**UNIVERSITÉ D'AIX-MARSEILLE**

**FACULTE DE MEDECINE DE MARSEILLE**

**ECOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ**

**THÈSE DE DOCTORAT**


# Mouna HAMEL


**Présentée et publiquement soutenue devant**

LA FACULTÉ DES SCIENCES MÉDICALES ET PARAMÉDICALES

Le 01 juillet 2021

# Développement Et Utilisation d'Outils Génétiques Innovants Pour La Caractérisation Moléculaire De La Résistance À La Colistine

**Pour obtenir le grade de Docteur d'Aix-Marseille Université**

Pathologie Humaine, Spécialité Maladies Infectieuses et Microbiologie

**Membres du Jury de la Thèse :**

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| **Président du Jury** | **Professeur Philipe COLSON** | **Marseille** |
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| **Directeur de Thèse** | **Professeur Jean-Marc ROLAIN** | **Marseille** |
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Laboratoire d'accueil

*Microbes Evolution Phylogeny and Infections MEϕI- Aix Marseille Université IRD, IHU Méditerranée Infection*, Marseille, France 2020/2021

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**Avant-propos**

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie du Master des Sciences de la Vie et de la Santé qui dépend de l'École Doctorale des Sciences de la Vie de Marseille. Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue publiée dans un journal scientifique afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse. Par ailleurs, la thèse est présentée sur article publié, accepté où soumis associée d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

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**SOMMAIRE**

**Résumé**

La colistine est un antibiotique de dernière ligne utilisée contre les bactéries à Gram-négatif multi-résistantes et dont l'augmentation récente de la résistance est préoccupante. Bien que de nombreux mécanismes aient été élucidés, les mécanismes qui sous-tendent cette résistance sont jusqu'à présent largement incompris. Le développement d'approches et de technologies innovantes a ouvert une nouvelle ère dans la compréhension des mécanismes d'action et de résistance à la colistine. L'objectif de cette thèse est d'utiliser ces technologies innovantes afin d'identifier et de caractériser de nouveaux gènes et voies de régulation liées à la résistance à la colistine.

La première partie de cette thèse a été consacrée à retracer l'histoire de la colistine et des méthodes utilisées pour découvrir ses principaux mécanismes d'action et de résistance. Nous avons également développé les techniques les plus récentes dans ce domaine et discuté du potentiel des nouvelles technologies dans la découverte de nouveaux mécanismes de résistance à la colistine. Dans une seconde partie, nous avons étudié l'épidémiologie moléculaire de la résistance à la colistine de souches cliniques de *Klebsiella pneumoniae* dans deux contextes différents. En Grèce, où la consommation et la résistance à la colistine sont élevées, nous avons démontré que la résistance à la colistine est endémique et multiclonale, favorisée par la pression de sélection. Les mécanismes médiant cette résistance sont principalement d'origine chromosomique due à l'inactivation du gène régulateur *mgrB* par des séquences d'insertion. En France, où la consommation et la résistance à la colistine restent faibles, nous rapportons l'émergence et la sélection d'un clone de *K. pneumoniae* résistant aux carbapénèmes, appartenant au ST307, résistant à la colistine. Enfin, dans une troisième partie, notre travail s'est focalisé sur le

développement et la mise au point de technologies innovantes telles que la mutagénèse aléatoire associée au séquençage à haut débit pour étudier les mécanismes de résistance à la colistine chez des souches présentant un phénotype de résistance atypique. Ainsi, nous avons mis en évidence le rôle important de plusieurs voies métaboliques dans la résistance à la colistine et nous avons illustré la complexité des mécanismes de résistance à la colistine et leur rapport avec la survie dans des environnements difficiles.

Nos travaux montrent la nécessité de la mise en place de systèmes de surveillance pour détecter de nouveaux clones résistants susceptibles de se propager dans la population humaine, et du développement de nouvelles technologies évoquées tout au long de cette thèse pour une meilleure compréhension de l'émergence de la résistance à la colistine.

**Mots clés** : Colistine, *Klebsiella pneumoniae,* clones, technologies innovantes, résistance atypique, voies métaboliques.

**Abstract**

Colistin is a last-line antimicrobial used against multidrug-resistant Gram-negative bacteria for which the recent increase in resistance is of concern. While some underlying mechanisms of resistance have been elucidated, so far, they still remain largely poorly understood. The development of innovative approaches and technologies has initiated a new era in the comprehension of the mechanisms of action and resistance to colistin. The objective of this thesis consists in the use of these innovative technologies to identify and to characterize new genes and regulatory pathways associated with colistin resistance.

In the first part of this thesis, the history of colistin and the methods used to discover its main mechanisms of action and resistance were outlined. We have also developed the latest technologies used in this field and discussed their potential in the discovery of new mechanisms of colistin resistance. In a second part, we studied the molecular epidemiology of colistin resistance in clinical strains of *Klebsiella pneumoniae* within two different contexts. In Greece, where colistin consumption and resistance are both high, we demonstrated that colistin resistance is endemic and multiclonal, enhanced by the selection pressure. The mechanisms mediating this resistance are mainly of chromosomal origin due to the inactivation of the *mgrB* regulatory gene by insertion sequences. In France, where consumption and colistin resistance remain low, we report the emergence and the selection of a carbapenem-resistant *K. pneumoniae* clone, ST307, resistant to colistin. Finally, in a third part, our work focused on the development of innovative technologies such as random mutagenesis combined with high-throughput sequencing to investigate the colistin resistance mechanisms in strains showing an atypical resistance pattern. Thus, we highlight the important involvement of several metabolic pathways in colistin

resistance, and we illustrate the complexity of colistin resistance mechanisms and their association with survival in harsh environments.

Our work shows the requirement for the implementation of surveillance systems to detect new resistant clones likely to spread in the human population and the need for the development of new technologies for further comprehension of the emergence of colistin resistance.

**Keywords** : Colistin, *Klebsiella pneumoniae*, clones, innovative technologies, atypical resistance, metabolic pathways.

# INTRODUCTION

## INTRODUCTION

La colistine est un antibiotique polypeptidique de la famille des polymyxines du groupe E, découvert en 1947 par Y. Koyama à partir de cultures de *Bacillus polymyxa* sous-espèce *colistinus* (Stansly and Schlosser, 1947). Lors de sa mise sur le marché dans les années 1950, la colistine a été considérée comme un antibiotique " miracle " grâce à ses faibles niveaux de résistance et son efficacité bactéricide contre les bactéries à Gram négatif (BGN) (Baron *et al.*, 2016). La colistine agit d'abord en déplaçant les cations $Ca^{2+}$ et $Mg^{2+}$ qui stabilisent la membrane externe par des interactions électrostatiques avec des groupements phosphates anioniques de la fraction lipidique A du lipopolysaccharide (LPS) des BGN (Baron *et al.*, 2016). Elle s'insère ensuite dans la membrane externe, affectant négativement l'intégrité de cette barrière. Cependant, la déstabilisation de la membrane externe par la colistine peut ne pas être létale. En effet, l'activité bactéricide de la colistine semble être principalement médiée par la perméabilisation de la membrane interne par le biais d'interactions entre la colistine et les molécules du LPS qui sont situées dans le feuillet externe de la membrane interne (Sabnis *et al.*, 2018). Cette perméabilisation conduit à la libération du contenu intracellulaire et à la lyse bactérienne.

Dans les années 1980, la colistine a été abandonnée au profit d'autres antibiotiques à large spectre, moins toxiques, avant de resurgir au premier plan comme une alternative thérapeutique pour un traitement de dernier recours contre les BGN multi-résistants dans les années 2000, et son usage est en augmentation dans le monde entier (Olaitan, Morand and Rolain, 2014). Cependant, l'utilisation excessive de la colistine à des doses inappropriées en médecine humaine et animale a accru la pression de sélection et a conduit à l'émergence de la résistance à la colistine (El-Sayed Ahmed *et al.*, 2020). Néanmoins, la prévalence de la résistance à la colistine

reste basse dans de nombreux pays notamment en France. En effet, en Europe, des données issues du programme de surveillance EARS-Net de l'ECDC ont indiqué en 2015 que le taux moyen de résistance à la colistine était d'environ 1 % pour *Escherichia coli* et de 8 à 9 % pour *Klebsiella pneumoniae*, sachant que cet antibiotique est systématiquement testé dans un nombre très limité de pays (Antimicrobial resistance surveillance in Europe, 2015). En médecine humaine, cette situation est très variable et la prévalence de la résistance à la colistine semble être étroitement corrélée avec la résistance aux carbapénèmes (Olaitan, Morand and Rolain, 2014). De ce fait, la prévalence de la résistance à la colistine est actuellement basse dans les pays où la propagation des entérobactéries productrices de carbapénémase (EPC) est encore faible. En contrepartie, la situation est bien plus préoccupante dans certains pays considérés comme endémiques pour les EPC comme la Grèce ou l'Italie. En effet, de multiples épidémies de *K. pneumoniae* résistantes à la colistine ont été signalées, avec un taux allant jusqu'à 43 % des *K. pneumoniae* résistants aux carbapénèmes en Italie, 20,8 % en Grèce, et jusqu'à 31 % en Espagne (Monaco *et al.*, 2014; Pena *et al.*, 2014; Meletis *et al.*, 2015).

Cette évolution a suscité un regain d'intérêt pour la recherche clinique relative à cet antibiotique, et a attiré l'attention sur les mécanismes de résistance à la colistine chez d'importants agents pathogènes opportunistes multirésistants, comme *K. pneumoniae*, l'espèce la plus fréquemment associée au développement de résistances, *E. coli* ou plus récemment *Enterobacter cloacae* (Janssen and van Schaik, 2021). Actuellement, les mécanismes de résistance à la colistine ont été essentiellement étudiés chez *Salmonella, E. coli* et *K. pneumoniae,* et ont permis d'identifier plusieurs mécanismes (Baron *et al.*, 2016). Les mécanismes les plus fréquemment évoqués concernent les modifications du lipide A réduisant l'affinité de

la colistine. Ces modifications peuvent être médiées par des mutations dans les gènes des systèmes à deux composants *pmrA-pmrB* et *phoP-phoQ,* conduisant à une surexpression de ces systèmes et à la synthèse des sucres 4-amino-4-désoxy-L-arabinose (L-Ara4N) et/ou phosphoéthanolamine (PEtN) qui vont remplacer les cations divalents en neutralisant les charges des phosphate du lipide A. L'inactivation du gène *mgrB* (également connu sous le nom de *YobG*) codant pour une petite protéine transmembranaire de 47 acides aminés, par mutation, insertion de séquence ou délétion, engendre la surexpression du système à deux composants PhoP/PhoQ, qui à son tour active l'opéron *arnBCADTEF* codant pour la biosynthèse de L-Ara4N, augmentant ainsi la résistance à la colistine (El-Sayed Ahmed *et al.*, 2020). Les modifications peuvent être également médiées par l'acquisition de gènes de résistance à la colistine à médiation plasmidique *mcr* (mobile colistin resistance), dont 10 homologues ont été décrits à ce jour grâce aux outils de séquençage (El-Sayed Ahmed *et al.*, 2020). De nombreux autres mécanismes de résistance ont été décrits à ce jour, traduisant la complexité des mécanismes médiant la résistance à la colistine, et de nombreuses souches bactériennes ont à ce jour un mécanisme de résistance à la colistine non élucidé.

La compréhension des divers mécanismes d'action et de résistance aux antibiotiques a progressé parallèlement à l'évolution des technologies mises en œuvre. Il y a longtemps, la détection de la résistance aux antibiotiques reposait essentiellement sur la culture, qui convenait bien à des fins épidémiologiques et de recherche, cependant, le déploiement des techniques et des équipements de biologie moléculaire dans les années 1990 a permis de caractériser et d'identifier de nouveaux gènes de résistance et de nombreux variants associés (Hadjadj *et al.*, 2019). Dernièrement, les techniques biotechnologiques modernes, le séquençage à haut

débit, les technologies "omiques" et les technologies d'édition du génome tel que la mutagénèse, la génomique fonctionnelle et la technologie CRISPR-Cas ont ouvert une nouvelle ère de découverte de gènes de résistance aux antibiotiques permettant ainsi une meilleure compréhension des mécanismes d'action et de résistance et ainsi une identification de nouvelles cibles potentielles pour combattre la résistance. La compréhension et la découverte de nouveaux mécanismes de résistance est un axe de recherche qui doit être intégré dans une problématique transversale pour une meilleure compréhension de la résistance. Cette thèse s'inscrit dans ce contexte.

Dans une première partie de notre travail de thèse, nous avons fait le point sur les différentes techniques qui ont été employées ou qui présentent un intérêt pour la description des mécanismes de résistance à la colistine. Ainsi, nous relatons l'historique de la description des mécanismes d'action et de résistance de la colistine, au travers des différentes avancées technologiques dans ce domaine **(Article 1)**.

Dans une deuxième partie, nous avons décrit les mécanismes de résistance à la colistine chez *K. pneumoniae* dans deux pays où la prévalence de la résistance à la colistine sont opposés : en Grèce, pays où la résistance à la colistine est devenue endémique **(Article 2)**, et en France, pays où cette résistance reste limitée. Nous nous intéressons notamment aux facteurs médiant la résistance à la colistine dans un pays, la France, où la pression de sélection par la colistine est très faible **(Article 3)**. A ce sujet, nous abordons les enjeux liés à l'émergence d'un clone de *K. pneumoniae* résistant aux carbapénèmes, appartenant au ST307, et qui a montré également une résistance à la colistine à Marseille, France.

Enfin, dans une troisième partie, nous avons utilisé des techniques innovantes décrites dans notre revue pour essayer de comprendre les mécanismes responsables de la résistance ou de la sensibilité à la colistine chez des souches d'entérobactéries

présentant un phénotype de résistance atypique. Nous nous sommes en particulier intéressés à une souche d'*E. coli* isolée chez un patient hospitalisé au sein des hôpitaux de Marseille qui présentait la particularité d'avoir une résistance isolée à la colistine pour laquelle aucun mécanisme de résistance connu n'avait été détecté **(Article 4)**. Enfin, durant notre étude épidémiologique des souches provenant de Grèce, nous avons détecté des souches de *K. pneumoniae* qui étaient sensibles à la colistine alors qu'elles présentaient un gène *mgrB* tronqué par une séquence d'insertion. Cette situation paradoxale nous a amené à poser l'hypothèse de l'existence d'un autre système de régulation de la synthèse des sucres (phosphoéthanolamine et aminoarabinose), que nous avons étudié **(Article 5)**. Notre travail de thèse se termine dans une dernière partie de perspectives relatives à l'émergence de la résistance à la colistine et plus généralement aux facteurs liés à la résistance.

# Chapitre I

# Technologies au service des mécanismes de la résistance à la colistine : une chronologie.

**Article 1: Revue: The History of Colistin Resistance Mechanisms in Bacteria: Progress and Challenges.**

Hamel, M., Rolain, J.-M. and Baron, S. A. (2021) 'The History of Colistin Resistance Mechanisms in Bacteria: Progress and Challenges', Microorganisms. MDPI AG, 9(2), p. 442. doi : 10.3390/microorganisms9020442.

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**Avant-propos**

La colistine est un ancien antibiotique principalement destiné à traiter les infections à BGN, et dont l'utilisation a longtemps été abandonnée en pratique clinique en raison de sa toxicité (Baron *et al.*, 2016), limitant également son intérêt sur le plan de la recherche. Ainsi, plus de soixante-dix ans après son introduction, de nombreuses questions subsistent concernant les mécanismes d'action et de résistance à la colistine. À ce jour, un certain nombre de mécanismes de résistance chromosomiques ont été identifiés, mais ne permettent d'expliquer qu'une partie de la résistance (Baron *et al.*, 2016). L'élucidation de nouveaux mécanismes de résistance à la colistine ces dernières années est corrélée à l'intérêt croissant que suscite cet antibiotique demeurant actif sur un grand nombre de BGN multirésistants, mais également à la démocratisation et à l'accessibilité grandissante à de nouvelles technologies, notamment les techniques de spectrométrie de masse, de Séquençage Nouvelle Génération (NGS) ou de modification de l'ADN bactérien. En 2015, grâce aux outils de séquençage, un gène de résistance à la colistine d'origine plasmidique et transférable a été décrit pour la première fois, le gène *mcr*-1 (Liu *et al.*, 2016). D'autres études de transcriptomique et de protéomique suggèrent l'implication d'un grand nombre de voies métaboliques dans les processus de résistance à la colistine chez les BGN (Keasey *et al.*, 2019; Li *et al.*, 2019; Sun *et al.*, 2020). Cette capacité constante d'adaptation de la bactérie, et notamment sa capacité à remodeler sa membrane plasmique, représente une nouvelle piste dans la découverte de nouveaux mécanismes de résistance. Toutefois, la complexité de ces mécanismes et la génération de multiples données biologiques vont nécessiter des outils informatiques puissants pour appréhender les liens et les interactions existant entre ces phénomènes.

Il nous a semblé important de retracer la genèse de la découverte des mécanismes de résistance, mais aussi celle des mécanismes d'action de la colistine à travers les méthodes et les technologies employées depuis 70 ans. Ainsi, de l'observation de l'effet de la colistine sur les bactéries par microscopie électronique dans les années 1960, à l'utilisation des technologies « omics » telles que la protéomique ou la génomique ou encore la manipulation de l'ADN par mutagénèse ou plus récemment par la méthode CRISPR-Cas9, nous avons cherché à identifier et à recenser les technologies novatrices ou encore les technologies abandonnées qui nous permettent de faire progresser nos connaissances dans ce domaine. Le défi qui subsiste aujourd'hui consiste à appréhender et à analyser les informations générées par ces outils.

*microorganisms*

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*Review*

# The History of Colistin Resistance Mechanisms in Bacteria: Progress and Challenges

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**Abstract:** Since 2015, the discovery of colistin resistance genes has been limited to the characterization of new mobile colistin resistance (*mcr*) gene variants. However, given the complexity of the mechanisms involved, there are many colistin-resistant bacterial strains whose mechanism remains unknown and whose exploitation requires complementary technologies. In this review, through the history of colistin, we underline the methods used over the last decades, both old and recent, to facilitate the discovery of the main colistin resistance mechanisms and how new technological approaches may help to improve the rapid and efficient exploration of new target genes. To accomplish this, a systematic search was carried out via PubMed and Google Scholar on published data concerning polymyxin resistance from 1950 to 2020 using terms most related to colistin. This review first explores the history of the discovery of the mechanisms of action and resistance to colistin, based on the technologies deployed. Then we focus on the most advanced technologies used, such as MALDI-TOF-MS, high throughput sequencing or the genetic toolbox. Finally, we outline promising new approaches, such as omics tools and CRISPR-Cas9, as well as the challenges they face. Much has been achieved since the discovery of polymyxins, through several innovative technologies. Nevertheless, colistin resistance mechanisms remains very complex.

