belonging to 51 genera, 31 families, 18 orders, 9 classes and 3 phyla were isolated using five different culture media and identified by barcode-based Sanger sequencing or MALDI-TOF mass spectrometry. Notably, the isolates included rare Ascomycota species such as *Collariella carteri* and *Bartalinia robillardoides* [34]. To the best of our knowledge, this is the first study to report the isolation of *Bartalinia robillardoides*, *Stagonosporopsis valerianellae*, *Discosia ravennica*, *Didymella prosopidis*, *Pseudorobillarda phragmitis*, *Fusarium coriorum*, *Aspergillus puulaauensis*, *Aspergillus montoensis*, *Aspergillus creber*, *Fusarium gamtoosense*, *Penicillium arabicum*, *Trichoderma lixii*, *Penicillium philippinense*, *Talaromyces rugulosus*, *Ovatospora pseudomollicella*, *Aspergillus westerdijkiae*, *Pseudopithomyces rosae*, *Coniochaeta mutabilis*, *Trichoderma nothescens*, *Penicillium magnielliptisporum*, and *Fusarium parceramosum* directly from soil samples. Furthermore, this approach allowed the isolation of a novel fungal species, currently undergoing taxonomic characterization. *Aspergillus*, followed by *Penicillium* and *Fusarium* were the predominant cultivable genera, accounting for over 50% of the fungal species isolated using this approach. Of the five culture media used in this approach, FastFung, a relatively new

477 medium [23], was found to be particularly effective in isolating soil fungi. Indeed, FastFung
478 yielded a significant proportion of the total number of fungal species (47.3%, or 78/165) and
479 recovered the highest number of exclusive fungal species (32.7%, 32/98), suggesting that
480 FastFung is superior to the other four media in supporting the growth of diverse soil fungal
481 communities. Interestingly, however, each of the five media allowed the isolation of a unique set
482 of species not captured by the other four. For example, MEA, CA, PDA and SDA yielded
483 exclusive isolates at rates of 14.3%, 12.2%, 18.4% and 22.4% respectively. Therefore, the
484 combination of all five media is likely to provide the most comprehensive characterisation of soil
485 fungal community structure by capturing and recovering a wider range of fungal isolates. While
486 the application of fungal culturomics combined with ITS1/ITS2 amplicon-based metagenomics
487 has recently been explored to study the human gut mycobiome [21] and the fungal communities
488 associated with the natural habitat of the caterpillar fungus, *Ophiocordyceps sinensis* [35], this
489 approach has not been extensively applied to directly investigate the composition of soil fungal
490 communities. In this respect, our work represents one of the first attempts to use this combined
491 strategy to comprehensively characterise the soil mycobiome. Next-generation sequencing
492 (NGS)-based metabarcoding approaches have revolutionised the study of environmental
493 microbial communities, especially in soil environments [9], [11], [36], [37]. Over the past
494 decades, the internal transcribed spacer region of ribosomal DNA has emerged as the gold
495 standard barcode marker for the characterisation of fungal communities [11], [12], [38].
496 Therefore, in the present study, amplicon-based metagenomics was used in parallel with the
497 culturomics approach to investigate the composition of fungal communities in 19 soil samples
498 with different contamination profiles. Analysis of the NGS data revealed a rich diversity of
499 fungal taxa within the soil samples. A total of 595 and 1,062 assigned OTUs were recovered

500 from the ITS1 and ITS2 datasets, respectively. When combined, a total of 1,087 assigned OTUs
501 were obtained, of which 25 were unique to the ITS1 dataset, 524 were unique to the ITS2
502 dataset, while 538 taxa were common to both datasets. Interestingly, combining the results from
503 both datasets provided a more comprehensive picture of the soil mycobiome, encompassing a
504 total of 1,087 assigned OTUs, 657 genera, 282 families, 116 orders, 45 classes and 12 phyla.
505 This integrative approach revealed a remarkable degree of disparity between the fungal
506 communities recovered using ITS1 and ITS2. Most fungal taxa identified using the ITS1 region
507 were also recovered in the ITS2 dataset, but not vice versa, suggesting the existence of a core
508 mycobiome captured by both regions. We observed that ITS2 sequencing yielded a greater
509 number of unique taxa at lower taxon levels, allowing for a broader and more comprehensive
510 understanding of the soil mycobiome, and therefore most of the visualisations shown in the
511 results section were configured based on the ITS2 rather than the ITS1 datasets.
512 For a direct comparison with the culturomics approach (Fig. 6), only ITS1/ITS2
513 metabarcoding datasets from the same 14 soil samples subjected to culturomics were considered.
514 While both approaches identified the same dominant taxa at the top of each taxonomic level, ITS
515 metabarcoding provided a broader view of the overall microbial community structure. However,
516 it is important to recognise the limitations associated with the short Illumina reads generated by
517 this approach, as they may not provide sufficient information for accurate identification at lower
518 taxonomic levels, particularly at the species level. Furthermore, relying solely on the ITS region
519 for fungal identification presents additional challenges. For example, several fungal genera,
520 including *Penicillium*, *Talaromyces*, *Aspergillus*, *Trichoderma* and *Fusarium*, require sequencing
521 of additional barcode regions such as the β-tubulin (β-tub) gene fragment, translation elongation
522 factor 1-alpha (TEF) or the RNA polymerase II subunit 2 (rpb2) gene for accurate species