**Keywords:** polymyxins; colistin history; mechanisms of action and resistance; technological approaches; innovative technologies



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## 1. Introduction

Over the last 70 years, the polymyxin family of antibiotics, including polymyxin B and colistin (also called polymyxin E), has experienced an uncommon fate. The polymyxins were initially considered "miracle" antibiotics when they were first commercialized in the 1950s, with a bactericidal efficacy against Gram-negative bacteria (GNB) and a low level of resistance [1]. Colistin was subsequently abandoned in the 1980s in favour of other less toxic broad-spectrum antibiotics before regaining the forefront in the 2000s for the treatment of multidrug-resistant GNB infections [2]. As a result, the pharmacokinetic (PK) and pharmacodynamic (PD) properties, as well as the resistance mechanisms developed by the target bacteria, remain poorly understood [3]. Several studies deciphered the mechanism of action of colistin without being able to elucidate it completely in the 1950s. At that time, polymyxin resistance was revealed by the detection of in vitro resistant mutants [4]. Moreover, 30 years of clinical disuse resulted in a lack of knowledge of the minimum inhibitory concentration (MIC) determination in vitro and its optimal use in the clinic [3]. In 2007, the reclassification of polymyxins as a major agent for the treatment of multidrug-resistant GNB infections by the World Health Organization (WHO) revived interest in clinical research on this antibiotic [1]. Consequently, data on PK/PD were collected, and new resistance mechanisms were elucidated. The discovery of the first transferable colistin resistance gene in 2015, the *mcr-1* gene (for *mobile colistin resistance*

gene), is the most significant example. This finding highlighted the major role of the animal reservoir in the transmission and diffusion of this antibiotic-resistance gene [5]. In fact, colistin has been used for many years in veterinary medicine as a growth factor and in the prophylaxis and treatment of livestock infections [3]. Investigations into colistin resistance mechanisms revealed the complexity of the pathways by which bacteria defend themselves against colistin activity [2]. It appears that the first targets identified as being responsible for colistin resistance were not sufficient to explain resistance in every isolate, suggesting the existence of other mechanisms involved in polymyxin resistance [1].

New technological tools have become available for researchers over the last few years and have generated a multitude of data to analyse. The remaining challenge is to understand and analyse such information in order to identify new pathways involved in colistin resistance. The aim of this review, through the scientific and clinical history of colistin, is to identify technological methods and interesting targets currently responsible for colistin resistance. In this review, we will resituate the current knowledge of colistin resistance mechanisms by the methods used for their discovery. We will then focus on the contribution of genomics in increasing our knowledge of colistin resistance and on the issues raised by genomic analysis and the limitations of this method. Proteome analysis is a method currently used to answer these questions, and its advantages and limitations will be detailed. Finally, we will discuss the benefits of new genetic tools such as the CRISPR-Cas9 technique, and how it might be useful in this field.

## 2. Pathways Leading to Colistin Action and Mechanisms of Resistance

### *2.1. Mode of Action*

Colistin is an old polypeptide antibiotic of the group E, discovered in 1947 by Y. Koyama from *Paenibacillus polymyxa* subspecies *colistinus* cultures [6]. It is a bactericidal, narrow-spectrum molecule directed against most GNB, but ineffective against Gram-positive bacteria, anaerobic bacteria, and mycoplasmas [3]. The main target of the polymyxins is the lipopolysaccharide (LPS) of GNB membranes [1]. The lipid A of the outer part of the LPS is negatively charged and interacts with divalent cations (mainly $Mg^{2+}$ and $Ca^{2+}$), allowing an overall stabilization of the outer membrane [2]. Colistin, a net positively charged molecule, has therefore a strong affinity to bind to the LPS, leading to a displacement of cations by electrostatic interaction. It results in a disorganization of the membrane structure, with release of the LPS [1]. Colistin is then introduced into the outer membrane through its lipophilic acid-fat chain. Colistin alters the permeability of the outer membrane, allowing it to insert itself and reach the inner membrane. A disorganization of this inner membrane then occurs by breaking the integrity of the phospholipid bilayer [7]. Eventually, lysis of this membrane results in the release of intracellular contents and death of the bacteria (Figure 1). This process is the most commonly used mechanism to explain the antibacterial action of colistin, but the ultimate mechanisms leading to cell death are still not well understood [7].

Other potential mechanisms of action have been identified, such as vesicle-vesicle contact. After colistin crosses the outer membrane, lipid exchanges between the inner and outer membrane take place, causing structural changes in the membranes with a loss of phospholipids, leading to an osmotic imbalance that lyses the bacteria [8]. The accumulation of free radicals linked to the oxidative stress induced by colistin is also a pathway responsible for DNA, protein, and lipid damage, leading to bacterial death. Inhibition of vital respiratory enzymes and endotoxin activity of lipid A have been described as well. Endotoxin activity involves the inhibition of this activity of lipid A of LPS by colistin, by binding to LPS molecules and neutralizing them, resulting in the release of tumour necrosis factor-alpha (TNF-a) and Interleukin 8 (IL-8) cytokines and thus the suppression of the shock. [7] (Figure 1).

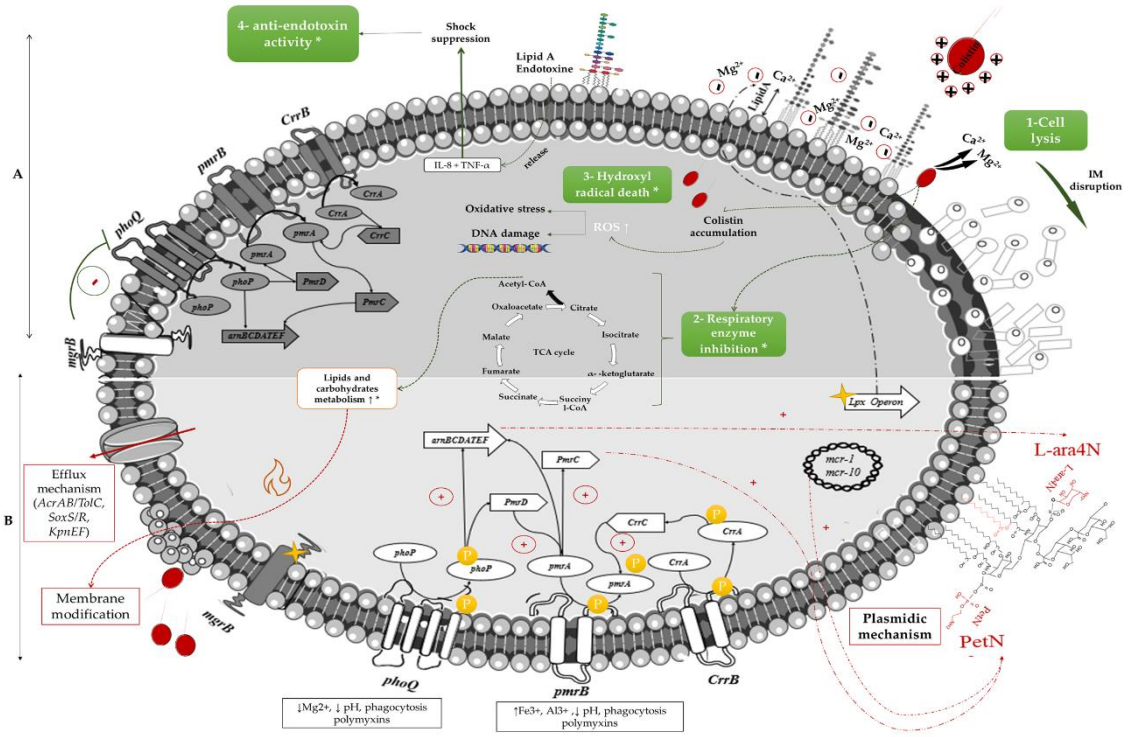


**Figure 1.** Mechanisms of action and resistance to colistin in Gram-negative organisms. (**A**): mechanisms of action of colistin, (**B**): colistin-resistance mechanisms, * on respiratory enzyme inhibition, Hydroxyl radical death, anti-endotoxin activity and the metabolism of lipids and carbohydrates represent the recently described mechanisms of action and resistance, which remain unclear so far; The yellow stars represent mutations resulting in the inactivation of regulatory genes. Green arrows indicate the mechanism of action for a susceptible strain, the red arrows indicate the different pathways involved in colistin resistance, the grey shades demonstrate the differences in the gene expression between the mechanism of action and resistance

### *2.2. Mechanisms of Resistance*

The most common mechanism of colistin resistance is due to chromosomal mutation in genes associated with the modification of the lipid A of LPS, the primary target of colistin, as an adaptative strategy [2]. Such alterations can be obtained by the addition of phosphoethanolamine (PEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) to the phosphate groups of lipid A [1]. The genes that encode enzymes involved in lipid A synthesis are *pmrHFIJFKLM* (also known as *arnBCADTEF-pmrE*). These genes are up-regulated by the two-component systems (TCS) PmrAB and PhoPQ, the latter being negatively regulated by the *mgrB* gene [9] (Figure 1). Other strategies include the use of efflux pumps and capsule formation [1]. Moreover, the horizontal transfer of the plasmid-carried gene *mcr-1* encoding for PEtN has become an important cause of the spread of polymyxin resistance among various GNB [5]. The origin of *mcr* enzymes can be retraced back to the 1980s in China [10].

In the following, we will focus on the methods that enabled discovery of these major mechanisms and on the methods that are currently of the greatest interest in enabling us to understand the unresolved mechanisms.

## 3. From Electron Microscopy to the Discovery of Regulatory Genes

Electron microscopy was the first tool used to explain colistin's mechanism of action, demonstrating morphologic changes such as loss of nuclear material and granularity of the cytoplasm [11]. Observations in *Escherichia coli* and *Pseudomonas aeruginosa* demonstrated that treatment with polymyxin B or E modifies the cell wall, forming cell wall projections or "blebs" and the release of cytoplasmic contents through splits in the cell envelope [12]. Their

presence grew with the polymyxin concentration, and they were inhibited by magnesium ions [13]. Using a fluorescent method, Newton et al. demonstrated in 1954 that $Mg^{2+}$ and other divalent cations antagonized the activity of polymyxin B at a negatively charged site on *P. aeruginosa* [14].

Several other studies examined this phenomenon using alternative techniques as evidence that they were not artefacts. Schindler and Teuber examined *Salmonella typhimurium* treated with polymyxin B by freeze-etching, a less destructive technique for the cell envelope [15]. This technique revealed three layers, which were interpreted as the outer and inner monolayer of the outer membrane and the outer surface of the cytoplasmic membrane. The formation of numerous blebs extended only to the outer monolayer of the outer membranes [15]. It was demonstrated that these particles were outer membrane fragments with a phospholipid/lipopolysaccharide/protein ratio similar to that of the outer membranes [13]. The researchers compared polymyxins to cationic detergents, in that they are charged similarly at neutral pH, since detergents disorganize the cell membrane and denature certain proteins. They suggested that polymyxin bactericidal activity is due to its ability to combine with bacterial cell structures and disorganize them, leading to loss of the cell's osmotic balance [16]. Based on this, little information was available concerning the mechanism by which polymyxin E exerts its bactericidal action. To elucidate it, lipid extraction procedures and chromatography showed that polymyxins not only bind to these lipids but also significantly alter their structures [17].

Polymyxins rapidly became the treatment of first choice for GNB infections, as it was supposed that one of the characteristics of polymyxins was bacterial difficulty in developing resistance, and that resistant strains were unstable and do not easily occur in vitro; bacteria become easily susceptible in the absence of polymyxins [18]. Later, facultative resistance to colistin from a genetic trait emerged; homologous recombination tests have shown that it was due to frequent reverse mutations that vary from species to species. The selection media seems to play a role in maintaining the frequency of mutants [4]. Conrad et al. showed that culture of *P. aeruginosa* on different media containing various sources of carbon affects the sensitivity of the cells to polymyxins. An adaptive resistance was observed when the carbon source was D-glucose or L-glutamate, and the latter was also associated with alterations in unsaturated fatty acids [19]. Thus, the mechanism for the development of polymyxin B resistance has been shown to be one of metabolic readjustment and media-dependent [20].

### *3.1. Intrinsic Resistance Bacteria*

Studies of strains naturally resistant to colistin contributed to the understanding of colistin activity and the basic mechanisms of colistin resistance. *Proteus* species and *Serratia marcescens* are among the *Enterobacteriaceae* and are regularly resistant to the action of polymyxins [21,22]. The extraction and chromatography of lipids from a wild type *Proteus sp.*, highly resistant to polymyxin B, and its polymyxin B-sensitive mutant, demonstrated that phospholipid compositions were nearly similar. Each organism contained similar amounts of N-methyl-phosphatidylethanolamine, in addition to similar amounts of phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin [22]. This finding demonstrates that the composition of the lipid of the envelope was not responsible, but rather the cell envelope, which restricts polymyxin access to sensitive lipid target sites [22]. Douglas et al. employed electron microscopy to observe the effect of polymyxin B on the polysaccharide components of the outer membrane in *S. marcescens*. A dense, dark granule was observed in sensitive and resistant *S. marcescens*, due to the action of polymyxin B on the polysaccharide part of the LPS components in the outer membrane [23].

To locate the polysaccharide components in *S. marcescens* cells treated with polymyxin B, the silver proteinate method was used [24]. Blebs are usually formed on the cell surface, due to the aggregation of polymyxin B with LPS and/or phospholipids from the outer membrane of GNB. These blebs contain lipopolysaccharide components, which demonstrates that polymyxin B releases polysaccharide components in the form of lipopolysaccharide [25].

Studies to overcome colistin resistance showed that EDTA may act directly on *P. aeruginosa* to increase its sensitivity to antibiotics and has synergistic potential with colistin or polymyxin B in the treatment of *Pseudomonas* skin or urinary tract infections [26]. EDTA induces an increase in the permeability of the outer membrane in a broad range of GNB. Its mode of action relies on its function as a divalent cation chelator, whereas polymyxin can displace divalent cations by competition [27]. EDTA-resistant mutants release up to 30% of their LPS without dramatic changes in permeability. The released LPS is then split into two parts, one containing the proteins and lipids associated with the LPS, the other containing sugars different from those of the LPS, the latter being responsible for the outer membrane's permeability. According to this proposal, polymyxin B and EDTA cause similar permeabilization events [27]. The efflux pump inhibitor, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), has also recently demonstrated its efficacy in re-establishing colistin sensitivity in GNB having various resistance mechanisms [28].

### *3.2. Enzymatic Inactivation*

Studies carried out several decades ago identified an enzyme capable of inactivating colistin. This enzyme, called colistinase, is a putative serine protease produced by *P. polymyxa*. Briefly, crude colistinase was fractionated into two components (colistinase I and II) by Sephadex G-50 gel filtration. After purification, it was characterized as a single band by polyacrylamide disc gel electrophoresis [29], and it appears to degrade colistin by cleavage of the peptide bond between the tripeptide side chain and the heptapeptide ring [29]. As *P. polymyxa* is a Gram-positive bacterium and therefore lacks LPS, why it develops an apparently secreted enzyme that inactivates colistin remains an intriguing question. Therefore, colistinase may be necessary for survival in the presence of polymyxin [30]. To date, colistin degradation has not been related to colistin resistance in other organisms [30]. Recently, a polymyxin inhibitory enzyme of *Bacillus licheniformis* strain DC-1 has been identified [31]. It is an alkaline Apr protease, which cleaves colistin at two peptide bonds: one between the tripeptide side chain and the heptapeptide ring, and the other between l-Thr and l-Dab within the heptapeptide ring [31]. One of the main contributors to development of antibiotic resistance in bacteria is the acetylation of antibiotics. Recently, Czub et al. described *P. aeruginosa* Gcn5-related N-acetyltransferases (GNAT) which acetylate polymyxin B and polymyxin E. The acetylation process occurs on a single diaminobutyric acid closest to the cyclic peptide [32]. This GNAT appears to be the first enzymatic acylation mechanism with ability to modify polymyxin. Consequently, some recent work has been carried out on GNAT inhibitors [33]. However, it is not known if such enzymatic modification may confer resistance to colistin.

### *3.3. Regulatory System Discovery*

The first evidence of *pmrA* (polymyxin resistance A) mutants dates back 42 years, following the analysis of *Salmonella* polymyxin-resistant in vitro mutants [34]. The mutant gene was transferred by conjugation in a colistin-susceptible *S. typhimurium* rough (rfaJ), confirming the role of the mutation in colistin resistance [34]. At that time, research for an altered membrane component in the *pmrA* mutants had so far only given negative results [35]. Vaara et al. showed that polymyxin-resistant *pmrA* strains bind only about 25% of polymyxin compared to the polymyxin-sensitive strain and caused an alteration of the LPS, probably in the lipid A or deep core [35]. Such substitutions reduce the negative charge of the LPS, thus decreasing the accessibility of the positively charged polymyxin to lipid A [36]. Cloning and characterization of a *pmrA* mutant revealed a single base alteration that led to a change in the amino acid in *pmrA* [36]. The mutants were also more resistant to membrane-damaging effects of other cationic agents and EDTA [37]. In 1993, the PmrAB two-component system was characterized from a polymyxin B-resistant mutant strain [38]. An R81H mutation in the PmrA regulator induced an activation constitutive of PmrAB regulation and increased levels of polymyxin resistance [38]. Genetic mapping and DNA sequence analysis subsequently revealed that PmrA is encoded in the *pmrCAB*

operon [38]. PmrC, also called *pagB* (*phoP*-activated gene), appears to be a protein involved in LPS modification, while the response regulator PmrA and the kinase sensor *pmrB* form a TCS [39]. The PmrC protein, later known as *EptA*, was described as a phosphoethanolamine transferase acting on lipid A [40].

Microarray, mutagenesis and in silico analysis have demonstrated that the PmrAB system regulates more than 20 confirmed genes, up to 100 genes in *Salmonella*, and orthologs also occur in several Gram-negative pathogens [39]. This TCS seems to be involved predominantly in modifying the cell surface by the addition of Ara4N and pEtN to the LPS. However, it is still somewhat unusual in many ways; it reacts directly to uncommon environmental conditions (high $Fe^{3+}$ content) and is activated indirectly by PhoPQ via the PmrD protein [39], resulting in LPS covalent modifications [36].