identification [39]-[41]. The culturomic approach used in this study provided a different perspective on soil fungal community structure compared to the amplicon-based metagenomic approach. While these approaches proved to be complementary, they provided unique insights into the soil mycobiome. These observations are consistent with reports from other studies using a combined ITS metabarcoding and culturomics approach to assess fungal diversity [21], [35]. Of the 51 fungal genera isolated by culturomics, 27 were common to all three datasets, 12 genera overlapped with the ITS2 dataset, while a remarkable 12 genera were exclusive to the culturomics approach. These results once again highlight the importance of culturomics as a complementary approach to ITS metabarcoding. The inability of the ITS1/ITS2 amplicon sequencing approach to detect all fungal isolates recovered by culturomics can be attributed to biases introduced during the DNA extraction step and PCR amplification within amplicon metabarcoding methods. While the use of Amplicon Sequence Variants (ASVs) is gaining traction, OTU analysis remains a potent tool for studying fungal community structures in soil. In fact, established methodologies with defined sequence similarity thresholds enable easier comparison with previous works and studies on the soil mycobiome. Moreover, OTU analysis provides sufficient resolution and offers computational efficiency when analysing broader microbial community patterns like diversity metrics, richness, and family/genus abundance, whereas ASVs provides a finer resolution for studies requiring species-level differentiation.

The selection of fungal genera consistently detected in all three datasets revealed a core mycobiome. This compact community was dominated by Dikarya, particularly Ascomycota, which accounted for the vast majority of total reads and abundance in each dataset. The dominance of Ascomycota in soil environments is well documented. A recent global study using ITS2 amplicon-based metagenomic comparisons of 235 soil samples worldwide found that only

546 83 Ascomycota phylotypes dominated these soils [11]. Notably, these dominant Ascomycota
547 phylotypes mainly belonged to the classes Sordariomycetes, Eurotiomycetes, Leotiomycetes and
548 Dothideomycetes. Remarkably, our findings in the soil environments of the South of France are
549 in perfect agreement with this global pattern.

550 Both approaches used in this study revealed notable shifts and dissimilarities in fungal
551 community structure between PAH-contaminated and unpolluted soils. However, in our study,
552 alpha diversity indices (species richness and Chao1) were not consistently and significantly
553 higher in all unpolluted soils compared to PAH-contaminated soils. While this lack of significant
554 difference in fungal richness has been reported in previous studies [36], other studies dealing
555 with petroleum hydrocarbon contamination have reported contrasting results [42]. Interestingly,
556 the culturomic approach highlighted the genus *Penicillium* as potentially sensitive to pollution
557 and thus a potential indicator genus. The number of *Penicillium* species isolated from unpolluted
558 soils was significantly higher, despite fewer total samples in the PAH-polluted group. On the
559 other hand, differential abundance analysis using ITS2 datasets identified Mortierellales,
560 Mortierellaceae and their associated genera, *Mortierella* and *Linnemania*, as exclusively enriched
561 in PAH-polluted soils. Further analysis with LEfSe revealed that the order Orbiliales and
562 families Cephalothecaceae and Orbiliaceae were associated with an unpolluted soil profile, while
563 Lulworthiales and Lulworthiaceae correlated with PAH contamination. Similarly, recovered
564 genera *Coniosporium*, *Ovatospora*, and *Phialemonium* correlated with unpolluted samples,
565 whereas *Gliomastix*, *Emericellopsis*, and some unclassified genera were associated with PAH
566 contamination. Our observations on the effect of PAH contamination on soil fungal communities
567 provide new insights that complement previous work. While some fungal genera, such as
568 *Penicillium*, showed sensitivity to PAH-contamination, another study reported a positive

association between PAH contamination and the genera *Alternaria*, *Trichoderma*, *Penicillium*, *Absidia*, *Acremonium*, *Cadophora* and *Aspergillus* [36]. In the same study, the orders Sordariales and Glomerellales were also positively correlated with elevated PAH levels.

5. Conclusions

In conclusion, our results highlight the complementary nature of culturomics and amplicon metagenomics in the elucidation of the complete fungal repertoire in soil environments. In particular, culturomics provided critical insights at lower taxonomic levels, especially at the species level, allowing a more accurate characterisation of the soil mycobiome. Furthermore, the culture-dependent nature of culturomics allowed isolation and description of novel fungal species, potentially unattainable by amplicon-based metagenomics and NGS approaches. In addition, the fungal isolates obtained using this culture-dependent approach hold promise for future applications in bioremediation and other biotechnological fields. Utilising the unique potential of culture-based approaches to discover undescribed fungal communities, together with the power of ITS metabarcoding to provide a broader understanding of the mycobiome, future studies using this combined approach have great promise to unravel the complex soil mycobiome and expand our knowledge of the fungal community structure of this important ecosystem.

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Data Availability Statement:

Nucleotide Sequence Accession Numbers: Raw ITS metabarcoding sequence data are publicly available in the NCBI Sequence Read Archive (SRA) under accession numbers SRX24753009-SRX24753046. Accession to cite for these SRA data: PRJNA1118208.
Taxonomy table and read counts recovered from the ITS1 and ITS2 datasets are displayed in Supplementary Table 4 and Supplementary Table 5, respectively.

Sequence data resulting from Sanger sequencing are publicly available in the NCBI GenBank and BankIt and in Supplementary Table 6. ITS (PP851664-PP851799), β-tub (PP892421-PP892523), TEF (PP869633- PP869652), D1D2 (PP860821- PP860826), and RPB2 (PP869628-PP869632).

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Article 7

Metagenomic analysis of gut microbiota diversity across treatment stages in algerian patients with colorectal cancer

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(En cours de préparation)

Article 8

Genomic features of carbapenem-resistant *Klebsiella pneumoniae* isolated in French Polynesia and novel transposon responsible for $bla_{\text{NDM-1}}$ dissemination

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(En cours de préparation)

Title: Description of a novel transposon responsible for *blaNDM-1* dissemination in carbapenem-resistant *Klebsiella pneumoniae* from French Polynesia

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Abstract

Background

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP), a significant nosocomial pathogen, causes severe infection diseases that constitute a critical threat to global health. The genomic characterization of CRKP isolated in French Polynesia and the mobile genetic elements (MGEs) responsible for their dissemination remains incompletely explored.

Objective

The aim of this study was to characterize CRKP strains isolated in Tahiti, to analyze their phenotypic antibiotic resistance, complete genomic sequence, resistance gene profile and MGEs associated with carbapenemase genes.

Materials and Methods

We studied seventeen CRKP isolates from 2006 to 2021 in Tahiti to evaluate the antimicrobial susceptibility. Whole-genome sequencing was performed to identify antibiotic-resistant genes, comparative genomic analysis and MGE analyses.

Results

These 17 CRKP strains exhibited high levels of resistance to ampicillin, third- and fourth-generation cephalosporins and to ertapenem, two of which were resistant to imipenem. The carbapenemase gene $bla_{\text{NDM-1}}$ and $bla_{\text{NDM-9}}$ were found in five and two CRKP strains, respectively, while ten lacked these genes. Phylogenetic analysis revealed polyclonal dissemination of NDM-producing *K. pneumoniae* among the isolates that belonged to seven different sequence types (ST). Interestingly, a novel transposon associated with four genes ble_{MBL}, $bla_{\text{NDM-1}}$, *qnrS1*, *aph(3')-VI*, conferring resistance to bleomycin, carbapenem, fluoroquinolone and aminoglycoside were found in five plasmids that belonged to two incompatibility types IncF, IncR. Similar genetic sequences

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have been determined in other carbapenem-resistant *Escherichia coli* isolates from Tahiti but also on plasmids of CRKP isolates from other continents. Our findings indicate the potential transmission of multidrug-resistant genes including $bla_{\text{NDM-1}}$ by the novel transposon across various regions worldwide. Additionally, the $bla_{\text{NDM-9}}$ gene is carried on two identical ~ 260-kb plasmids of IncHI2 type in two CRKP strains belonging to two different STs (ST34, ST307). These plasmids also contain many additional genes for resistance to several antibiotics and conjugative transfer functions suggesting *in vivo* transfer of these plasmids among *K. pneumoniae* isolates by conjugation.

Conclusion

This study highlights the potential risk of dissemination of NDM producers among CRKP isolates mainly by various MGEs in French Polynesia. Genome analysis stands as a robust method for monitoring of antimicrobial resistance and should be used continuously to track the evolution of epidemiology of CRKP strains.

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Introduction

Klebsiella pneumoniae is one of the most important pathogens causing many severe infectious diseases, such as abdominal infections, pneumonia, and bloodstream infections (1). Carbapenem-class antibiotics are considered crucial therapeutic options for treating serious infections by *K. pneumoniae*, in view of their broad antimicrobial spectrum and high stability (2). However, the rise of carbapenem-resistant *K. pneumoniae* (CRKP) strains constitutes critical threat to global health, involving high mortality and morbidity rate (1,3). The most concerning mechanism of resistance in CRKP is the acquisition of carbapenemase genes, mainly KPC, NDM, OXA-48 encoding enzyme which facilitate the degradation of cephalosporins, monobactams, carbapenems and even β-lactamase inhibitors (4). New Delhi metallo-β-lactamases (NDM), one of the carbapenemase of greatest concern, was initially discovered in *K. pneumoniae* in 2008 and can hydrolyze almost β-lactams including carbapenem (5). Originating from a Swedish patient who travelled to New Delhi, NDM-encoding gene bla_{NDM} has rapidly been found in a wide variety of gram-negative bacterial species (e.g, *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas*, *Acinetobacter*). Thus far, 40 variants of NDM have been identified (6), among which NDM-1 and NDM-5 variants remain the most prevalent, especially in the Idian subcontinent, China, Europe (3,7,8). In addition, other NDM variants, such as NDM-4, NDM-6 and NDM-7, have been identified sporadically in various regions, indicating ongoing genetic evolution and dissemination (8,9).

The rapid dissemination of NDM among different bacterial species and across various geographical locations is mainly due to successive horizontal genetic transfer, mediated by plasmids and transposons (9,10). To date, more than 20 different Inc types plasmids were found to carry NDM gene, predominantly IncFIB, IncFII, IncA/C, IncX3, IncH and IncL/M (9). In

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addition, even within the same plasmid type, NDM can be located in a variety of genetic environment, often surrounded by multiple insertion sequences (ISs) and composite transposons (10,11). Many MGEs are believed to play a significant role in dissemination of NDM, including IS*26*, IS*Aba125*, IS*3000*, IS*CR1*, Tn*3*, Tn*125* and Tn*3000* (11,12). The spread of NDM gene is evidently a complex and multi-layered process involving variable MGEs such as genetic recombination, transposition, plasmid conjugation (9). However, in French Polynesia, the epidemiology of carbapenemase- producing *K. pneumoniae*, particularly with regard to NDM-producing strains is not as well documented as in other regions. A recent study highlighted the emergence of polyclonal strains of NDM-1 and NDM-9- producing *E. coli* and *K. pneumoniae* in Tahiti (13). The geographical isolation of French Polynesia, along with its distinct healthcare problems, influx of tourists and limited healthcare resources makes it challenging to implement robust infection control procedures. This work aims to analyze the genomic characteristics of CRKPs isolated in Tahiti over a 16-year period (2006-2021) surveillance defining their phenotypic antibiotic resistance, resistance gene profile and focusing specifically on elucidating the role of MGEs in dissemination of carbapenemase genes and other resistant determinants.