The first report describing the PhoP/PhoQ TCS reported that this system regulates expression of several genes involved in the virulence and survival of *S. typhimurium* in macrophages and confers enhanced susceptibility to the action of cationic antimicrobial peptides (CAMP), called *pag* genes (polymyxin activated genes) [41]. This system has been identified as being a mutation affecting *Salmonella* resistance to raw neutrophil granule extracts, subsequently characterized as a TCS in 1989 [42] (Figure 2). It was believed that the function of this system was to control the expression of a non-specific acidophosphatase [42], based on sequence similarity with homologues of the sensor family. It has been suggested that the protein PhoQ acts as a protein kinase associated with the membrane phosphorylating PhoP in response to environmental signals, in turn activating promoters for *pag* genes. The Pho in PhoP typically refers to loci involved in the metabolism of phosphate [43]. This system reacts to limited concentrations of $Mg^{2+}$ and other divalent cations to activate virulence, and to polymyxin B by interfering with the transcription of more than 40 genes in *Salmonella* [44]. The latter has been identified using either classical genetic methods, Matrix-Assisted Laser Desorption/Ionization—Time of Flight (MALDI-TOF) analysis, or high-density DNA chips [43]. Indeed, transposon mutagenesis has shown that this system represents a regulator, and that it was necessary for the expression of a panel of genes involved in virulence with different chromosomal locations, such as *pagC* [41]. In an *E. coli* K-12 and upstream of the transcription initiation site of three PhoP-activated genes, namely *phoPQ*, *mgtA,* and *mgrB* at 25 bp, a direct repetition, (T / G) GTTTA, has been identified, representing a PhoP liaison site [43]. This pattern has also been identified in the promoter region of the *pmrA* gene [43]. The analysis of these various genes regulated by PhoP leads to the following conclusions: this PhoP/PhoQ regulator is involved in the adaptation to $Mg^{2+}$ restrictive environments, it regulates virulence in several bacterial species; several genes regulated by PhoP are species-specific, and this system regulates the modification of many components of the bacterial cell envelope [43]. The TCS PhoP/PhoQ regulates LPS changes by the addition of aminoarabinose and 2-hydroxy myristate through the extraction of lipid A and LPS from different strains of *S. typhimurium* [45]. The fatty acid content of LPS and whole bacteria has been investigated by gas chromatography (GC) mass spectrometry [45].

### *3.4. Arn Operon Discovery*

Gunn et al. reported the identification of two PmrA/PmrB regulated loci necessary for the addition of aminoarabinose to lipid A and thus for polymyxin resistance in *S. typhimurium* [46]. Analysis of the DNA sequence extending the transposon showed that the insertions were located in *pagA* (now called *pmrE*), a gene regulated by PhoP/PhoQ and activated by the previously identified PmrA/PmrB, which is a UDP-glucose dehydrogenase [46]. Mass spectrometry of the mutant *pmrE* showed that it is impossible to add aminoarabinose to the LPS, and that it is transcribed as an individual unit [46]. For insertion into the second gene, it appears to be driven by a seven-gene operon, named *pmrHFIJKLM* [47], an active component in naturally colistin-resistant GNB [48] (Figure 2).

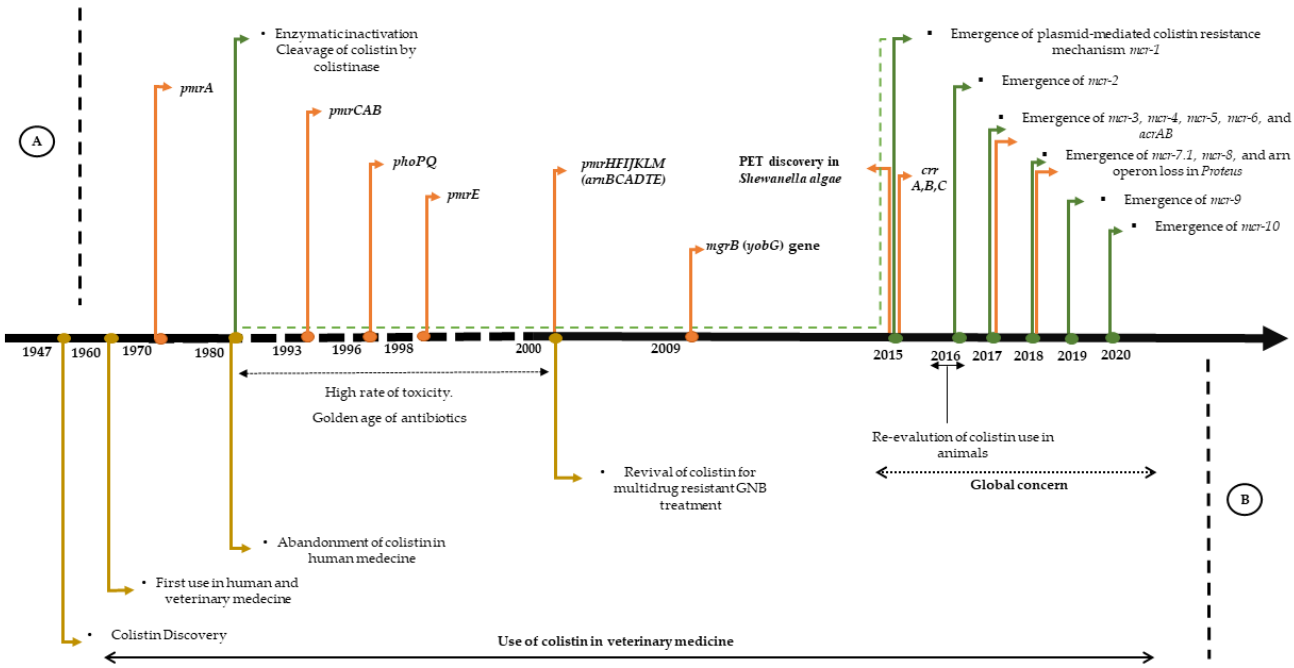


**Figure 2.** Timeline of the main events related to colistin, from the discovery of colistin to the development of resistance (**A**) represents the described mechanisms, the green arrows symbolize the enzymatic mechanisms, the orange arrows symbolize the chromosomal mechanisms. (**B**) represents the chronology of colistin.

### *3.5. mgrB (yobG) Discovery*

The *mgrB* was first described in 1999 in *E. coli* as a short lipoprotein, induced by low extracellular levels of magnesium and controlled by the PhoP/PhoQ two-component system [49]. It was further demonstrated that the *phoP* protein binds in vitro to the *mgrB* promoter region responding to $Mg^{2+}$ [49]. The *mgrB* gene, also known as *yobG*, identified in *Salmonella enterica*, has been shown to be activated by PhoP through microarray experiments and transcription reports [50] (Figure 2). The *mgrB* gene was discovered to be a regulator of the PhoP/PhoQ pathway, based on the hypothesis of feedback inhibition in terms of stress response. The researchers screened *phoP*-regulated genes individually in *E. coli* by knockout [50]. The deletion of *mgrB* coding for a small membrane protein of only 47 amino acids led to upregulation of the PhoP/PhoQ regulator system in *E. coli*, *S. typhimurium*, *Yersinia pestis*, and other related bacteria [50]. The sequence alignments showed multiple conserved residues, suggesting a well-conserved negative regulatory system [50]. The proper annotation of genes encoding for small proteins such as the *mgrB* gene remains a major challenge. The open reading frame of the *mgrB* gene was predicted based on the existence of a ribosomal binding site within an intergenic region [51]. Inactivation of *mgrB* through intergenic sequences represents one of the most common colistin resistance mechanisms and is regarded as an emerging epidemic [52,53].

### *3.6. CrrAB Discovery*

Investigation of colistin resistance mechanisms in nine *K. pneumoniae* clinical isolates, through a combination of genome sequencing and RNA sequencing transcriptional profile analysis (RNA-Seq) [54], illustrated a range of genetic variations. This enabled the characterization of distinct mutations within two strains in a previously uncharacterized histidine kinase gene belonging to a TCS named CrrAB (Figure 2), which was found to be highly expressed, as well as *pmrCAB* and an adjacent glycosyltransferase gene [54]. Complementation assays with the wild-type gene restored colistin sensitivity in both strains. The *crrAB* genes are common in most *K. pneumoniae* genomes and the resistance mechanisms are genetically background-dependent [54].

## 4. Exploitation of Colistin Resistance: Methods and Challenges

### *4.1. Lipid A Extraction and Mass Spectrometry*

Historically, mass spectrometry has been used in colistin resistance to highlight lipid A modifications in colistin-resistant bacteria. MALDI-TOF MS directly assesses the biochemical cause of colistin resistance and the presence, absence and modification of lipid A, the capsule and the LPS structure, which are components of colistin resistance [9], as lipid A has a specific peak of 1796.2 m/z [55]. Chromosomal mutations cause mass shifts of m/z +131, +123, and/or +161 in lipid A through the substitution of at least one phosphate with 4-amino-4-deoxy-L-arabinose (L-Ara4N), phosphoethanolamine (pEtN) and/or galactosamine, respectively [56]. As for the *mcr-1* gene, a displacement of m/z+123 has been observed in the lipid A peaks of Gram-negative colistin-resistant pathogens [56].

The development of MALDI-TOF MS technology, combined with automation and the implementation of a user-friendly interface in clinical laboratories, has considerably changed bacteriological diagnoses and opened the possibility of developing new rapid tools for the detection of colistin resistance. First, MALDI-TOF ionization for clinical microbiology laboratories was developed in positive ion mode, and despite numerous studies devoted to the mechanistic understanding of the ionization principle, few studies have been devoted to analysis in negative ion mode [57]. Recently, some new machines used the negative mode, which makes it possible to analyse lipids and therefore lipid A [57,58]. The analytical method requires a more elaborate sample preparation than that applied for routine MALDI-TOF identification, involving hydrolysis of LPS and extraction of lipid A by solvents [56]. This method has recently been simplified and made chemically safer, done in 30 min [59].

Thus, three studies evaluated MALDIxin, a test based on new MALDI-TOF machines [60–62]. The MALDIxin assay was developed to detect pEtN modification in lipid A directly from bacterial colonies in less than 15 min. Dortet et al. (2018) evaluated MALDIxin on *Acinetobacter baumannii* isolates. Peaks m/z 2033.3 and m/z 1935.3 were associated with pEtN-modified lipid A in all colistin-resistant isolates and were not observed in any of the colistin-sensitive isolates [60]. The optimization of MALDIxin allowed identification of L-Ara4N and pEtN-modified lipid A in *E. coli* and *K. pneumoniae* isolates. In addition, the optimized methods were able to discriminate chromosomally encoded colistin resistance and *mcr*-mediated colistin resistance, by comparing the percentage of modification of Lipid A by either L-Ara4N or pEtN [61,62]. For chromosomal resistance, a 100% of modification through the addition of L-Ara4N was observed, while for *mcr*-encoded resistance, up to 100% of the modified Lipid A was related to the addition of pEtN [62].

### *4.2. The Discovery of New Variants and Mutations in a Common Gene*

The PCR-based approach (conventional PCR, real-time qRT-PCR) is the most common molecular method used by clinical laboratories to understand resistance mechanisms underlying the observed phenotypic resistance to colistin [9]. Such approaches are used to amplify the known genes involved in colistin resistance: *mgrB*, *phoPQ*, *pmrAB*, *pmrHFIJKLM/arnBCADT*, and *mcr* genes. The amplicons are sequenced and analysed by comparing the sequences to wild-type strain sequences for possible mutation of the amplified gene. In addition, qRT-PCR is also used to study the expression levels of these genes in both strains to determine the transcription rate of the respective resistance genes in the resistant phenotype [9].

### *4.3. Genetic Toolbox and Colistin Resistance Research*

Functional genomics: enables the identification of new antibiotic resistance genes encoding for an enzyme based on their function in an expression vector. However, it becomes meaningless if the mechanisms of resistance involve several components or regulatory genes [63,64]. Functional genomics is based on the extraction and fragmentation of DNA enabling the creation of a specific library of a homogeneous size. This library is then cloned into an expression vector, which is then screened depending on the studied

antibiotic [64]. This strategy has been used to decipher the colistin resistance mechanism in *Shewanella algae* MARS 14, suggesting the involvement of a phosphoethanolamine transferase *EptA* coding for *pmrC* in polymyxin resistance [63].

Mutagenesis: variations in microorganism genomes are often the result of homologous recombination that allows the exchange of genes without reorganizing the genome. Other sources of variations come from the environment, such as plasmids or from linear DNA sequences that can move through the genome (transposon) [65]. Transposon-based mutagenesis of bacterial genomes is a powerful method for indirectly identifying genetic elements associated with specific functions and phenotypes by the creation of mutations at random locations in genomes [66]. Tn5 and Tn10 transposases, both used to generate non-redundant mutant libraries, are among the most frequently used transposon tools [67–69].

Random mutagenesis methods have been widely used since the 1980s to elucidate the main genes involved in colistin resistance, as described above. At the time, these methods were complex, due to the difficulty of sequencing the genes of interest and analysing the proteins involved. Thus, colistin resistance mechanisms have been studied mainly in *E. coli* and *S. enterica* [3]. These techniques are now used to identify the molecular mechanisms of colistin resistance in other species. The transposon is randomly inserted into the bacterial chromosome, generating a random library of mutants which can be screened by modifying the culture medium, depending on the studied phenotype [64]. Recently, transposon mutagenesis has highlighted the role of certain efflux pumps in colistin resistance mechanisms [64]. Telke et al. demonstrated that heteroresistance is linked to overexpression of the *acrAB-tolC* efflux pump in *Enterobacter spp*. after the screening of mutant clones generated by transposon mutagenesis [70]. More recently, Huang et al. showed that the *phoP* gene, the *dedA*(*Ecl*) gene (coding for an inner membrane protein of the *DedA* family) and the *tolC* gene (coding for part of the *AcrAB-TolC* efflux pump) are required for colistin heteroresistance, and they identified a new gene, *ecr*, coding for a transmembrane protein of 72 amino acids, which mediates colistin heteroresistance in *Enterobacter cloacae* [71].

The Himar1 mariner is a novel transposon isolated from the barn fly *Haematobia irritans*, identified by Lampe et al. and has become popular in bacterial genetics [72,73]. This system encodes a transposase and a transposon within one or two suicide plasmids that are electroporated on host cell-free bacteria. Transposase Himar1 randomly integrates the transposon, which contains an antibiotic resistance cassette and fluorescent markers that are flanked by repeats into the AT dinucleotide sites of the bacterial genome through a cut and paste process, which allows dual selection. The himar1 system has so far remained unexploited in the field of colistin resistance, despite its advantage of genetic markers in the transposon which facilitate selection, visual detection, and identification of insertion loci in the bacterial genome [65].

### *4.4. Whole Genome Sequencing: A Modern Approach for a Growing Challenge*

The development of genomic techniques allowing the study of diverse environmental microbial communities independently of culture has been a key factor in the paradigm changes in resistance studies [74]. Modern high-throughput DNA sequencing technologies have the potential to contain the growing antibiotic resistance trend. Analysis of the genome sequences of large collections of strains has elucidated the pathways by which antibiotic-resistant bacteria propagate [75]. Furthermore, metagenomic sequencing has revealed a large reservoir of antibiotic resistance genes present in the microbial community complex that populate the gastrointestinal tract of animals, humans, and natural environments such as surface water or soil [76]. This is demonstrated by the growing number of known resistance genes, which has occurred in parallel with the rapid decrease in sequencing costs [74]. Genomic analysis combined with updated databases is an emerging alternative approach that provides access to large amounts of information and has helped in studying resistance and better elucidating bacterial behaviour [55].

In late 2015, a Chinese team for the first time demonstrated the emergence of a colistin resistance gene carried by a plasmid, the *mcr-1* gene [5] (Figure 2). The existence of this plasmid-mediated colistin-resistance gene was demonstrated by conjugation. The *mcr-1* gene was then identified by WGS and homology modelling of the extracted plasmid pHNSHP45, using an IncI2 plasmid (pHN1122-1) as reference for annotation [5]. The *mcr-1* gene was ligated to a pUC18 cloning vector and transformed into *E. coli*. This transformant had a 4-fold increase in MIC, indicating that *mcr-1* resistance was conferred for colistin. The researchers undertook conjugation and transformation experiments to confirm the transferability of this gene [5]. Using an amino acid homology sequence, they identified *mcr-1* codes for a plasmid phosphoethanolamine transferase that catalyses the addition of pEtN to lipid A, increasing the cationic charges on LPS, thus decreasing the affinity of colistin for LPS [5]. Through genetic and bioinformatic analyses, the origin of the gene could be linked to the genus *Moraxella*. The *mcr-1* gene appears to be carried by various types of plasmids (IncX4, IncI2, IncHI2, IncF, IncY, and IncP) [7]. Since then, *mcr-1* has been reported worldwide in over 40 countries in 5 continents, and among 11 *Enterobacteriaceae* species [77]. International genome databases (NCBI, EBI) have facilitated access to genomic data, studying the source and identifying new variants of this *mcr* gene. Thus, recent publications have suggested different groups of origin: *Moraxella*, *Enhydrobacter*, *Methylophilaceae*, *Limnobacter,* and *Vibrio* as primary sources of the gene [78]. Most of the described mcr gene variants originate from animals or humans and bacteria present in water sources, which may suggest defensive functions against bacteriophages or antimicrobial peptides [78]. The pathway by which the *mcr-1* gene circulates and spreads has remained ambiguous [7,77]. The use of colistin in veterinary medicine has probably accelerated the worldwide dissemination of the *mcr-1* gene among animals and humans, which is consistent with the hypothesis that cattle, and particularly pigs, are most likely the primary source of *mcr-1* producers [7,77]. After the prohibition of colistin use as a growth promoter in animals, a considerable reduction in colistin resistance in animals and humans has been observed [79].

*mcr-2* has been discovered in an *E. coli* strain isolated from pigs and cattle in Belgium. Exclusively carried by a type IncX4 plasmid, the progenitor of the gene coding for *mcr-2* is most likely *Moraxella pluranimalium* [80] *mcr-3*, carried by an IncHI2 type plasmid. A bacterial species of the genus *Aeromonas* would be the progenitor of the *mcr-3* [81]. *mcr-4*, carried by a colE10 plasmid, was discovered in a strain of *S. enterica* serovar *Typhimurium* isolated from pigs in Italy and in *E. coli* strains from pigs in Spain and Belgium and may have *Shewanella frigidimarina* as progenitor [82]. The *mcr-5* gene was detected in an *S. enterica* serovar *Paratyphi B* carried by a ColE plasmid [83]. Until now, the progenitor is unidentified, but the *mcr-5* protein was identified in *Cupriavidus gilardii*, suggesting that this bacterial species could be the progenitor [7]. The variant *mcr-2.2* has been discovered in *Moraxella spp*. and renamed *mcr-6* [7]. One variant has been identified. The *mcr-7* gene has been identified in *K. pneumoniae* hosted on an IncI2 type conjugated plasmid. Like *mcr-3*, *mcr-7.1* comes from the *Aeromonas* species. One variant of *mcr-7* (*mcr-7.1*) has been identified to date [84]. *mcr-8* is carried on an IncFII-type conjugative plasmid in *K. pneumoniae*. Four variants of *mcr-8* have been identified, *mcr-8.1* to *mcr-8.4* [85]. *mcr-9* was discovered during an in silico screening of antimicrobial resistance gene of *S. typhimurium* sequenced genomes; the gene was harboured by IncHI2 plasmids [86]. *mcr-10* has been identified in an *Enterobacter roggenkampii* strain carried by an IncFIA plasmid. It seems the proteins *mcr-10* and *mcr-9* derived from a common ancestor of the *Buttiauxella* genes [87]. Generally, only one *mcr* determinant is contained in a bacterial isolate. However, recent studies have demonstrated the co-existence of different *mcr* variants in the same bacterial strain [88].