Materials and Methods

Isolate collection and antimicrobial susceptibility tests

The 17 CRKP isolates described in this study were collected in Tahiti hospital from 2006 to 2021 based on the criteria of resistance to carbapenem and species identification as *K. pneumoniae* (Table S1). These CRKP isolates were sent to the IHU-Méditérranée Infection research laboratory in Marseille, France, for investigation of resistance mechanisms. Isolate species were identified using Matrix Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF) mass spectrometry (BRUKER Daltonic, Bremen, Germany) (14).

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Antimicrobial Susceptibility Testing (AST) was performed using disk diffusion method on Mueller-Hinton agar and interpreted according to EUCAST 2023 guidelines (https://www.eucast.org/). Sixteen antibiotics were used for AST, amoxicillin (20 μg), amoxicillin/clavulanic acid (20/10 μg), piperacillin/tazobactam (36 μg), mecilinam (10 μg), ceftriaxone (30 μg), cefepime (30 μg), ceftazidime (10 μg), ertapenem (10 μg), imipenem (10 μg), meropenem (10 μg), aztreonam (30 μg), ciprofloxacin (5 μg), amikacin (30 μg), gentamicin (10 μg), fosfomycin (200 μg) and trimethoprim/sulfamethoxazole (25 μg). The minimum inhibitory concentrations (MICs) for ertapenem, imipenem, meropenem was determined by epsilometry (E-test) (BioMérieux, Marcy l'Etoile, France), whereas MIC of colistin was determined by microdilution (Biocentric, Bandol, France). *K. pneumoniae* ATCC 13883 was used as quality control strain.

Molecular analysis of carbapenem genes

DNA from 17 CRKP isolates was extracted by the automated EZ1 method (Qiagen BioRobot EZ1-, Tokyo, Japan) using "Bacterial DNA" program and kit (EZ1 DNA, Qiagen, Hilden, Germany) (15). A quantitive PCR (qPCR) or standard PCR were performed to investigates class A, B and D carbapenemase genes including (bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{OXA-48}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-58}).

Biochemical Detection of Carbapenemase Activity and Efflux Pump Inhibition Testing

Carbapenemase activity was detected using the CARBA NP test (Biorad, Hercules, California, United States) (16).

Whole-genome sequencing and bioinformatic analysis of CRKP isolates.

Genomic DNA of CRKP isolates was extracted via an automated EZ1 strategy (Quiagen, Hilden, Germany) and sequenced by MiSeq technology (Illumina Inc., San Diego, CA, USA)

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using a paired-end strategy. FastQC v0.12.1 (17) and Trimmomatic v0.36 (18) were performed for quality check and trimming. Genomes were also sequenced using long-read techniques (Oxford Nanopore Technology, Oxford, United Kingdom). Unicycler v0.5.0 was used for hybrid assembly with the short and long reads as input (19), enabling us to obtain the complete chromosomal and plasmid sequences of CRKP isolates. The genomic assemblies were evaluated by QUAST v 5.2.0 (20) and annotated with Prokka v1.14.6 (21). Multilocus sequence typing was performed by MLST v2.22.0 available on Galaxy Australia platform (https://usegalaxy.org.au/).

Phylogenetic tree of *K. pneumoniae* genome database

We constructed a phylogenetic tree including 17 CRKP isolated in our study and extended our analysis by comprising sequences previously assembled and downloaded from NCBI to explore the global evolutionary relationships of CRKP isolates in Tahiti. Roary v3.13.0 was used for pangenome analysis with default parameter of 95% identity setting for BLASTp and 99% for core genome definition. The output file of core gene alignment was then used for maximum likelihood phylogenetic construction using RaXML software v8.2.12 with the default settings. The phylogenetic tree was displayed by iTOL (https://itol.embl.de/).

Identification of resistance genes and virulence genes

The Resfinder (22) and CARD (23) databases included in the Abricate v1.0.1 software was used for antibiotic resistance prediction with a minimum identity threshold of 90% and a minimum coverage of 70%. Virulence genes were determined using VFDB database (24).

Outer membrane protein (ompk) genes analysis

The coding sequence of the *ompK35* and *ompK36* of *K. pneumoniae* ATCC 13883 (https://www.atcc.org/products/13883#product-references) were used to align with those of 17 CRKP isolates to determine frameshift alterations.

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MGE analyses

The identification and typing of the plasmids were determined using Plasmidfinder tool available in the Abricate v1.0.1 software, the MOB-suite mob_typer module v3.0.3 with defaut parameters (25). We used oriTfinder to identify transfer origins (oriT) and conjugation genes (26). Integron and insertion sequences were detected using IntegronFinder v2.0.2 (27) and ISfinder (28). Transposons associated with ARGs (Antibiotic resistance genes) were predicted using Tncentral (29) and Transposon registry (https://transposon.lstmed.ac.uk/tn-registry). Identification of prophage was made using Phigaro v2.3.0 (30).

Results

Epidemiological characteristic of CRKP isolates

We studied carbapenem-resistant *K. pneumoniae* detected in Tahiti focusing on genome features, antimicrobial resistance, virulence factors and MGEs. In the Tahiti hospital, over a 16-years period from 2006 to 2021, as part of the policy of screening carbapenemase-producing *Enterobacterales* for all hospitalized patients once a week, we detected 17 CRKP isolates from screening and clinical samples for complete genome sequencing and phenotypic characterisation. These CRKP isolates were collected from seventeen different patients hospitalized in different hospital departments, most originating from the intensive care unit (n=6) (Table S1). Among the patients, the majority were male (n=12) and aged between 50 and 60 (n=7). These isolates were primarily recovered from various samples, including rectal swab (n=12), followed by urine (n=3), blood (n=1), tracheal sample (n=1) (Table S1).