The analysis of 64,628 genomes deposited in NCBI databases and screened for *mcr* sequences predicted a total of 5265 putative *mcr*-like sequences that may soon be described in pathogenic bacteria, as was done for all the previously mentioned mcr variants. The study of the genetic environment demonstrated that the three less commonly found genes

lack pap2- or dgkA-linked sequences, like *mcr-4* and *mcr-5*, or are clustered to a truncated pap2 coding sequence in the even more rarely found *mcr-2* gene [89]. Furthermore, *mcr-9* and *mcr-10*, the two last and most closely related *mcr* genes identified so far, lack both hpap2 and dgkA sequences [89].

### *4.5. Transcriptomics and Proteomics*

Barczak and colleagues suggest that when using genome sequencing, the prediction of antimicrobial resistance requires a prior knowledge of all the variables that can induce phenotypical resistance (inactivating enzymes, porin mutations, influx systems, binding site mutations, gene inactivation, promoter mutations, etc.) [90]. They underline that sequence-based known resistance markers represent a small proportion and believe that transcriptome analysis could possibly afford a more comprehensive phenotypic profile [90]. They also showed that changes in bacterial expression patterns in the presence or absence of antibiotics may differ, offering the possibility of detecting resistance at the RNA level as a function of stress responses. The study of RNA provides a viable alternative to the research for resistance genes in genome sequences [91]. High throughput RNA sequencing is a modern "omics" technology [91], which employs deep sequencing technologies and is based on the sequencing of an RNA population converted into a library of cDNA fragments [92]. The read sequences are then assembled bioinformatically to reconstruct the complete transcription sequence and quantify the expression levels of the annotated genes [92].

The study of transcriptome profile variation has emerged as a widely used method to explore the genetic mechanisms that confer colistin resistance. Using a combination of genome sequencing and transcriptional profiling by RNA sequencing analysis (RNA-Seq), the identification of *crr* genes previously described as an uncharacterized histidine kinase as additional regulators of colistin resistance broadens the available genes that regulate the phenotype and illustrates the multiplicity of ways in which bacteria respond to antimicrobial peptides [54]. The increased expression of cation transporters and other efflux pumps are among the transcription modifications commonly found in colistin-resistant strains [54].

Proteome profiling has always started with immobilized pH gradient two-dimensional gel electrophoresis (2-DE) to identify differential protein abundance between samples, with validation by qPCR and/or western blot [93]. Proteomics and metabolomics, other new "omics" approaches, are used to elucidate the development process of specific biologicals and regulatory mechanisms, as well as the expression of the entire protein and changes in metabolites in specific tissues or cells at the system level [94]. The use of these "omics" techniques has enabled the large-scale discovery of new potential targets to investigate colistin resistance [95]. Fernández-Reyes et al. studied the cost of colistin-resistant *A. baumannii* resistance. They revealed that 35 differently expressed proteins, including phosphorodiamidate morpholino oligomers (PMOs), chaperones, enzymes involved in metabolism, and protein biosynthesis factors, were down-regulated in colistin-resistant strains [96]. An unlabelled quantitative proteomics study compared the proteomes of *mcr-1*-mediated colistin-resistant and colistin-sensitive *E. coli*. The authors identified a large amount of differentially expressed proteins that may contribute to *mcr-1*-mediated antimicrobial resistance through regulation of glycerophospholipid metabolism, LPS biosynthesis and phosphoethanolamine substrate accumulation [94]. Additionally, to adapt to colistin stress, some proteins involved in the TCA cycle and pentose phosphate pathway were highly expressed in *K. pneumoniae* strains [97]. These recent advances in proteomics highlight significant metabolic pathways and suggest that the processes involved in the acquisition of the colistin response are related to energy, carbohydrate, and lipid metabolism [96,97]. Thus, the inhibition of the metabolic process may potentially offer a way to overcome colistin resistance [97].

Proteomics offers a more profound, global perspective on the molecular mechanisms of polymyxin resistance and should be applied in conjunction with other omics approaches:

genomics, transcriptomics, and metabolomics, in order to understand polymyxin resistance [93]. It is still widely unexplored, due to the complexity of the strains, the cost of running proteomics on a large number of samples, the amount of generated data, and the difficulty of interpreting results [93].

### *4.6. Application of CRISPR-cas 9-Based Genome Editing*

Over the past 10 years, clustered regularly interspaced short palindromic repeats, the CRISPR-Cas Type II system of Streptococcus pyogenes, have become the easier, faster and predominant choice for engineering applications of the genomes [98]. This system requires the co-expression of the Cas9 protein and two RNAs that guide Cas9 to the target site in the intrusive DNA for recognition and subsequent cleavage. Genome editing with Cas9 has become easier since both RNAs have been enhanced to become a chimeric single-guided RNA (sgRNA). The gRNA targeting specific sequences recruits the Cas9 protein to form the compound, and the Cas9 protein acts as a nuclease and generates a blunt end double-strand break [99].

So far, the CRISPR/Cas9 system has been used as a new microbial gene therapy technology to combat colistin resistance, to confirm its use among resistant bacteria [100] and to investigate determinants of antimicrobial resistance. However, this technique is limited to in vitro treatment, due to its inability to penetrate the cell membrane by itself. There is also the lack of an effective delivery system for CRISPR/Cas9 systems, limited to phage based CRISPR/Cas9 dissemination strategies, with a risk of recombination of drug resistance genes, a narrow host range, diffusion barriers and bacterial resistance to phages [100,101]. Recently, bacterial conjugation seems to be one of the ways to release CRISPR/Cas9 into bacteria through the construction of a host-independent conjugated plasmid, which has effectively and selectively eliminated antibiotic resistance genes in *E. faecalis* [102]. Some recent original work provides a convincing demonstration of the potential of CRISPR/Cas technology to overcome the problem of multi-drug resistance, by demonstrating that existing bacterial CRISPR/Cas systems can limit the spread of drug resistance genes by countering multiple horizontal gene transfer pathways [103]. This can limit the spread of plasmid resistance by targeting antibiotic resistance genes and destroying bacterial plasmids conferring resistance [104,105]. Combating antibiotic-resistant bacteria by eliminating resistance genes contained in a bacterial community without affecting it has become possible with the advent of CRISPR/Cas9, through anti-plasmid approaches [106]. Recently, the curing of specific plasmids in multiple plasmid-carrying bacteria through the Cas9 gRNA system has been developed [106]. This system has demonstrated high efficiency, and it has been effective in destroying the plasmid harbouring *mcr-1* in *E. coli* [100] and in overcoming the resistance to other antibiotics, such as the carbapenemase and extended-spectrum-lactamase (ESBL) eradication in clinical isolates of Enterobacteriaceae [106,107]. Sun et al. have developed the CRISPR/Cas9 editing system to delete the *ramR*, *tetA* for tigecycline, and the *mgrB* gene for colistin [108] and to study the effect of these genes in carbapenem-resistant *K. pneumoniae*. More recently, McConville et al. built a CRISPR system to insert a single nucleotide point (SNP) mutation in *crrB*, *arnT* and to disrupt *crrA*, *crrB*, *mgrB*, and *pmrA* genes. *crrB* gene mutations lead to resistance to polymyxins and mediate the addition of both L-Ara4N and pEtN to lipid A, thus efficiently validating the functions of these genes [109]. This technology has the advantage of incorporating the desired mutations that enable a specific and precise modification at a particular site of the bacterial genome, without affecting the rest of the genome [110].

## 5. Conclusions

Due to its disuse in clinical medicine for years, the mechanisms of action and resistance to colistin remained unclear for a long time. This delay is compounded by a complex mechanism involving multiple metabolic pathways. However, the scientific impetus of recent years has enabled us to increase our knowledge in this field, supported by the use and development of innovative tools. For example, whole genome sequencing

represents a real added value to more traditional in vitro methods. It also enables faster detection of the presence of mutations in genes known to be involved in colistin resistance, although in vitro confirmation is still required. High throughput transcriptomics tools have considerably increased the data pool on colistin resistance. However, they remain expensive, difficult to interpret and isolate-dependent. Only a few such studies have been conducted, and the accumulation of data, combined with genome sequencing data, may allow us to identify the pathways involved in resistance. Finally, new knock-in/knock-out strategies and mutagenesis methods represent powerful tools for research in this field. Modern biotechnology techniques, high-throughput sequencing, omics techniques, and genome manipulation have facilitated a new era of discovery. Despite spectacular progress, challenges to the detection and understanding of antimicrobial resistance persist, especially for colistin, due to the unexpectedly large number of mechanisms described in various bacterial species.

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# Chapitre II

# Epidémiologie et mécanismes de résistance à la colistine chez les souches de *K. pneumoniae*.

**Article 2: Inactivation of *mgrB* gene regulator and resistance to colistin is becoming endemic in carbapenem-resistant *Klebsiella pneumoniae* in Greece: A nationwide study from 2014 to 2017.**

Mouna, H. et al. (2020) 'Inactivation of *mgrB* gene regulator and resistance to colistin is becoming endemic in carbapenem-resistant *Klebsiella pneumoniae* in Greece: A nationwide study from 2014 to 2017', International Journal of Antimicrobial Agents. Elsevier B.V., 55(4). doi: 10.1016/j.ijantimicag.2020.105930.

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**Article 3: Colistin-resistant *Klebsiella pneumoniae* ST307 clone without colistin usage: a matched case-control study and whole genome sequence comparison of bacterial genomes linked to an outbreak in Marseille, France.**

S. Baron, N. Cassir, M. Hamel, L. Hadjadj, N. Saidani, G. Dubourg, J. Rolain. Accepted Eurosurveillance (2021).

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**Avant-propos**

La propagation des souches de *K. pneumoniae* productrices de carbapénémases représente un problème de santé publique majeur au niveau mondial car les options thérapeutiques sont souvent limitées pour le traitement des infections dues à ces germes (Gomez-Simmonds and Uhlemann, 2017). Cette résistance aux carbapénèmes est souvent attribuée à un nombre limité de clones à haut risque, qui prédominent dans les populations bactériennes en milieu clinique, provoquant des infections locales et des épidémies (Lee *et al.*, 2016). L'un des clones les plus connus est le séquence type (ST) 258, et ses variants apparentés appartenant au complexe clonal 258 (CG258) (Heiden *et al.*, 2020).

La colistine reste souvent un des rares antimicrobiens encore actifs contre les souches de *K. pneumoniae* résistantes aux carbapénèmes. Cependant, certaines souches ont également acquis une résistance à la colistine (El-Sayed Ahmed *et al.*, 2020). En effet, le taux de résistance à la colistine parmi les souches de *K. pneumoniae* résistantes aux carbapénèmes a graduellement augmenté de moins de 2% à 9% dans le monde entier au cours de la dernière décennie (Petrosillo, Taglietti and Granata, 2019). Cependant, peu de données sont disponibles, car les laboratoires ne testent pas la colistine de manière systématique, ce qui limite l'interprétation de ces données. En revanche, de multiples épidémies de *K. pneumoniae* résistantes à la colistine ont été rapportées (Petrosillo, Taglietti and Granata, 2019), et des rapports récents font état de données plus inquiétantes dans certains pays européens, avec une résistance à la colistine allant jusqu'à 43 % des *K. pneumoniae* résistantes aux carbapénèmes en Italie, 20,8 % en Grèce, et jusqu'à 31 % en Espagne (Monaco *et al.*, 2014; Pena *et al.*, 2014; Meletis *et al.*, 2015). La diffusion de ces souches résulte d'une combinaison de facteurs, dont la pression de sélection exercée, la transmission de souches

résistantes, la transmission croisée, et enfin la propagation des supports génétiques de résistance (tels que les plasmides et les transposons). Cependant, ces facteurs ont été essentiellement étudiés dans des pays où l'utilisation de colistine est élevée, tandis que les facteurs induisant l'émergence de cette résistance restent mal connus dans les pays où l'utilisation de colistine est faible.

Chez *K. pneumoniae*, la régulation à la hausse des deux systèmes PhoP/PhoQ et PmrA/PmrB, conduit à l'ajout de L-Ara4N ou PEtN au LPS induisant ainsi la modification du lipide A (El-Sayed Ahmed *et al.*, 2020). Ces modifications sont aussi associées à des mutations dans les gènes *pmrD, pmrC, ccrA* et *ccrB* (Sánchez *et al.*, 2021). Les mutations du gène *mgrB* dont l'inactivation ou la délétion engendre la surexpression du système à deux composants PhoP/PhoQ, qui à son tour active l'opéron arnBCADTEF induisant la biosynthèse de L-Ara4N, augmentant ainsi la résistance à la colistine (El-Sayed Ahmed *et al.*, 2020). Il semble que la désactivation du gène *mgrB* soit le principal mécanisme de résistance à la colistine parmi ceux décrits jusqu'à présent.

L'objectif de ce chapitre était donc d'évaluer la résistance à la colistine et ses mécanismes dans une collection de souches de *K. pneumoniae* isolées en Grèce où la résistance à la colistine est endémique, et en France où la résistance à la colistine reste faible. Ainsi, dans un premier article intitulé "Inactivation of *mgrB* gene regulator and resistance to colistin is becoming endemic in carbapenem-resistant *Klebsiella pneumoniae* in Greece : A nationwide study from 2014 to 2017" (Article 2), nous avons documenté la résistance à la colistine et son épidémiologie moléculaire sur une collection de 288 isolats cliniques de *K. pneumoniae* résistantes aux carbapénèmes isolés en Grèce. Dans un deuxième article intitulé "Colistin-resistant *Klebsiella pneumoniae* ST307 clone : Epidemiological, risk factors and massive molecular

analysis of bacterial genomes linked to an outbreak in Marseille, France", nous rapportons l'émergence d'un clone de *K. pneumoniae* résistant aux carbapénèmes et à la colistine, appartenant au ST307, isolé de patients hospitalisés à l'AP-HM de Marseille entre 2014 et 2017, et nous évaluons les facteurs de risque associés à l'acquisition de ces souches résistantes (Article 3).

**Article 2: Inactivation of *mgrB* gene regulator and resistance to colistin is becoming endemic in carbapenem-resistant *Klebsiella pneumoniae* in Greece: A nationwide study from 2014 to 2017**.

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**Résumé**

La Grèce compte parmi les pays où la propagation des entérobactéries résistantes aux carbapénèmes a pris une grande ampleur. Les travaux antérieurs relatifs à la prévalence réelle de la résistance à la colistine en Grèce étaient limités et fondés principalement sur une analyse phénotypique, sans exploration du mécanisme moléculaire de la résistance à la colistine. Ces travaux observaient une augmentation rapide du niveau de la résistance à la colistine, en raison notamment de l'endémicité des souches de *K. pneumoniae* résistantes aux carbapénèmes. Ainsi, nous avons réalisé dans un premier temps une étude épidémiologique multicentrique rétrospective afin d'évaluer la prévalence de la résistance à la colistine et aux carbapénèmes parmi une collection représentative de 973 souches cliniques de *K. pneumoniae* isolées de huit sites hospitaliers en Grèce. Dans un deuxième temps, nous avons caractérisé la résistance aux carbapénèmes et à la colistine par une analyse des mécanismes moléculaires impliqués en utilisant des approches phénotypiques et génotypiques. Cette étude rapporte également l'utilisation de la technologie du séquençage du génome complet pour étudier le cas d'une souche de *K. pneumoniae* dépourvu d'une grande région au sein du locus *mgrB*.

Sur les 973 souches cliniques de *K. pneumoniae,* 288 souches résistantes au méropénème, ont été recueillies. 226 (78%) de ces souches étaient des bactéries difficiles à traiter (DTR), qui correspondent aux bactéries résistantes aux antibiotiques de 1ère ligne, notamment les β-lactamines et les fluoroquinolones (Kadri *et al.*, 2018). 256 (88,8 %) étaient porteuses d'un gène codant pour une carbapénémase. L'enzyme KPC était la carbapénémase la plus fréquemment rencontrée (116 ; 40,3 %), suivi par VIM (41 ; 14.2 %) et NDM (33 ; 11.5 %). Alors que l'enzyme OXA-48 n'avait jusqu'alors été rapportée que dans des cas sporadiques, nous avons noté la présence non

négligeable de souches productrices d'OXA-48 (22 ; 7,6 %), témoignant ainsi d'un changement d'épidémiologie. En outre, 44 (15,3 %) souches coproduisaient deux types de carbapénémases.

Parmi les souches résistantes aux carbapénèmes, la co-résistance à la colistine était élevée, de l'ordre de 73% (N= 213), suggérant ainsi que cette résistance était devenue endémique en Grèce. La concentration minimale inhibitrice de la colistine variait de 4 mg/L à > 256 mg/L, ce qui suppose que ce haut niveau de résistance est dû principalement à des mutations chromosomiques. En effet, de manière surprenante aucun gène *mcr* n'a été détecté dans cette collection de souches, mais des mutations dans les gènes chromosomiques ont été détectées. En revanche, le gène *mgrB* était inactivé dans 148 souches (69,5 %), majoritairement par des séquences d'insertion (N=94, 44,1%). Les séquences d'insertion appartenant à la famille IS5 étaient le mécanisme le plus commun retrouvé. Nous n'avons pas pu amplifier le gène dans 21 (10 %) des souches, suggérant que le gène était délété ou présentait une grande insertions de séquence. Pour explorer ce phénomène, un génome d'une souche avec un gène *mgrB* non amplifiable a été séquencé, assemblé, annoté et mappé contre un génome de référence. Une recherche par BLAST effectuée sur une zone de 7kb incluant le locus *mgrB* a révélé que le gène était tronqué par une insertion de séquence. La souche présentait une délétion d'une région de 500 pb contenant 3 gènes, dont une grande partie du gène *mgrB,* expliquant la négativation de la PCR. Nous ignorons cependant le mécanisme qui sous-tend la délétion observée dans un locus chromosomique particulièrement conservé chez *K. pneumoniae.* Enfin, 30,5% (N=65) des souches résistantes à la colistine présentaient un gène *mgrB* intact, suggérant la présence d'un autre mécanisme de résistance.

Dans notre étude, la dissémination de la résistance à la colistine dans les isolats cliniques de *K. pneumoniae* est probablement multi clonale. Ainsi en Grèce, il apparait que la pression de sélection liée à l'utilisation de la colistine chez l'homme a conduit à l'émergence et à la sélection de différents clones bactériens résistants à la colistine par mutations chromosomiques dans les gènes de la régulation. La modification des stratégies thérapeutiques pour traiter les infections à BGN apparait nécessaire pour réduire la dissémination de ces clones multi-résistants. Une surveillance épidémiologique continue en Grèce permettra d'identifier les changements épidémiologiques, et d'évaluer l'efficacité des mesures prises pour réduire la prévalence de la résistance aux carbapénèmes et à la colistine chez ces entérobactéries.

Notre étude sur une période relativement courte montre que l'épidémiologie d'infections comme celles dues à *K. pneumoniae* peut changer de manière extrêmement rapide lorsqu'une pression de sélection est présente. L'émergence actuelle dans le monde d'infections dues à *K. pneumoniae* montre à quel point cette bactérie est capable de s'adapter rapidement aux changements liés aux interventions humaines.

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# Inactivation of *mgrB* gene regulator and resistance to colistin is becoming endemic in carbapenem-resistant *Klebsiella pneumoniae* in Greece: A nationwide study from 2014 to 2017

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ABSTRACT

*Introduction:* In Greece, the spread of carbapenem-resistant Enterobacteriaceae in humans has led to the reintroduction of colistin as a therapeutic agent. Unfortunately, colistin resistance with different mechanisms has emerged. The present work aims to determine the prevalence of carbapenem and colistin resistance and the corresponding mechanisms in *Klebsiella pneumoniae* clinical isolates from Greece.
*Methods:* From 2014 to 2017, 288 carbapenem-resistant *K. pneumoniae* clinical strains were gathered from a collection of 973 isolates from eight different hospitals in Greece. Antibiotic susceptibility testing was performed using three different methods. Screening of carbapenem and colistin resistance genes was conducted using polymerase chain reaction (PCR) amplification and sequencing.
*Results:* Among the 288 (29.6 %) carbapenem-resistant isolates, 213 (73.9%) were colistin-resistant (minimum inhibitory concentration [MIC] >2 mg/L). The KPC type was the most common carbapenemase gene (116; 40.3%), followed by VIM (41; 14.2%), NDM (33; 11.5%) and OXA-48 (22; 7.6%). Moreover, 44 (15.3%) strains co-produced two types of carbapenemases. No *mcr* genes were detected for colistin resistance but mutations in chromosomal genes were found. These included inactivation of the *mgrB* gene for 148 (69.5%) strains, including insertion sequences for 94 (44.1%), nonsense mutations for 4 (1.9%) and missense mutations for 24 (11.3%). Moreover, PCR amplification of *mgrB* gene was negative for 26 (12.2%) strains. Finally, 65 (30.5%) colistin-resistant strains exhibited a wild-type *mgrB*, the mechanisms of which remain to be elucidated.
*Conclusion:* This study shows that *K. pneumoniae* clinical strains in Greece are resistant to both carbapenems and colistin and this is endemic and is likely chromosomally encoded.


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## 1. Introduction

Carbapenems have long been considered the treatment of choice for human infections caused by multidrug-resistant Gram-negative bacteria (GNB), and their use has increased due to the spread of extended-spectrum $\beta$-lactamases (ESBLs) among Enterobacteriaceae [1]. Nevertheless, carbapenem efficacy has been challenged by the emergence of carbapenem-resistant Enterobacteriaceae, which have spread worldwide, including to Greece [2].

A national outbreak of carbapenem-resistant *Klebsiella pneumoniae* carrying the VIM gene was reported in Greece in the 2000s [3], followed by the emergence of KPC-producing *K. pneumoniae* isolated for the first time in August 2007 [4]. Since then, these KPC-producing strains have spread rapidly, causing initial outbreaks that evolved into an endemic situation [5]. A first isolate of NDM-1-producing *K. pneumoniae* was reported in 2012; since then, several outbreaks of NDM-producing Enterobacteriaceae have been reported in different hospitals in Greece [6]. The isolation of OXA-48-producing Enterobacteriaceae is still uncommon [3]. Surveillance data in 2017 from the European Centre for Disease Prevention and Control (ECDC) showed a carbapenem resistance rate of 64.7% in *K. pneumoniae* strains recovered from blood samples in Greece; this was the highest resistance rate in Europe [7]. A European survey of carbapenemase-producing Enterobacteriaceae conducted from 2013 to 2014 in 35 European countries reported an average of 1.3 patients per 10 000 hospital admissions carrying carbapenemase in Europe [8]. In Greece, this incidence was 5.78 patients per 10 000 admissions, second only to Italy (5.96/10 000) [8]. Cassini et al. estimated that 33 000 deaths per year in Europe could be attributed to multidrug-resistant (MDR) bacteria because of the high prevalence of resistance in GNB, particularly in Greece and Italy [9]. However, the true rate of deaths attributed to antibiotic resistance is unclear [10]. The high prevalence of carbapenem resistance in Greece may lead to difficult-to-treat (DTR) infections [11].

Under these circumstances, colistin has been reintroduced as a last resort treatment for infections caused by multidrug-resistant carbapenemase producers [12]. However, the emergence of colistin resistance in GNB has been reported in several countries, with resistance mediated via genetic variations represented by chromosomal mutations in genes involved in lipopolysaccharide synthesis, namely *phoP* / *phoQ, pmrA* / *pmrB* or *crrA* / *crrB* as well as on the *mgrB* regulatory gene [12]. These mutations lead to overexpression of these systems and an increased synthesis of phosphoethanolamine (pEtN) and 4-amino-4-deoxy-L-arabinose (LAra4N) [12]. Moreover, colistin resistance can also be plasmid-mediated: the first colistin resistance gene involving a conjugative plasmid, *mcr*-1 (mobilized colistin resistance) was reported by Yi-Yun Liu et al. in China in December 2015 [13]. The *mcr*-1 was identified in GNB isolates of human, animal and environmental origin [13]. The location of the plasmid gives it the potential to be laterally transferable, which explains its wide diffusion (confirmed by numerous studies) [14]. Seven other *mcr* variants have since been reported worldwide [15]. The use of an efflux pump and the formation of capsules may also be involved in colistin resistance [14].

Studies conducted in Greece on *K. pneumoniae* isolates from different clinical samples have reported a significant increase in colistin resistance from 3.5% (4/115) in 2010 to 20% (25/120) after 2010 [16]. In addition, a recent study reported a 19% resistance rate to last-line antibiotics, including colistin, in human clinical isolates [3]. This rapid increase, coupled with the lack of molecular data, motivated this current study project, which was conducted to evaluate the prevalence of colistin- and carbapenem-resistance among a collection of *K. pneumoniae* clinical strains isolated between 2014 and 2017 from eight hospitals in Greece, and to understand the molecular mechanisms involved.

## 2. Materials and methods

### 2.1. Bacterial isolates

Between 2014 and 2017, 973 *K. pneumoniae* clinical strains were collected from eight Greek hospitals, six in Athens, one in Thessalia (central Greece) and one in Peloponnese (Fig. S1). Strains were tested against carbapenems (meropenem) by Etest (BioMérieux, France), and the resistant strains with a minimum inhibitory concentration (MIC) >8 mg/L were selected and stored for further analysis. Identification at the species level was performed by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry (Bruker Daltonik, Bremen, Germany) [17].

### 2.2. Antimicrobial susceptibility test

Antimicrobial susceptibility testing was performed on meropenem-resistant isolates using the disk diffusion method on a Mueller-Hinton agar (MHE BioMérieux, France) and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, except for cyclines, which were interpreted according to Clinical and Laboratories Standard Institute (CLSI) recommendations. The imipenem MIC determination was performed by the Etest method (BioMérieux, France), and data were interpreted according to EUCAST criteria. MICs of colistin were determined by the broth microdilution method and the results were interpreted according to EUCAST guidelines.

DTR bacteria were defined as previously described [11]. Strains were considered as DTR when they had an intermediate or resistant status to three antibiotic classes ($\beta$-lactams, carbapenems, and fluoroquinolones).

### 2.3. Molecular characterization

DNA extraction was performed on BioRobot EZ1 (Qiagen, Venlo, Netherlands) using a commercial EZ1 DNA extraction kit (Qiagen) according to the manufacturer's instructions.

#### 2.3.1. Carbapenemase-encoding genes

All strains were screened for the presence of bla$_{KPC}$, bla$_{NDM}$, bla$_{VIM}$, bla$_{OXA-48}$, and bla$_{IMP}$ by quantitative polymerase chain reaction (qPCR) (Table S_1).

#### 2.3.2. Colistin resistance genes

All strains were screened for the presence of plasmid-mediated colistin resistance genes represented by *mcr-1, mcr-2, mcr-3, mcr-4, mcr-5* and *mcr-8* by PCR using specific primers and probes (Table S_1). Those strains were also investigated to detect possible genetic alterations associated with colistin resistance in the *mgrB* genes. The *mgrB* genes were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA). Sequences of the colistin resistance genes were recovered and compared to those of the reference strain, *K. pneumoniae* MGH78578 carrying wild-type genes (GenBank accession number NC_009648). PROVEAN software (http://provean.jcvi.org/index.php) was used to check whether amino acid sequence changes could alter protein function [18]. In addition, the ISfinder database was used to identify the insertion sequences types (https://www-is.biotoul.fr/) [19].

### 2.4. Whole-genome sequencing

Genomic sequencing was performed with the MiSeq sequencer (Illumina Inc., San Diego, CA, USA) in a pair-end to study certain atypical phenotypes. D*e novo* assembly was performed with A5

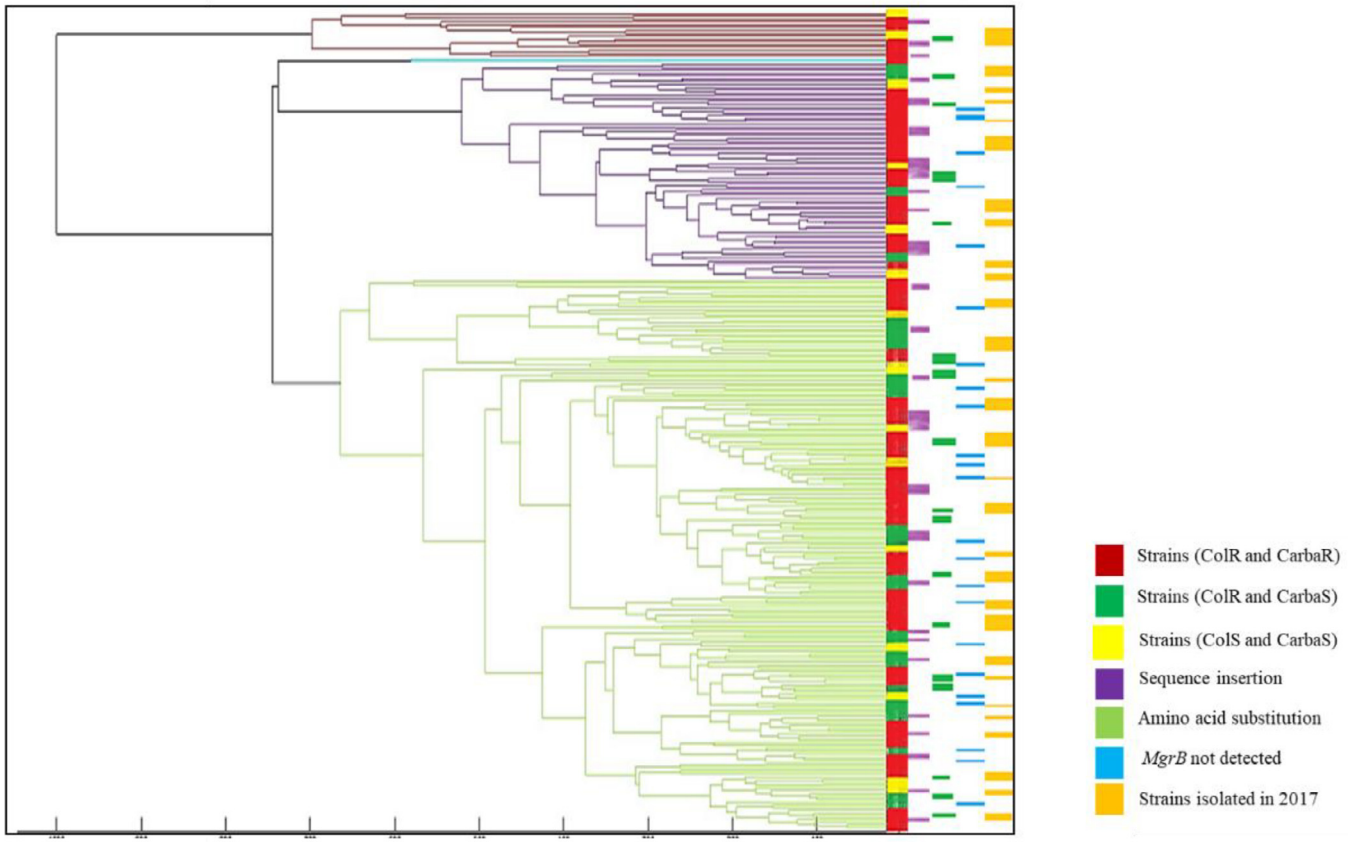


**Fig. 1.** MALDI-TOF MS Dendrogram of 288 meropenem-resistant *K. pneumoniae* clinical strains, 2014 to 2017.

pipeline, then reorganized by mapping on a reference genome of *K. pneumoniae* MGH785 using MAUVE.

### 2.5. Statistical analyses

Statistical analyses were performed using the Chi-squared test. A *P*-value $<0.05$ was considered statistically significant.

## 3. Results

### 3.1. Bacterial identification

The 973 *K. pneumoniae* strains were isolated from 196 (20.1%) blood, 569 (58.5%) urine and 153 (15.7%) respiratory samples, and the remaining 55 (5.6%) were from other sources. The 288 meropenem-resistant *K. pneumoniae* strains were isolated from 148 (44%) blood, 89 (27%) urine and 44 (13%) respiratory samples, and the remaining 7 (2.43%) were from other sources. The majority of strains (213, 74%) were isolated in 2017.

The MSP dendrogram of 288 meropenem-resistant *K. pneumoniae* strains did not reveal any distinct groups or clusters (year, origin, hospital) or (phenotype of carbapenem or colistin resistance) (Fig. 1).

### 3.2. Phenotypic profile of the bacteria studied

Antibiotic results from the 288 strains of *K. pneumoniae* showed a very remarkable resistance to the $\beta$-lactam family (97%) (Fig. 2). *K. pneumoniae* strains were resistant to aminoglycosides, with 57% and 69% resistance for gentamicin and amikacin, respectively. Resistance to quinolones, sulfamides, nitrofurans and cyclines was observed in 96%, 78%, 75% and 74% of strains, respectively. However, fosfomycin was the most active antibiotic on the majority of strains tested (28%). A total of 226 of the 288 (78%) strains were DTR.

The imipenem Etest showed that of 288 *K. pneumoniae* strains, 256 (88.8%) were resistant to imipenem with MICs ranging from 3 mg/L to >32 mg/L, 156 (16%) strains of which had an MIC >32 mg/L. The 32 remaining strains were resistant to both ertapenem and meropenem but not to imipenem. Colistin MIC revealed that of 973 *K. pneumoniae* strains, 213 (21.9%) were resistant to colistin, with MICs ranging from 4 mg/L to >256 mg/L.

Statistical analyses showed that resistance to colistin was significantly associated with resistance to carbapenems for the meropenem-resistant strains (n=288) ($P<0.05$).

### 3.3. Molecular analyses

#### 3.3.1. Molecular mechanisms of carbapenem resistance

Of the 288 meropenem-resistant *K. pneumoniae* strains, 256 (88.9%) carried a gene encoding a carbapenemase, and 32 (11.1%) strains were carbapenem-resistant and negative for all tested carbapenemase genes in this study. The KPC enzyme was the most common carbapenemase and was identified in 116 strains (40.3%), followed by the VIM and NDM detected in 41 (14.2%) and 33 (11.5%) strains, respectively. OXA-48 enzymes were found in 22 strains (7.6%) (Table 1). Four OXA-48-positive strains remain susceptible to imipenem (MICs ≤2 mg/L). The IMP enzyme was not detected in any strain. A total of 44 (15.3%) strains harboured two types of carbapenemases, with five combinations, namely $bla_{KPC+NDM}$, $bla_{KPC+OXA-48}$, $bla_{KPC+VIM}$, $bla_{NDM+OXA-48}$, and $bla_{NDM+VIM}$ (Table 1).

Sequence analysis of the $bla_{KPC}$, $bla_{NDM}$, and $bla_{OXA-48}$-positive strains revealed that these strains belonged to the KPC-2, NDM-1,

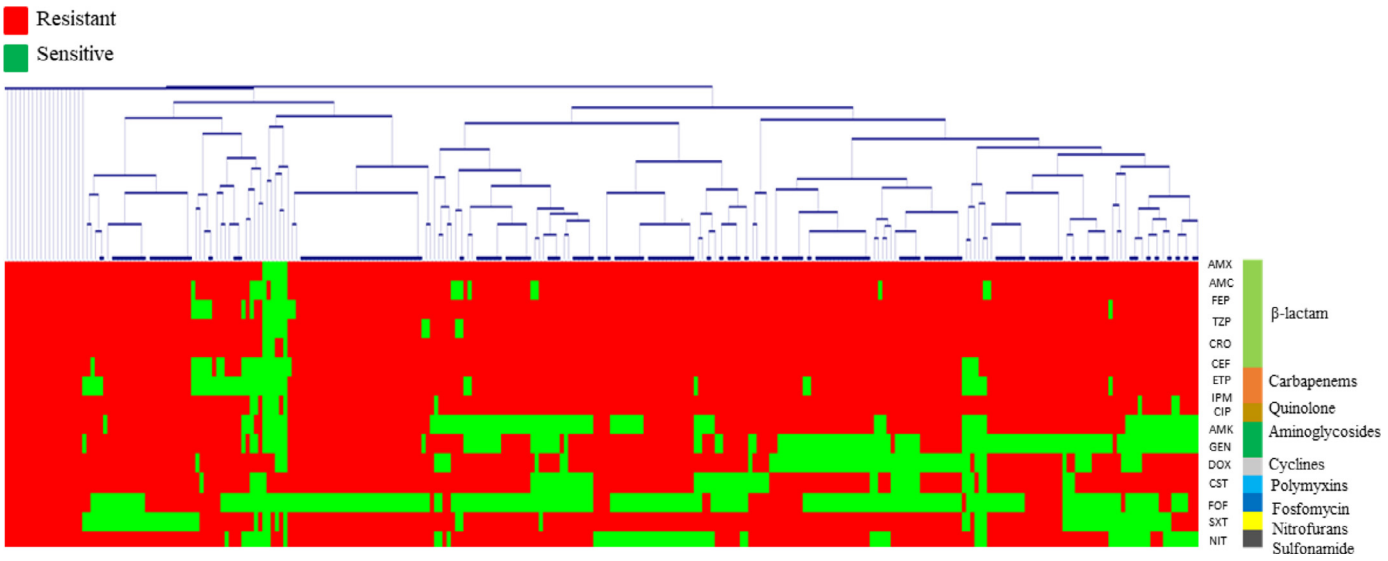


**Fig. 2.** Hierarchical clustering of antibiotic susceptibility profiles (disk diffusion method) representing the activity of the 16 antibiotics tested against the 288 meropenem-resistant *K. pneumoniae* strains isolated in this study. AMX: Amoxicillin; AMC: Amoxicillin + clavulanic acid; FEP: Cefepime; TPZ: Piperacillin + Tazobactam; CRO: Ceftriaxone; CEF: Cefalotin; ERT: Ertapenem; IMP: Imipenem; CIP: Ciprofloxacin; AMK: Amikacin; GEN: Gentamicin; DOX: Doxycycline; CST: Colistin; FOF: Fosfomycin; SXT: Trimethoprim / sulfamethoxazole; NIT: Nitrofurantoin.