Phenotypic antibiotic resistance

All of 17 CRKP isolates showed resistance to all β-lactams, including the third generation (ceftriaxone and/or cefepime), fourth generation cephalosporins (ceftazidime) and ertapenem,

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while five were resistant to imipenem, ten to meropenem and eight to aztreonam (Table 1). Seven CRKP isolates were confirmed carbapenemase-producing strains by a positive Carba NP test and PCR analysis detected the presence of NDM carbapenemase gene. In contrast, the other ten isolates harbored a negative Carba NP test and lack of carbapenemase genes (Table 1). In addition, most were resistant to ciprofloxacin (n=12), fosfomycin (n=16), trimethoprim/sulfamethoxazole (n=11) and amikacin (n=9). Most of CRKP isolates stayed susceptible to gentamycin (n=11). Colistin also remained a treatment option for most isolates (15/17). Thus, all of 17 CRKP Tahiti isolates were identified as extensively multi-drug resistance with high resistance to carbapenems and other last line antimicrobial drugs.

Genome resistance gene profiles

We investigated the complete resistome present in the CRKP Tahiti isolates by performing a genome-wide ARG search, revealing the presence of multiple ARGs. The β-lactamase genes were the most identified, with at least two and up to six genes per isolates (Fig 1, Table S2). Among multiple β-lactamase genes detected, ESBL variants are the most prevalent with at least one SHV variant gene found in all of CRKP isolates (Fig 1). CTX-M-15 was identified in seven isolates, while the less frequent CTX-M-3 was found in one isolate (Table S2). Additionally, other acquired β-lactamase genes, including CMY-2 and an ampC β-lactamase DHA-1 were detected, though less frequently, in two and four isolates, respectively (Table S3). In particular, five isolates were found to carry carbapenemase genes NDM-1 (Q8529, Q8533, Q8535, Q8539, Q8540), while two isolates contained NDM-9 (Q8532, Q8534). These seven isolates were found to carry the combinations NDM and ble(MBL) encoding BRP(MBL) bleomycin resistance protein (Figure 1). In addition, we detected mutation in the OmpK35 and OmpK36 porins in nine isolates, which would be responsible for the carbapeneme resistance in Q8528, Q8536, Q8538, Q8541 lacking

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carbapenemase genes (Table S3). Accordingly, these isolates exhibited high MIC of Ertapenem (12-32 mg/L) (Table 1).

Regarding aminoglycoside resistance genes, most isolates (16/17) harbored at least one variant of aminoglycoside acetyltransferases (AACs), nucleotidyltransferases (ANTs) and phosphotransferases (APHs). In particular, aac(6')-Ib-cr, ant(3'')-Ia and aph(3') were the most prevalent (Figure 1). Consequently, more than half of the CRKP isolates (12/17) were resistant to gentamicin and/or amikacin (Table 1). Besides, most isolates (16/17) carried *oqxA* and *oqxB* encoding the RND efflux pump conferring resistance to fluoroquinolone (Figure 1). On the other hand, we found other quinolone resistance genes mainly QnrS1, QnrB4 in five and four isolates, respectively. Accordingly, most isolates exhibited resistance to ciprofloxacin (Table 1).

While Q8538 and Q8541 isolates showed colistin resistance, they did not carry *mcr* genes. Conversely, Q8528 isolate harboring *mcr*-9 demonstrated sensitivity to colistin (MIC=0.5 mg/L). We therefore investigated known mutation associated with colistin resistance in *K. pneumoniae* (31). However, these two Q8538 and Q8541 isolates did not possess mutations in any of the genes examined, including MgrB, PmrA, PmrB, PhoP, PhoQ and CrrB. Thus, no clear genetic basis could be determined for the resistance to colistin observed in Q8538 and Q8541 isolates.

Plasmids associated with carbapenemase genes and other resistance determinants.

Among the seventeen CRKP isolates analyzed in this study, a total of 66 plasmids were identified. These plasmids belonged to more than ten compatibility groups with IncFIB, IncFII, IncR and the combination of them accounting for the most prevalent (Table S2). Out of these, 26 plasmids were predicted as conjugative, 38 mobilizable and 27 non-mobilizable. One mobilizable plasmid pQ8539-3 and three conjugative plasmids pQ8529-1, pQ8533-2 and pQ8540-2 were found to carry NDM-1 encoding carbapenemases. pQ8539-3 was 63.4 kbp in length and had a

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53% GC content. It carried a class 1 integron containing multidrug-resistant genes aac(6')-Ib-cr, two gene cassettes (*dfrA*27 and *aadA*16) and qacE encoding resistance to antiseptic (Figure 2). Comparative analysis of pQ8539-3 with available sequences in NCBI showed 99.9% identity and 79% coverage with a (IncR) plasmid, pC069_NDM1 (LC613144.1), originating from the *K. pneumoniae* C069 strain, recovered in Thailand.

The NDM-1 conjugative plasmids pQ8529-1, pQ8533-2 and pQ8540-2 were highly identical and possessed IncFIB and IncFII replicons. They were approximately 126 kbp in length and had a 52.2% GC content (Figure 2). They carry other ARGs conferring resistance to β-lactam, aminoglycoside, quinolone, bleomycin including TEM-150, OXA-9, CTX-M-15, *ant(3'')-IIa*, *aac(6')-Ib-1*, *aph(3')-VIa*, *qnrS1*, *ble*(MBL), respectively (Figure 2, Table S3). They showed 99.9% identity and 89% coverage with a (IncFIB+IncFII) plasmid, pK66-45-3 (CP020904.1), originating from the *K. pneumoniae* K66-45 strain, ST11, recovered in Norway (GCF_002113865.1). The pK66-45-3 is 120 kbp in length and has a 52.5% GC content yet it lacks the 16 Kbp region containing various ARGs including NDM-1, ble(MBL), aph(3')-VI and qnrS1 (Fig 3).

Implication of transposition in the transfer of NDM-1 gene

The NDM-1 region had its best match with a sequence flanked by two IS26 from pK66-45-1 (CP020902.1) which is a 338 Kbp plasmid very similar to pK66-45-3, both from the *K. pneumoniae* K66-45 strain. These data showed that NDM-1 is within a composite transposon. The comparison of this transposon with other transposons in the Transposon registry databases (https://transposon.lstmed.ac.uk/tn-registry) revealed that it is a novel transposon that has been named Tn7722. Altogether, NDM-1 seem to have been acquired through transposition between pK66-45-1 and pK66-45-3 as shown by the presence of 5-bp target site duplications (AAAAT)

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observed in pK66-45-1 (Figure 2B). Likewise, we found that *K. pneumoniae* Q8539 harbored, besides the mobilizable plasmid pQ8539-3, a conjugative plasmid, pQ8539_1, which did not carry NDM-1 gene. pQ8539_1 showed high similarity, as much as 98.54%, with the conjugative plasmid p1 containing Tn7722-like transposon from *K. pneumoniae* strain 110821 (CP132632.1) ST395. This finding suggested the possibility of transposition of Tn772 between pQ8539_1 and pQ8539_3. Interestingly, similar transposons were found within 57 plasmid sequences in PLSDB (https://ccb-microbe.cs.uni-saarland.de/plsdb/) including 54 plasmids from *K. pneumoniae*, one from *K. variicola*, one from *Enterobacteriaceae bacterium* and one from *E. coli* (Table S4). In addition, Tn7722 was 99.9% identical to sequences identified on plasmids from incompatibility groups IncF, IncH and IncR originating from *K. pneumoniae* across various continents and countries in the world, including Russia, USA, Italy, Germany, Korea, China …(Table S4).

Tn7722 is 16,246 bp long and is bracketed by two copies of IS*26* (Figure 2). It harbored qnrS1, aph(3')-VIa conferring resistance to fluoroquinolone and aminoglycoside, located upstream of NDM-1 as well as additional transposases like Tn2 transposase, IS*Spu2*, IS*Ab125*, IS*Aba14*, IS*Kpn19*. The transposon conserved a 3,395 bp segment similar to Tn3000 with the presence of ble_{MBL}, followed by gene encoding a phosphoribosylanthranilate isomerase (*trpF*), a twin-arginine translocation pathway signal protein (*tat*) and a divalent ion tolerance protein (*cutA*), downstream of NDM-1 (Figure 2B). Tn7722 showed high similarity to Tn6368, yet Tn6368 lacked aph(3')-VIa. It is noteworthy that the IS26 of Tn7722 was truncated at its 5' extremity by insertion of unit ISEcp1-blaCTX-M-15 in pQ8529-1, pQ8533-2, pQ8540-2 and pQ8539-3. Altogether, the genetic environment of NDM-1 rather indicated an acquisition by transposition from a plasmid to another plasmid followed by sequence rearrangement that might compromise future transfer events.

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Many ARGs on plasmids and chromosomes

Multiple ARGs were identified within both chromosomes and plasmids in 17 CRKP strains (Table S2). NDM-9 was found on conjugative plasmids pQ8532_1 and pQ8534_1 that showed high identity, approximately 126 kbp in length. They both carried IncH12 and repA replicons and had GC content of 47.5%. These plasmids showed 99% identity and 88% coverage compared to a 254 kbp-plasmid from NCBI database, including pK121-1(CP031850) from *K. pneumoniae* strain 121 (GCF_004801235.1) isolated in China. Remarkably, pQ8532_1 and pQ8534_1 differed from pMCR1-59496 in bearing an additional region 14 kb containing *qacE* encoding quaternary ammonium counpound efflux transporter, NDM-9, *mer* operon encoding mercury resistance protein and a single IS26 elements at the 3'extremity (Figure 2). In addition, we found two class 1 integrons containing gene cassettes (aadA2, cmIA1, ant(3'')-IIa and qacH) and (sul, fosA, dfrA12, aadA2) in pQ8532_1 and pQ8534_1.

bla$_{\text{CTX-M-15}}$ was detected in seven CRKP strains: Q8538, Q8529, Q8533, Q8540, Q8532 isolates, each carrying one copy of *bla*$_{\text{CTX-M-15}}$ in different incompatibility types of plasmids (Table S2). Q8536 and Q8541 isolates harbored two and one copies of *bla*$_{\text{CTX-M-15}}$ on its chromosome, respectively. The alignment of sequences containing *bla*$_{\text{CTX-M-15}}$ in these isolates revealed that *bla*$_{\text{CTX-M-15}}$ was found to be associated with the same insertion sequence IS*Ecp1* within both chromosomes and plasmids (Figure 4). Thus, insertion of *bla*$_{\text{CTX-M-15}}$ in the Q8536 and Q8541 chromosomes was consistent with transposition events mediated by ISEcp1, occurring between plasmids (pQ8536_2, pQ8541_2) and chromosomes. In particular, we identified two 5-bp target site duplication (TSD) sequences, including TATAA and TATAT, for each copy in the Q8536 chromosome (Figure 4). However, the ISEcp1- *bla*$_{\text{CTX-M-15}}$ unit in the Q8541 chromosome was interrupted by an insertion of IS903 in the middle of ISEcp1 transposase at the 5' end (Figure 4).

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And a 5-bp TSD (AAATA) for this unit was identified in the Q8541 chromosome. Moreover, blaCTX-M-3 was also found to be associated with ISEcp1 on plasmid pQ8528_2 with three IncH2A, IncHI2, repA replicons.