**Table 1**
Carbapenemases detected among carbapenem-resistant *K. pneumoniae* strains isolated from Greek hospitals, 2014 to 2017. (* Several isolates contain more than one carbapenemase). KPC: *Klebsiella pneumoniae* carbapenemase; NDM: New Delhi metallo-beta-lactamase; VIC: Verona integron-mediated metallo-beta-lactamase; OXA-48: Oxacillinase-48.

| Species Imipenem R (MIC >2 mg/L | Carbapenem resistance genes | Positive results for carbapenemases N (%) | | MIC imipenem median value |
|---|---|---|---|---|
| | | N | Percentage (%) | |
| ***Klebsiella pneumoniae*** | $bla_{KPC}$ | 116 | 40.3 | 32 |
| | $bla_{NDM}$ | 33 | 11.5 | |
| | $bla_{OXA-48}$ | 22 | 7.6 | 8 |
| | $bla_{VIM}$ | 41 | 14.2 | 12 |
| | $bla_{KPC+NDM}$ | 12 | 4.2 | 32 |
| | $bla_{KPC+OXA-48}$ | 9 | 3.1 | 12 |
| | $bla_{KPC+VIM}$ | 10 | 3.5 | 32 |
| | $bla_{NDM+OXA-48}$ | 6 | 2.1 | |
| | $bla_{NDM+VIM}$ | 7 | 2.4 | |
| | Negative | 32 | 11.1 | |
| | **Total** | 288* | 100 | 32 |

MIC, minimum inhibitory concentration

and OXA-48 variants, respectively. Of the VIM-positive strains, five variants were identified, VIM-1, VIM-2, VIM-19, VIM-52 and VIM-55 (VIM-52 and VIM-55 are variants of VIM-1).

### 3.3.2. Molecular mechanisms of colistin resistance

*3.3.2.1. Plasmid-mediated colistin resistance genes.* PCR screening for plasmid-mediated colistin resistance genes (*mcr-1* to *mcr-8*) was performed on the 288 *K. pneumoniae* strains. Results show that no positive *mcr* isolates were detected.

### 3.3.3. Genetic alterations of the mgrB gene

The entire *mgrB* gene of the 213 colistin-resistant *K. pneumoniae* strains was amplified by standard PCR and sequenced. Among these isolates, 187 were *mgrB*-positive and 26 were *mgrB*-negative. Of the 187 positives, 94 (50.3%) strains generated amplicons larger than the normal size of the *mgrB* gene, indicating the presence of sequence insertions at the coding region for the *mgrB* gene.

ISK*pn*26 of the IS5 family was the most common insertion sequence found in *mgrB* and was identified in 42 (19.7%) isolates, followed by IS*Ec68* (19 strains; 8.9%), ISK*pn*14 (13; 6.1%), IS1R (10; 2.3%), ISK*pn*25 (6; 2.8%), IS903 (2; 0.9%) and IS5 (2; 0.9%) (Table 2). The ISK*pn*26 and IS*Ec*68 belonging to the IS5 family were 1200 bp and 1199 bp, respectively (Fig. 3). These ISs were inserted at the same position, between nucleotides 69 and 70. In contrast,

**Table 2**
Table summarizing the different genetic alterations of the *mgrB* gene in the 213 *K. pneumoniae* colistin-resistant strains isolated from Greek hospitals, 2014 to 2017.

| Alterations of the *mgrB* gene | | N | Percentage (%) |
|---|---|---|---|
| 94 sequence insertions | ISK*pn*26 - IS5 | 42 | 19.7 |
| | IS*Ec*68 - IS5 | 19 | 9 |
| | ISK*pn*14 - IS1 | 13 | 6.1 |
| | IS1R - IS1 | 10 | 4.6 |
| | ISK*pn*25 - ISL3 | 6 | 2.8 |
| | IS903 - IS5 | 2 | 1 |
| | IS5 - IS5 | 2 | 1 |
| Amino acid substitutions | | 24 | 11.3 |
| Premature stop codon | | 4 | 1.9 |
| Intact | | 65 | 30.5 |
| Not detected | Total deletion | 21 | 9.8 |
| | Partial deletion | 5 | 2.3 |
| Total | | 213 | 100 |

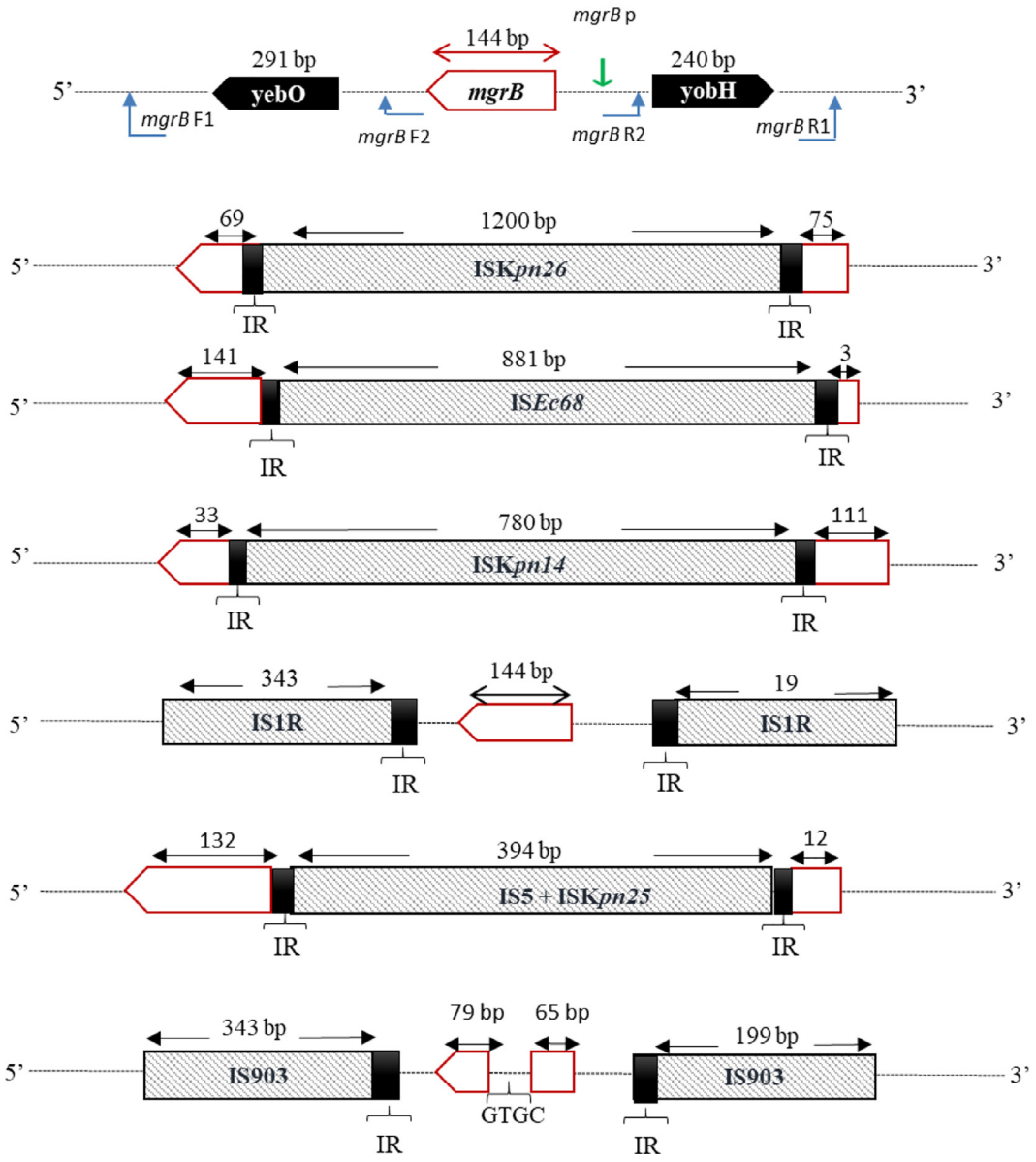


**Fig. 3.** Schematic representation of the *mgrB* gene of *K. pneumoniae* strains resistant to colistin, showing the different integration sites of sequence insertions (IR: inverted repeat; *mgrB* p: *mgrB* promotor).

an IS*Ec*68 of 881 bp was inserted between nucleotides 141 and 142. An ISK*pn*14 belonging to the IS1 family was found inserted at two different positions with sequence lengths of 780 bp and 778 bp (Fig. 3). The IS1R of the IS1 family was identified at different positions in the gene but at the promoter of the *mgrB* gene in one isolate. An IS903 was also found inserted at the level of the gene promoter in a strain that also had a 4-base insertion (GTGC) between nucleotides 80 and 81 (Fig. 3). A 394-bp ISK*pn*25 belonging to the ISL3 family was detected between nucleotides 132 and 133.


**Fig. 4.** Alignment of the protein sequences of the *mgrB* gene of *K. pneumoniae* colistin-resistant strains (a): using the external primers (the underlined areas indicate the presence of a mutation); (b): using the internal primers; KP: *Klebsiella pneumoniae*.

### *3.3.4.* MgrB *gene sequence changes by nonsense mutations*

In 4 (1.9%) strains, a single mutation induced the appearance of a premature stop codon (Table 2), which leads to the replacement of a glutamine by a stop codon at position 30 (Q30Δ). The resulting protein comprised only 29 amino acids, instead of a non-mutated protein of 47 amino acids (Fig. 4.A).

### *3.3.5.* MgrB *gene sequence change by missense mutations*

Twenty-four *K. pneumoniae* colistin-resistant strains had a single nucleotide substitution in the *mgrB* gene (Fig. 4.A). Mutations

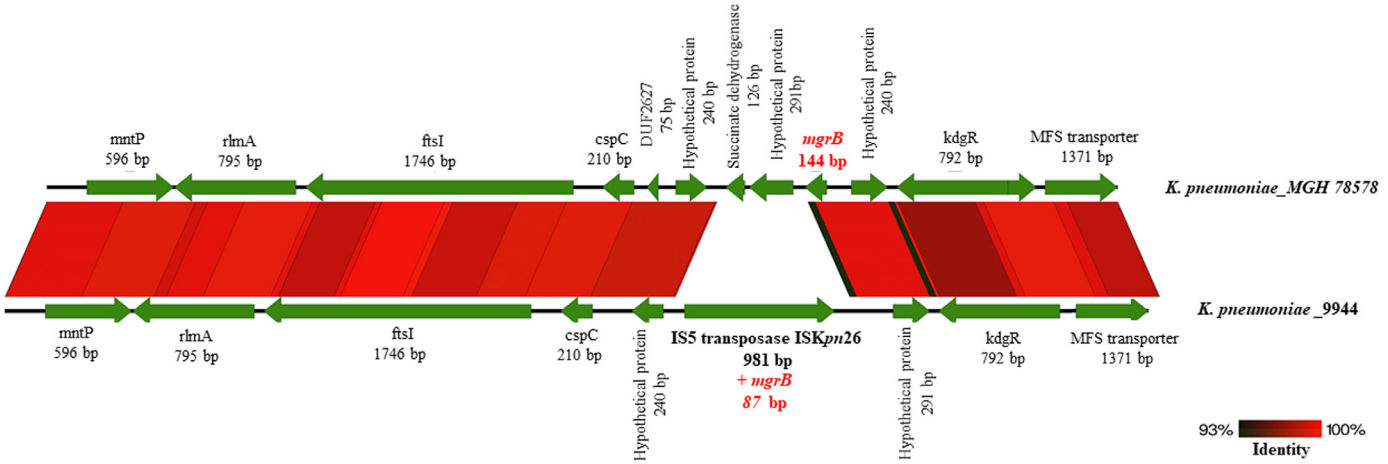


**Fig. 5.** Genetic location and linear comparison of the 7-kb containing the *mgrB* gene of the reference *K. pneumoniae_MGH 78578* and *K. pneumoniae 9944* using EasyFig software. The arrows indicate the positions and directions of the open reading frames (ORFs); direction indicates the gene orientation. The green arrows represent the genes included in the genetic environment, the *mgrB* gene is shown in red. The names of the proteins are shown above each gene.

induced the replacement of phenylalanine (F35I) by isoleucine at position 35 in one isolate and glycine by serine (G37S) at position 37 in three isolates. Substitution of lysine with valine at position 2 (K2V) was the most frequently encountered mutation (20 isolates) (Table 2). All these substitutions have been reported deleterious by PROVEAN software. One isolate had a four-base insertion (TGCG) between nucleotides 85 and 86, resulting in a frame shift and a non-functional truncated protein of 27 amino acids.

*3.3.6. Partial and total deletion of the* mgrB *gene*

For DNAs of 26 (12.2%) colistin-resistant *K. pneumoniae* strains that could not be amplified by standard PCR, additional amplification using internal primers of the *mgrB* gene was performed (Fig. 3). The latter made it possible to obtain amplicons for 5 of the 26 strains. The five strains had a partial deletion of the gene as well as significant alterations in the protein sequence of the *mgrB* gene (Fig. 4.B). For the remaining 21 strains, no sequence was obtained, indicating complete absence of the *mgrB* locus.

Genomic sequencing of one of those strains revealed that the *mgrB* gene was truncated by an ISK*pn*26 (981 bp), and the strains lacked a region of 500 bp containing 3 genes, including a part of a *mgrB* gene (57 pb), which explains the impossibility to amplify the gene by the two sets of primers available. A BLAST search along a 7-kb region containing the *mgrB* gene against *K. pneumoniae_MGH 78578* revealed a conserved chromosomal location and genetic environment of this gene (Fig. 5).

## 4. Discussion

The prevalence of carbapenem-resistant *K. pneumoniae* has increased in Greece since the first report of *K. pneumoniae*-producing VIM in 2001, and it is currently considered endemic [20].

In the present study, prevalence of carbapenem resistance in *K. pneumoniae* clinical strains was about 30% in 8 hospitals in Greece. The KPC type (40.3%) remains the most prevalent carbapenemase-encoding gene followed by VIM (12.5%), the latter are also known for their endemicity in Greece [21].

These results are consistent with a national surveillance study carried out between January 2011 and June 2012 in 119 hospitals in Greece: this study showed that in *K. pneumoniae* strains, the KPC enzyme was the most prevalent mechanism of resistance to carbapenems (82.6%), followed by the VIM (9.7%) [22]. However, a European survey of carbapenemase-producing Enterobacteriaceae from 2013 to 2014 showed that of 86 carbapenem-resistant *K. pneumoniae* strains isolated in Greece, the NDM-type enzyme was the second most prevalent carbapenemase with a rate of 14% after KPC (10.5%) [8].

Although the OXA-48 enzyme has been reported in sporadic cases [3], the emergence of strains carrying the OXA-48 gene has been noted. The true prevalence of this enzyme is poorly known in Greece [23]. Usually, OXA-48 confers a lower resistance than other carbapenemases and is not always detected in antibiotic susceptibility testing [24]. Interestingly, OXA-48-positive *K. pneumoniae* strains that were sensitive to carbapenems were found in the current study. The IMP enzyme was not detected in any of the isolates; indeed, in Greece there are no data regarding its isolation [25]. The co-production of different carbapenemases by the same strain is more frequently reported in the latest studies in several countries (India, Egypt, China and Greece) [23,24,26–29]. In the current study strain collection, 44 (15.3%) strains of *K. pneumoniae* co-producing two types of carbapenemases were identified. Recent studies in Greece have reported the presence of *K. pneumoniae* strains producing both KPC-2 and VIM-1 as well as NDM-1 and VIM-1 [28,29]. The KPC-2 and NDM-1 variants remain the most prevalent carbapenemases among the current study strains, confirming the previous Greek estimates [21,29]. For the VIM-type genes analysed, several variants were detected. VIM-1, VIM-2 and VIM-19 have already been described in Greece [21,29], in contrast to VIM-52 and VIM-55.

Colistin has regained a significant part of the therapeutic regimen for the treatment of carbapenem-resistant bacterial infections; however, it was rapidly followed by the emergence of resistance. In the current study strain collection, among the 973 *K. pneumoniae* strains we reported an overall colistin resistance rate of 21.9% and a rate of 73.9% of resistance among carbapenem-resistant *K. pneumoniae* strains. None of the *mcr* genes tested were detected, which confirms the data in the literature that show the prevalence of *mcr* genes worldwide is much higher in strains of animal than human origin [30]. This assumes that their reservoir is at least in animals and the environment, following the important use of colistin in animal production and in general agriculture [30]. In Greece, unlike other countries, the massive use of colistin in clinical practice following the spread of carbapenemase-producing Enterobacteriaceae has led to the selection of multidrug-resistant bacteria in hospital settings. According to data available on ECDC, the consumption of colistin was 0.004 DDDs (daily defined dose of antibiotics) / 1000 patients per day in 2000, 0.071 in 2010 and 0.135 in 2016 [7].

In the present study, of the 213 colistin-resistant *K. pneumoniae*, 148 (69.5%) had an inactivated *mgrB* gene, mediated either

by sequence insertions, absence or point mutations of the *mgrB* gene. The *mgrB* gene is a conserved, 144-nucleotide gene encoding a 47-amino acid transmembrane protein, a strong negative feedback from the *PhoQ* / *PhoP* regulatory system [14]. Inactivation by sequence insertions was the predominant cause of colistin resistance and the ISK*pn*26 element was the most prevalent.