Discussion

The ARGs including NDM-1 genes are a part of the mobilome. Diverse processes of genetic recombination (32), transposition, conjugation and transformation (33), transduction (34) and transfer through outer-membrane vesicles (35) play a major role in the transfer and spread of NDM genes. Many different transposons are thought to play important roles in dissemination of NDM-1, including Tn125, Tn3000, IS26, ISAba125. We have discovered a novel transposon that can be responsible of the emergence and spread of NDM-1 among *Klebsiella* sp. all over the world.

In addition, many reports also have outlined non-carbapenemase-producing *K. pneumoniae* strains through production of other enzymes such as ESBLs and AmpC β-lactamase associated with inactivation of outer membrane porins, overexpression of efflux pumps as well as increased expression of drug-degrading enzymes (36).

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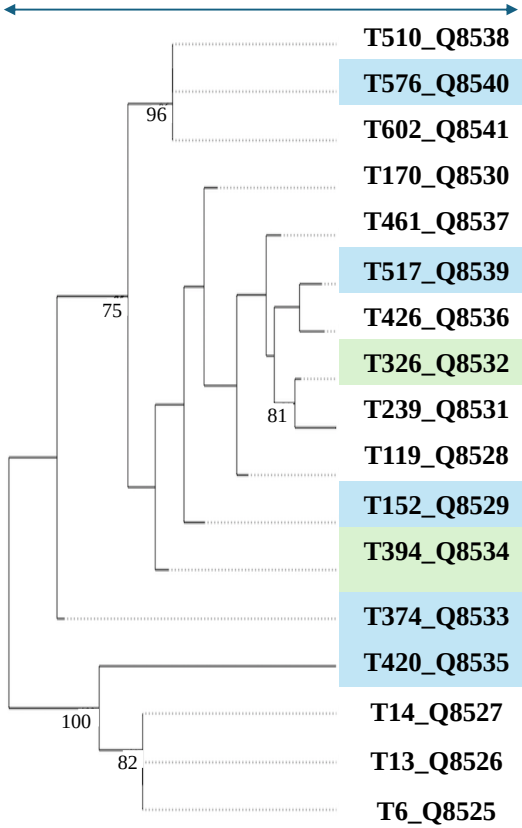
Characteristics of *17 K. pneumoniae* strains Tahiti



	Department	Year	Specimen Type	Etest MIC (µg/ml)		CARBA test	ST	Carbapenemase genes
				ETP	IPM			
T510_Q8538	Nephrology	2020	Rectal	> 32	16	-	15	-
T576_Q8540	Hematology	2021	Rectal	1.5	1	+	147	*blaNDM-1*
T602_Q8541	Internal medecine	2021	Rectal	> 32	1.5	-	15	-
T170_Q8530	Intensive care	2014	Rectal	2	0.75	-	252	-
T461_Q8537	Cohorting	2020	Rectal	1.5	0.25	-	5605	-
T517_Q8539	Intensive care	2020	Urine	1.5	12	+	111	*blaNDM-1*
T426_Q8536	Intensive care	2019	Blood	32	1.5	-	336	-
T326_Q8532	Emergency	2017	Urine	2	1.5	+	307	*blaNDM-9*
T239_Q8531	Emergency	2014	Urine	0.5	0.19	-	45	-
T119_Q8528	Orthopedic surgery	2013	Rectal	> 32	> 32	-	2136	-
T152_Q8529	Emergency	2014	Urine	2	2	+	22	*blaNDM-1*
T394_Q8534	Cardiological Intensive Care	2018	Rectal	1	0.38	+	34	*blaNDM-9*
T374_Q8533	Hematology	2018	Rectal	4	1.5	+	661	*blaNDM-1*
T420_Q8535	Hematology	2019	Rectal	8	> 32	+	1967	*blaNDM-1*
T14_Q8527	Intensive care	2008	Tracheal	3	0.25	-	1307	-
T13_Q8526	Nephrology	2008	Rectal	2	0.25	-	1307	-
T6_Q8525	Intensive care	2007	Rectal	1.5	0.25	-	1307	-

Figure 1. Phylogenetic analysis and chacterisations of CRKP isolates, French Polynesia, 2014 to 2021.

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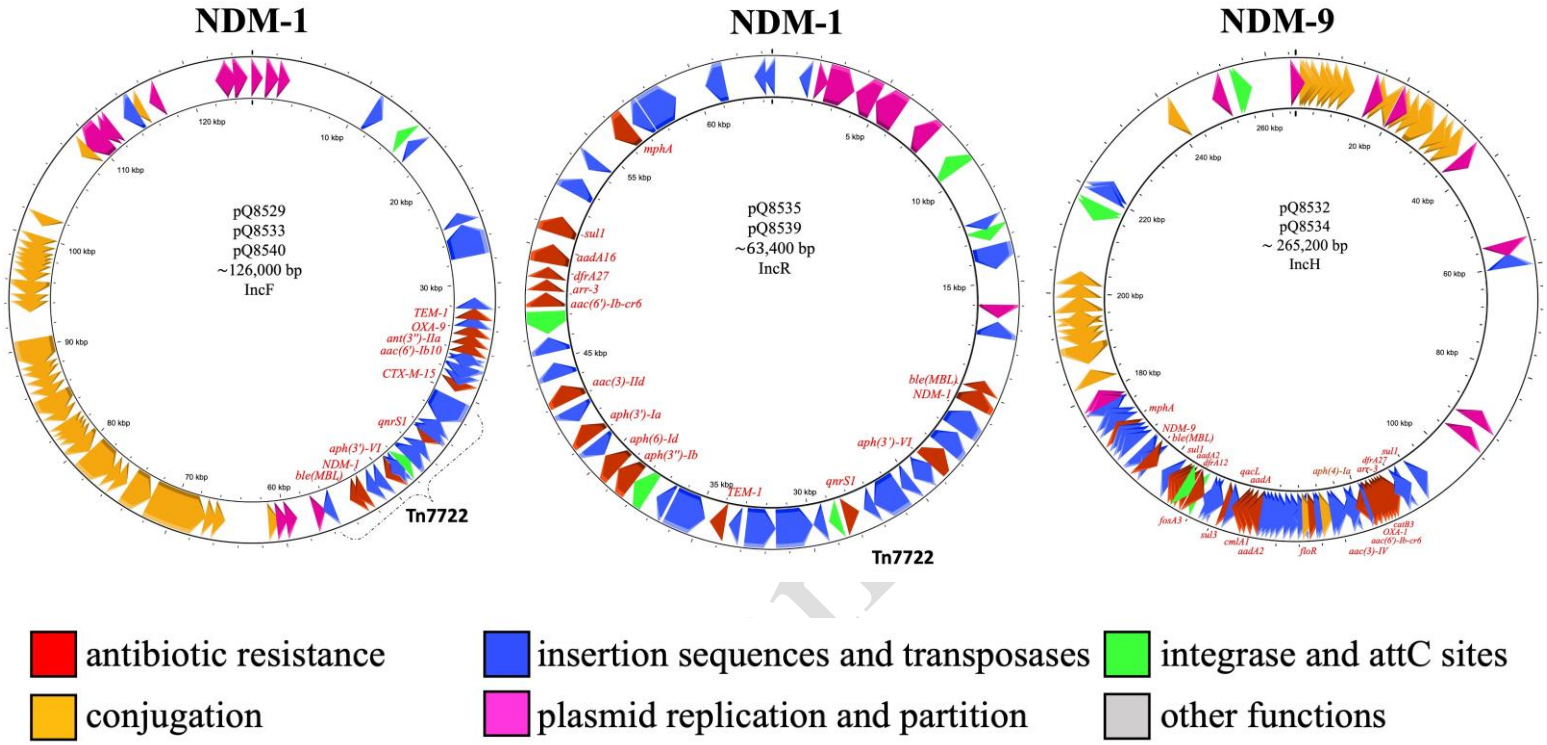

Figure 2. Plasmids carrying NDM genes and other resistance determinants NDM-producing *K. pneumoniae* isolates.

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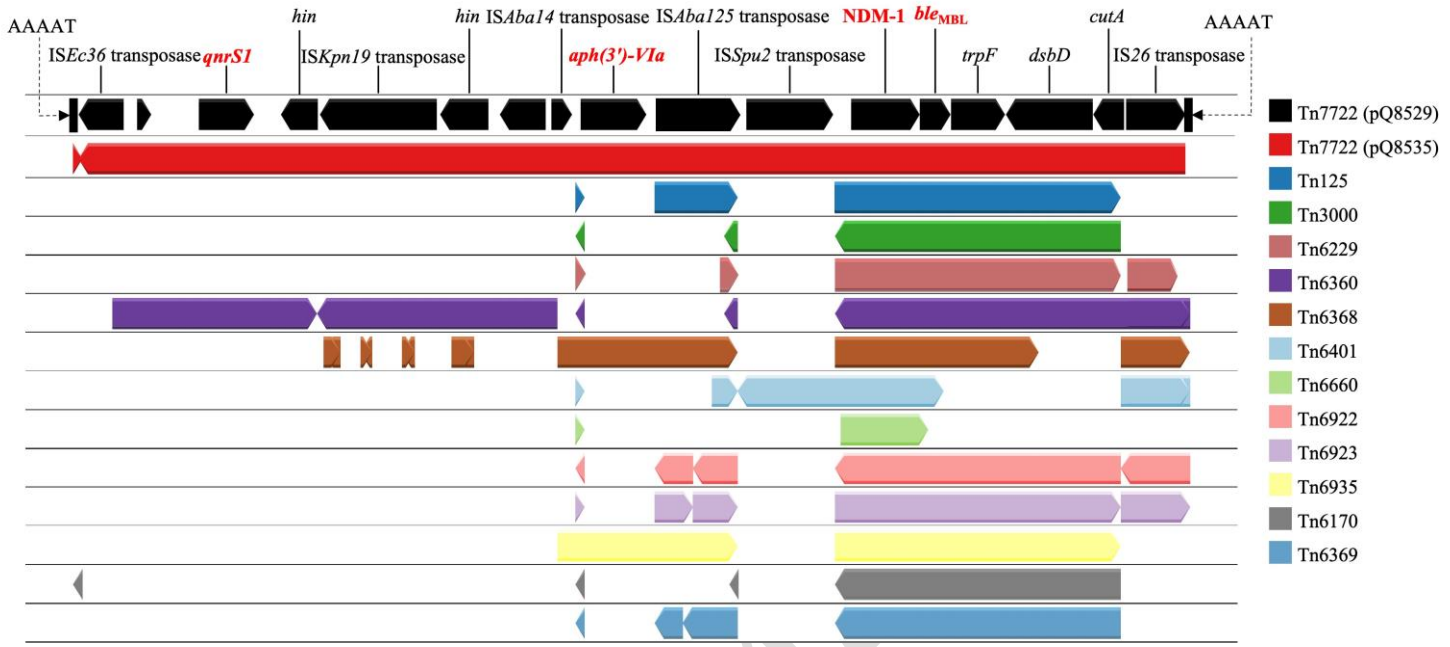

Figure 3. **Novel transposon containing NDM-1, qnrS1, aph(3')-VI, ble(MBL) identical between two groupe of plasmids.**

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