The interruption of the *mgrB* gene by ISK*pn*25, IS903 and IS*Cs*68, *IS5*, or ISK*pn*14 has already been reported, but has not been described so far in Greece [31–35]. Avgoulea et al. reported that insertional inactivation of the *mgrB* gene conferred resistance to colistin in all isolates tested [31]. The F35I and G30Del substitutions in the *mgrB* gene have been reported; mutations in the same amino acid position have also been reported in colistin-resistant *K. pneumoniae* isolates in several other studies [36]. This reinforces the hypothesis that these substitutions in the *mgrB* protein occur in a critical region that is likely to mutate upon the emergence of resistance. In addition, new amino acid changes in the *mgrB* gene were observed and probably led to a predicted truncated and non-functional *mgrB* protein.

The prevalence of colistin resistance remains low in many countries, unlike in Greece. Overall, this resistance is closely related to the dissemination of carbapenem-resistant bacteria. Thus, the current study statistical analyses have shown that resistance to colistin was significantly associated with the carbapenem resistance phenotype and correlated with the production of KPC and NDM carbapenemase genes in *K. pneumoniae,* contributing to their multiresistant phenotypes.

In conclusion, the current study reported a high rate of resistance to colistin (70%) in carbapenem-resistant *K. pneumoniae* clinical isolates. This was likely due to chromosomal mutations of target genes, particularly inactivation of the *mgrB* gene by sequence insertions, and not by spreading of plasmid-mediated *mcr* colistin resistance genes. This finding clearly supports the notion that colistin selection pressure in humans and animals led to the selection of different bacterial clones in colistin-resistant bacteria, with *mcr* variants in animals and environment and specific clones with chromosomal mutations in humans. However, the observed resistance could not be explained in a large proportion of samples (approximately 31% that harboured a wild-type *mgrB* gene and in about 10% where the *mgrB* gene could not be amplified), thereby limiting the conclusions that might be drawn. Finally, the current study indicates that colistin resistance is becoming endemic in Greece in carbapenem-resistant *K. pneumoniae* human isolates. The prevalence of resistance to carbapenems and colistin in Greece should be surveyed and new therapeutic strategies including old drugs should be evaluated and used in Greece. Among the different antibiotics tested, fosfomycin may be a valid alternative to treat carbapenem- and colistin-resistant bacteria in Greece.

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## Declarations

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**Competing Interests:** The authors declare that they have no competing interests.

**Ethical Approval:** Not required

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2020.105930.

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**Article 3: Colistin-resistant *Klebsiella pneumoniae* ST307 clone without colistin usage: a matched case-control study and whole genome sequence comparison of bacterial genomes linked to an outbreak in Marseille, France.**

S. Baron, N. Cassir, **M. Hamel**, L. Hadjadj, N. Saidani, G. Dubourg, J. Rolain. Accepted Eurosurveillance (2021).

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**Résumé**

L'émergence et la dissémination d'une résistance à un antibiotique est souvent liée à l'utilisation massive de cet antibiotique. Les premières études portant sur les facteurs d'émergence de la résistance à la colistine, ont confirmé cette hypothèse, notamment dans les pays où l'utilisation de colistine est importante, comme en Grèce ou en Italie (Olaitan *et al.*, 2014; Petrosillo, Taglietti and Granata, 2019; Mouna *et al.*, 2020). Plus récemment, d'autres rapports ont observé une émergence de cette résistance à la colistine chez l'homme en l'absence d'utilisation de colistine (Prim *et al.*, 2015; Olaitan, Morand and Rolain, 2016). En France, l'utilisation de colistine est limitée et les données de prévalence et des mécanismes de la résistance à la colistine ont été très peu étudiés. Ainsi, l'objectif de notre travail était d'étudier les facteurs associés à la résistance à la colistine chez *K. pneumoniae* en France et de décrire les mécanismes moléculaires impliqués dans cette résistance. Nous avons réalisé pour cela une étude rétrospective cas-témoins entre 2014 et 2017 en incluant les patients hospitalisés à l'AP-HM pour lesquels au moins une souche de *K. pneumoniae* résistante à la colistine avait été isolée. Nous avons apparié ces cas à des patients témoins pour lesquels une souche de *K. pneumoniae* sensible à la colistine avait été isolée, selon un ratio de 1 :2, en fonction de l'âge, du sexe, du service et de la période d'hospitalisation. Sur les 22 patients étudiés, 14 souches *de K. pneumoniae* résistantes à la colistine ont été séquencées afin de définir le support moléculaire de la résistance. Les mutations observées ont ensuite été comparées à 6412 génomes de *K. pneumoniae* disponibles dans la base de données de NCBI.

Dans notre étude, la consommation de colistine n'a pas été identifiée comme étant un facteur de risque pour l'acquisition de souches de *K. pneumoniae* résistantes à la colistine, mais plutôt le sexe masculin, la proximité avec un patient porteur d'une

*K. pneumoniae* résistante à la colistine ainsi qu'une récente exposition à une infection bactérienne comme facteurs indépendants significatifs. Tout comme dans l'étude réalisée en Grèce, la résistance à la colistine a été surtout retrouvée dans des souches productrices de carbapénémases, essentiellement des OXA-48 (N=2).

D'un point de vue génomique, nous n'avons pas mis en évidence de mécanisme de résistance majoritaire, deux souches avaient un gène *mgrB* inactivé. En revanche, nous avons identifié un clone épidémique ST307 majoritaire (N=9). Ce clone ST307 a émergé récemment, responsable d'épidémies de souches multi-résistantes en milieu hospitalier (Heiden *et al.*, 2020). Il est souvent associé à la présence de gènes transférables de type *blaCTX-M-15* et moins fréquemment des gènes *blaKPC-3, blaNDM-1* ou encore *blaOXA-48.* La résistance à la colistine a également été signalée chez ce clone.

Les souches appartenant à ce clone portaient la même association de 7 mutations non connues dans les gènes connus pour être impliqués dans la résistance à la colistine (*pmrA/B, phoP/Q, mgrB, crrA/B*). Afin de comprendre si ces mutations étaient associées à la résistance à la colistine ou au clone ST307, nous les avons recherchées dans les 6 412 génomes de *K. pneumoniae* disponibles dans la base de données NCBI. Ainsi, nous avons mis en évidence une spécificité de ces mutations au complexe clonal CC307 dans 192 génomes de bactéries ST307, ces souches pouvant être sensibles, résistantes ou de sensibilité inconnue à la colistine. De plus amples études sont nécessaires pour déterminer si ces mutations représentent une « signature » conférant au clone ST307 une faculté supérieure à acquérir la résistance à la colistine. Une surveillance de la résistance à la colistine en France est nécessaire pour détecter les clones résistants et l'isolement des patients positifs.

# Risk factors for acquisition of colistin-resistant *Klebsiella pneumoniae* and expansion of a colistin-resistant ST307 epidemic clone in hospitals in Marseille, France, 2014 to 2017

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**Background:** France is a low prevalence country for colistin resistance. Molecular and epidemiological events contributing to the emergence of resistance to colistin, one of the 'last-resort' antibiotics to treat multidrug-resistant Gram-negative infections, are important to investigate. **Aim:** This retrospective (2014 to 2017) observational study aimed to identify risk factors associated with acquisition of colistin-resistant *Klebsiella pneumoniae* (CRKP) in hospitals in Marseille, France, and to molecularly characterise clinical isolates. **Methods:** To identify risk factors for CRKP, a matched-case–control (1:2) study was performed in two groups of patients with CRKP or colistin-susceptible *K. pneumoniae* respectively. Whole-genome-sequences (WGS) of CRKP were compared with 6,412 *K. pneumoniae* genomes available at the National Center for Biotechnology Information (NCBI). **Results:** Multivariate analysis identified male sex and contact with a patient carrying a CRKP as significant independent factors ($p < 0.05$) for CRKP acquisition, but not colistin administration. WGS of nine of 14 CRKP clinical isolates belonged to the same sequence type (ST)307. These isolates were from patients who had been hospitalised in the same wards, suggesting an outbreak. Comparison of the corresponding strains' WGS to *K. pneumoniae* genomes in NCBI revealed that in chromosomal genes likely playing a role in colistin resistance, a subset of five specific mutations were significantly associated with ST307 ($p < 0.001$). **Conclusion:** A ST307 CRKP clone was identified in this study, with specific chromosomal mutations in genes potentially implicated in colistin resistance. ST307 might have a propensity to be or become resistant to colistin, however confirming this requires further investigations.

## Introduction

Since the mid-2000s, carbapenem resistance has led to the revival of colistin, a polymyxin, as a last resort antibiotic [1] to treat Gram-negative bacterial infections [2], including carbapenemase-producing *Klebsiella pneumoniae* [2]. In this regard, the recent emergence of colistin resistance in humans and animals, particularly via the mobile colistin resistance gene (*mcr*) is concerning [1]. For *K. pneumoniae,* a study between 2015 and 2017, concerning 18 European countries estimated an overall colistin resistance rate of 5.4% [3]. Other surveillance activities in Europe found resistance to polymyxins in this species varying from 2.6% in carbapenem-susceptible strains to 31.9% in carbapenem-resistant ones [4].

In the literature, previous use of colistin has been reported as a risk factor for colistin resistance. Nevertheless, colistin-resistant bacteria have also been isolated from people who had not previously been treated with this drug [5] and, for *K. pneumoniae,* some investigations point to other risk factors in patients, such as previous carbapenemase-producing *K. pneumoniae* colonisation, corticosteroid administration and prior hospitalisations. The aforementioned risk factors were mainly assessed in Greece [6] and Italy [7], where the rate of colistin resistance reached 30%, which is much higher than in France [8]. In our hospitals in Marseille, France, susceptibility to colistin is only tested in certain circumstances (see methods). As

**Table 1**

Univariate analysis of characteristics potentially presenting a risk factor for colistin-resistant *Klebsiella pneumoniae* acquisition, Marseille, France, 2014–2017 (n = 66 patients)

| Characteristics | | CRKP n = 22 | CSKP n = 44 | p value |
|---|---|---|---|---|
| Age of patients in years; median (range) | | 69 (53–76) | 66 (53–75) | NS |
| Number of patients of male sex | | 18 | 19 | 0.004 |
| Length of hospital stay in days; mean (standard deviation) | | 60.1 (56.6) | 44.7 (40.3) | < 0.001 |
| Delay before first positive sample in days; mean (standard deviation) | | 21.7 (22.1) | 13.2 (17.2) | 0.151 |
| Recent travel; number of patients (%) | | 2 (9) | 3 (7) | NS |
| Type of ward; number of patients (%) | Intensive care unit | 15 (68) | 30 (68) | NS |
| | Surgery services | 3 (14) | 6 (14) | NS |
| | Medical services | 3 (14) | 6 (14) | NS |
| | Neonatal intensive care unit | 1 (5) | 2 (5) | NS |
| Type of sample; number of patients (%) | Respiratory | 6 (27) | 13 (30) | 0.848 |
| | Urine | 6 (27) | 18 (41) | 0.279 |
| | Blood | 1 (5) | 4 (9) | 0.911 |
| | Bone | 1 (5) | 2 (5) | NS |
| | Liquid puncture | 2 (9) | 4 (9) | NS |
| | Cutaneous swab | 2 (9) | 6 (14) | 0.923 |
| | Rectal swab | 7 (32) | 14 (32) | NS |
| Comorbidities; number of patients (%) | Solid organ cancer | 8 (36) | 7 (16) | 0.062 |
| | Haematologic cancer | 1 (5) | 3 (7) | NS |
| | Cerebrovascular disease | 0 (0) | 6 (14) | 0.167 |
| | Liver disease | 1 (5) | 2 (5) | NS |
| | Chronic renal failure | 2 (9) | 7 (16) | 0.706 |
| | Chronic pulmonary disease | 2 (9) | 5 (11) | NS |
| | Diabetes mellitus | 5 (23) | 13 (30) | 0.770 |
| | Cardiovascular disease | 7 (32) | 12 (27) | 0.701 |
| | Solid organ transplantation | 0 (0) | 3 (7) | 0.545 |
| | Charlson score ≥ 3 | 16 (73) | 32 (73) | NS |
| History; number of patients (%) | Previous surgery | 10 (45) | 15 (34) | 0.370 |
| | Mechanical ventilation | 14 (64) | 20 (45) | 0.164 |
| | Central venous catheter | 16 (73) | 32 (73) | NS |
| | Intravenous home therapy | 3 (14) | 4 (9) | 0.678 |
| | Urinary catheter | 16 (73) | 25 (57) | 0.209 |
| | Haemodialysis | 4 (18) | 3 (7) | 0.210 |
| | Recent bacterial infection | 12 (55) | 13 (30) | 0.048 |
| | Previous isolation of an ESBL-producing bacteria | 3 (14) | 7 (16) | NS |
| | Previous isolation of a carbapenemase producing bacteria | 11 (50) | 6 (14) | 0.006 |
| | Contact with patient carrying a CRKP | 14 (64) | 12 (27) | 0.004 |
| Previous treatment; number of patients (%) | Amoxicillin + clavulanic acid | 4 (18) | 9 (20) | NS |
| | Piperacillin + clavulanic acid | 9 (41) | 17 (39) | 0.859 |
| | Third generation cephalosporin | 13 (59) | 15 (34) | 0.053 |
| | Carbapenems (imipenem, ertapenem or meropenem) | 9 (41) | 7 (16) | 0.025 |
| | Fluoroquinolones | 11 (50) | 21 (48) | 0.025 |
| | Aminoglycosides | 15 (68) | 15 (34) | 0.009 |
| | Colistin | 3 (14) | 2 (5) | 0.323 |

CRKP: colistin-resistant *Klebsiella pneumoniae*; CSKP: colistin-susceptible *Klebsiella pneumoniae*; ESBL: extended-spectrum beta-lactamase; NS: non-significant p value.

**Table 2**

Independent risk factors for acquisition of colistin-resistant *Klebsiella pneumoniae* found through multivariate analysis, Marseille, France, 2014–2017 (n = 66 patients)

| Risk factors | OR (95% CI) | p value |
|---|---|---|
| Male sex | 2.5 (1.5–12.5) | 0.023 |
| Contact with patient carrying a CRKP | 3.7 (1.8–17.0) | 0.014 |

CI: confidence interval; CRKP: colistin-resistant *Klebsiella pneumoniae*; OR: odds ratio.

a result, the prevalence of colistin resistance cannot be estimated. Moreover, the mechanisms of colistin resistance as well as the risk factors associated with the acquisition of colistin-resistant organisms have thus far not been assessed in our setting.

The *K. pneumoniae* sequence type (ST)307 was first reported in a clinical case in 2009 in Pakistan but subsequent findings indicated that this ST might have already emerged in the mid-1990s [9,10]. ST307 has been involved in local hospital outbreaks in Africa, the Americas, Asia and Europe [9]. It is frequently associated with multidrug resistance, especially with CTX-M-15 extended-spectrum beta-lactamase (ESBL) and carbapenemase (*K. pneumoniae* carbapenemase; KPC) and it has even replaced the multidrug-resistant international ST258 in some countries such as Canada and Italy [11]. In 2018, a whole genome sequencing (WGS) and epidemiologic analysis of KPC-*Klebsiella pneumoniae* isolates in France found the emergence of several high-risk clones in the hospital environment including ST307 [12]. Among studies focusing on ST307 multidrug resistance however, few have investigated colistin resistance [10,13,14]. On the other hand, of *K. pneumoniae* STs reported to be colistin-resistant, those belonging to clonal complexes (CC)258 (ST258, ST512 and ST11) and CC15 have most frequently been identified [10].

The aim of our work was to find colistin-resistant *K. pneumoniae* (CRKP) isolated from patients hospitalised in Marseille university hospitals between February 2014 and May 2017, to molecularly characterise the strains, and to determine risk factors associated with CRKP acquisition. Because strains of ST307 dominated among the CRKP identified, these were also the object of further focus and epidemiological investigations.

## Methods

### Collection of colistin-resistant *Klebsiella pneumoniae* strains and microbiological procedures

This retrospective study was conducted on patients who had been hospitalised in Marseille university hospitals, France, from February 2014 to August 2017. These four hospitals (Timone, Conception, North, and Sainte-Marguerite hospitals) have a total of 3,700 beds.

Our laboratory carries out the microbiological analysis of specimens collected in the four hospitals. Colistin susceptibility testing is performed on *K. pneumoniae* isolates that are susceptible to less than three families of antibiotics included in our routine antibiotic testing panel, or upon specific request. We selected all *K. pneumoniae* strains (one per patient) for which a colistin minimum inhibitory concentration (MIC) was > 2 µg/mL (performed by E-test or microdilution method) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations [15]. Antimicrobial susceptibility testing for other antibiotics was done according both the EUCAST and Comité de l'Antibiogramme – Société Française de Microbiologie (CA-SFM) recommendations using the disk diffusion method on Mueller–Hinton (MH) agar (BioMérieux, Marcy l'Etoile, France) enriched with commercial antibiotic disks, and/or E-test (Biomérieux) as previously described [16].

### Study of risk factors for colistin-resistant *Klebsiella pneumoniae* acquisition

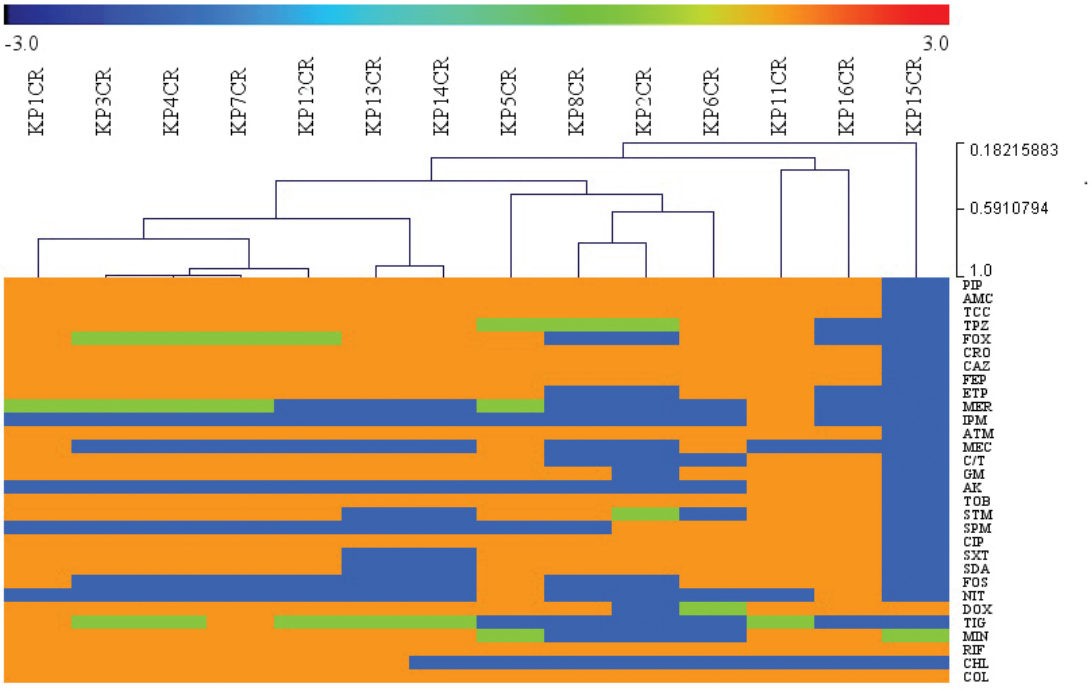
Cases were defined as patients for whom a CRKP had been isolated at least once in one sample, thus including patients colonised and/or infected with CRKP. Patients' clinical histories were obtained from their electronic medical records. Each case was included in the study only once, i.e. at the time of the first CRKP isolation. In order to assess the risk factors for CRKP carriage, cases were compared to control patients colonised or infected with colistin-susceptible *K. pneumoniae* (CSKP) strains isolated during the same study period. In this case–control analysis, controls were matched to cases (2:1) by age, type of ward (medical, surgical or intensive care) and type of sample. Comorbidities/conditions analysed were as follows: organ transplant recipient, chronic renal disease, haematologic or solid organ cancer (excluded if remission > 5 years), chronic pulmonary disease, diabetes mellitus, splenectomy, cerebrovascular disease, liver cirrhosis and heart disease. Modified Charlson scores ≥ 3 [17] were calculated and we considered several aspects of the patient's recent medical history such as: previous surgery (< 1 year prior to the current study), previous antibiotic therapy (< 3 months prior), recent history of bacterial infection (< 1 month prior), previous isolation of an ESBL or a carbapenemase-producing bacteria, use of mechanical ventilation, haemodialysis, urinary catheter and central venous catheter. The final diagnosis (e.g. infection or colonisation) defined by the practitioner in his final report was also retrieved.

### Statistical analysis

The selected variables were compared by the chi-squared test, Fisher's exact test and the Student's t-test as appropriate. Univariate analysis was two-sided and a significant p value was chosen at $p < 0.05$. Multivariate analysis was performed by conditional

**Figure 1**

Hierarchical tree of antimicrobial susceptibility testing results of sequenced colistin-resistant *Klebsiella pneumoniae* isolates, Marseille, France, 2014–2017 (n = 14 isolates)




AK: amikacin; AMC: amoxicillin + clavulanic acid; ATM: aztreonam; CAZ: ceftazidime; CHL: chloramphenicol; CIP: ciprofloxacin; COL: colistin; CRO: ceftriaxone; C/T: ceftolozane + tazobactam; DOX: doxycyline; ETP: ertapenem; FEP: cefepime; FOS: fosfomycin; FOX: cefoxitin; GM: gentamicin; IPM: imipenem; MEC: mecillinam; MER: meropenem; MIN: minocycline; NIT: nitrofurantoin; PIP: piperacillin; RIF: rifampicin; SDA: sulfadiazine; SPM: spectinomycin; STM: streptomycin; SXT: trimethoprim + sulfamethoxazole; TCC: ticarcillin + clavulanic acid; TIG: tigecycline; TOB: tobramycin; TPZ: piperacillin + tazobactam.

The colistin resistant (CR) *Klebsiella pneumoniae* (KP) isolates/strains are listed above the diagram and in each column under them, the susceptibility profile to specific antibiotics, which are listed to the right, is depicted. Orange indicates that the strain is classified as resistant to an antibiotic, green that the strain is categorised as intermediate and blue that the strain is classified as susceptible.

logistic regression, and only the risk factors with a p value < 0.05 in univariate analysis were used for the multivariate analysis. Statistical analysis was performed using the SPSS software (SPSS Inc, Chicago, Illinois, United States (US)).

## Genomic study

### Whole genome sequencing, bioinformatical analysis and clonal relationship

Genomic DNAs of *K. pneumoniae* strains were sequenced with the MiSeq Technology (Illumina, San Diego, US) with a 2 × 250 paired-end run strategy using the Nextera Mate Pair sample prep kit (Illumina). Genome assembly was done using the A5-miseq software (http://sourceforge.net/projects/ngopt/) and annotated with Prokka (https://github.com/tseemann/prokka). Plasmid replicons, virulence and resistance genes were sought with Abricate. Genome files were deposited in the National Center for Biotechnology Information (NCBI) database under the Bioproject number PRJNA520974 (Supplementary Table S1).

### Clonal relationship study

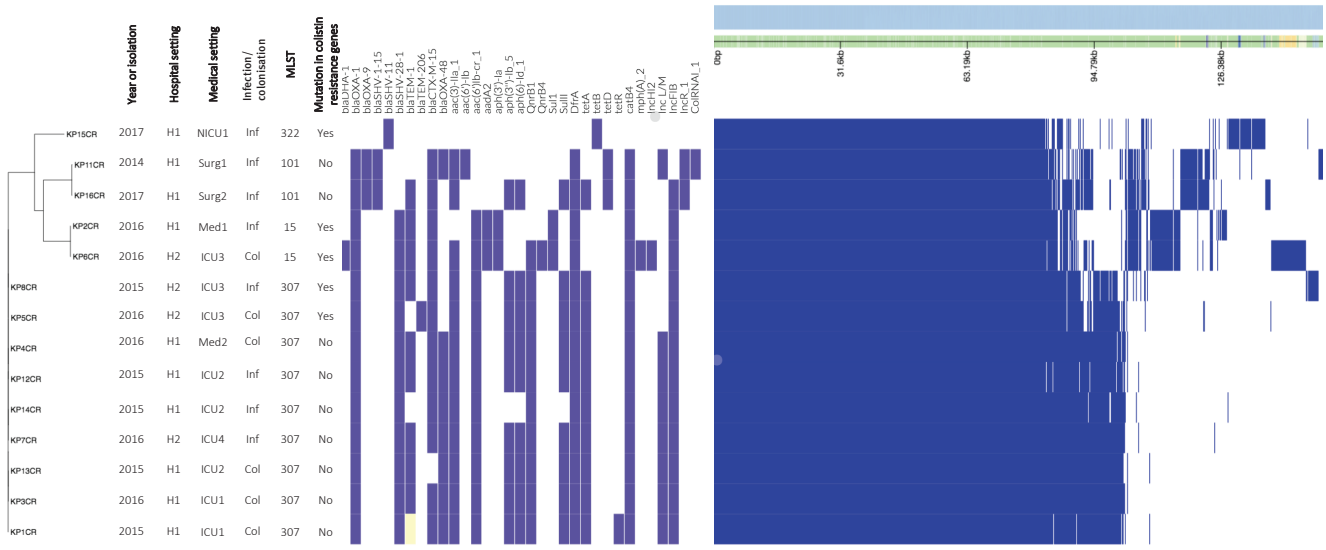
Multilocus sequence typing (MLST) analysis was performed in silico to determine the ST of collected strains using the Bacterial Isolate Genome Sequence Database (BIGSdb) database (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). The *wzi* locus type was also identified with information from the BIGsdb database. The pangenome of *K. pneumoniae* isolates was determined using Roary [18]. We used in a first step the core gene alignment output file to construct the maximum likelihood phylogenetic tree using the RaXML software. In a second step, we performed a genome comparison based on single nucleotide polymorphism (SNP)s variants using the CSI Phylogeny (version 1.4) available on the Center for Genomic Epidemiology website (https://cge.cbs.dtu.dk/services/CSIPhylogeny). An average of 10 SNP (range: 0–30) was chosen as cut-off to define isolates of a same clone as previously described [19].

### In silico mutations analysis

Sequences of proteins involved in colistin resistance were compared with the colistin-susceptible (MIC = 2 mg/L) [20] reference strain *K. pneumoniae* MGH78578 (GenBank accession

**Figure 2**

Phylogenetic tree based on core genome sequence alignment of colistin-resistant *Klebsiella pneumoniae*, Marseille, France, 2014–2017 (n = 14 sequences)




CR: colistin-resistant; ICU: intensive care unit; H: hospital; Inf: infection; KP: *Klebsiella pneumoniae*; Med: medical ward; MLST: multilocus sequence typing; Surg: surgery ward.

The following is indicated on the left side: year of isolation, the hospital, type of ward, stage of CRKP pathogenesis (colonisation/infection), MLST type and if a mutation was present in previously-studied colistin resistance genes. Other acquired resistant genes are indicated in purple if they are present in the genome. If a resistance gene is truncated, it is represented in yellow. On the right section of the Figure, the pangenomes of the K. pneumoniae strains are respectively represented, with on top a scale referring to the length of the pangenome. Each blue band represents a gene in a pangenome. Genes that are present in all pangenomes are toward the left of the section (core genome), whereas those toward the right, are not universally present in the pangenomes (accessory genome).

number: CP000647). The deleterious effect of detected mutations on the function of proteins was investigated using Protein Variation Effect Analyzer (PROVEAN; http://provean.jcvi.org/index.php). Moreover, we investigated the frequency of the mutations found in *PmrA/B*, *CrrA/B* [20] and *AcrR/S* [21-23] in all ST307 among the 6,412 genomes of *K. pneumoniae* available on NCBI (last upgrade April 2019) by Basic Local Alignment Search Tool (BLAST) protein (blastp) using as threshold expect (E)-value $10e^{-5}$. Next we performed MLST using PubMLST typing schemes by local BLAST on all *K. pneumoniae* genomes available in NCBI to compare the frequency of different STs in this database.

## Ethical statement

This study was qualified as an internal study not involving the human person, as it was conducted on the basis of data collected as part of the individual therapeutic or medical follow-up of patients, by the personnel providing this follow-up and for their exclusive use. It was approved by the sites' institutional review boards with a waiver of informed consent and registered under number RGPD/Ap-Hm 2020–02.

## Results

From February 2014 to August 2017, 22 patients carried a CRKP isolate among the 5,304 patients who had at least one *K. pneumoniae* isolated in our laboratory. During this period, 374 determinations of colistin MIC were performed. The median age of the 22 patients was 69 years (range: 53–76) and 18 were males (sex ratio = 4.5). Four strains were isolated in 2014, eight in 2015, seven in 2016 and three in 2017. Strains were recovered from five respiratory, six urine, and five stool samples as well as one blood culture, one bone biopsy, two liquid punctures and two cutaneous swabs. Colistin MIC varied from 4 to > 64 µg/mL (Supplementary Tables S2 and S3).

## Risk factors for colistin resistance acquisition

The 22 CRKP cases were matched by age, type of ward and sample type with 44 controls carrying a CSKP isolated during the same study period. Univariate analysis of the two groups identified as significant risk factors male sex, length of hospital stay, recent bacterial infection, previous isolation of an ESBL or a carbapenemase producing bacteria and contact with cases carrying a CRKP (Table 1). Finally, previous antibiotic therapy,

especially by fluoroquinolones, aminoglycosides or carbapenems was associated with an increased risk to acquire CRKP.

In the multivariate analysis, male sex ($p<0.05$) and contact with a case carrying a CRKP ($p<0.01$) remained the only independent factors for CRKP acquisition (Table 2).

In our study, only five patients had previously received colistin. Three cases with CRKP received at least intravenous colistin, while two patients with CSKP received only aerosolised colistin (Supplementary Table S4).
Twelve cases among the 22 in the CRKP group were infected vs 21 of 44 patients in the control group ($p=0.862$). Mortality rate reached 18% (4/22) in the CRKP group vs 25% (11/44) in the CSKP group ($p=0.770$). In the CRKP group, one death was attributable to *K. pneumoniae* infection.

## Clinical and biological features of cases with acquired colistin-resistant *Klebsiella pneumoniae*

We compared cases with a CRKP infection to those who were just colonised by CRKP. The length of stay in hospital (37 vs 88 days; $p=0.025$) as well as the delay before the first positive CRKP sample (14 vs 31 days; $p=0.0385$) were significantly shorter in the infected group than in the colonised group. However, while the delay in the colonised group was based on 10 cases, only five of them had tested negative for CRKP at admission. The remainder had not been tested at that time, but were nevertheless used to estimate delay, under the assumption that testing was not performed, due to absence of risk factors requiring this. No other significant difference was observed between the two groups. No specific ST was associated with infection or colonisation.

## Whole genome sequencing study and resistome

As this work was a retrospective study and all laboratory strains are not permanently stored, only 14 of the 22 CRKP isolates from the microbiology laboratory were available for WGS analysis. Genomes sizes ranged from 5,518,326 bp to 5,912,443 bp with a percentage of GC bases ranging from 56.3 to 57.3%. Genomes were assembled in 74 to 603 contigs with a 19 to 55 coverage. The main features of the different genomes are reported in Supplementary Tables S2 and S3.

Of 14 colistin-resistant *K. pneumoniae* strains, nine belonged to ST307, followed by two to ST15, two to ST101 and one to ST322. Antibiotic susceptibility testing was performed on the 14 sequenced strains (Supplementary Tables S2 and S3).

Resistance rate to each antibiotic is presented in Figure 1. The isolates were mostly susceptible to imipenem (13 isolates), nitrofurantoin (12 isolates), amikacin (12 isolates), minocycline (11 isolates), mecillinam (11 isolates), fosfomycin (nine isolates), chloramphenicol (eight isolates) and tigecycline (six isolates) (Figure 1). However, the combination of ceftolozane and tazobactam was active on only four isolates.

The nine ST307 CRKP carried $bla_{OXA-1}$, $bla_{SHV-28}$, *aac(3)-IIa, aac(6')Ib-cr, qnrB1, tetA*, and *catB4* genes (Supplementary Tables S2 and S3). All but two (KP5CR and KP8CR) of these strains carried a $bla_{OXA-48}$ . The two ST15 CRKP (KP2CR and KP6CR on Figure 1) were positive for $bla_{OXA-1}$, $bla_{SHV-28}$, $bla_{TEM-1}$, $bla_{CTX-M-15}$, *aac(6')Ib-cr, aadA2, aph(3')-Ia, sulI, dfrA* and *catB4*. One ST15 strain (KP6CR) was also carrying $bla_{DHA-1}$, *aac(3)-IIa, qnrB1, qnrB4, mph(A)* genes. The two CRKP ST101 (KP11CR and KP16CR) had a $bla_{OXA-1}$, $bla_{OXA-9}$, $bla_{SHV-15}$, $bla_{CTX-M-15}$, *aac(3)-IIa, dfrA, tetD* and *catB4* genes. The strain KP11CR also carried *aac(6')Ib*, $bla_{OXA-48}$ genes. The strain KP16CR was also positive for $bla_{TEM-1}$, *sulII, tetA*. Finally, the ST322 strain (KP15CR), which was susceptible to all antibiotic but cyclins, was carrying a *tetB* and a $bla_{SHV-11}$ gene.

## Spread of an epidemic ST307 colistin-resistant *Klebsiella pneumoniae* in intensive care units

The pangenome of the 14 isolates recovered 7,897 genes, including 4,136 genes belonging to the core genome and 3,761 to the accessory genome (Figure 2). A phylogenetic tree based on the core genome showed that the nine ST307 strains were closely related (2–114 SNPs) and suggested that they could possibly belong to the same clone.

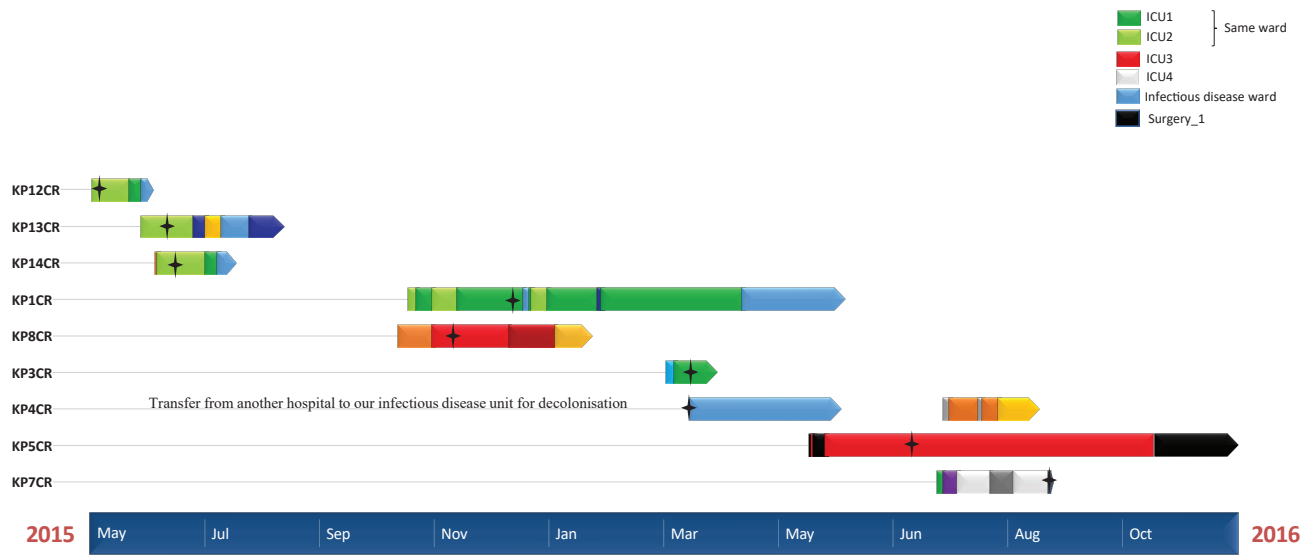
Interestingly, five of the nine ST307 cases (KP12CR, KP13CR, KP14CR, KP1CR and KP3CR) met at different times between 2015 and 2016 in the same ward, i.e. intensive care unit (ICU)1 and ICU2 (Figure 3, light green and green) and became positive for CRKP in this ward. One additional case was present in the same ward (KP7CR) but never met the other CRKP positive cases. Another case (KP4CR), who had been previously hospitalised in a nearby town ICU and identified as a KPC carrier there, tested positive for KPCR on the first day of hospitalisation in Marseille. Interestingly, this person's respective isolate and the isolates of the six other cases only presented between 2 and 13 SNPs (average 7 SNPs), confirming that all seven were from the same clone. However, two other cases (KP8CR and KP5CR) who were hospitalised in a common ward (ICU3, red), never met and genomes recovered from their isolates had between 91 and 114 different SNPs (average 102 SNPs), suggesting that they were affected by different strains. Moreover, these two strains were negative for the $bla_{OXA48}$ gene that was present in the other ST307 strains.

## Possible genetic basis of colistin resistance in *Klebsiella pneumoniae* strains

All strains were negative for the *mcr-1* to *mcr-8* gene variants. Mutations for the most common genes involved in colistin resistance were analysed (Table 3). All 14 strains analysed showed mutations in at least

**Figure 3**

Time of hospitalisations in different wards of cases of *Klebsiella pneumoniae* ST307 resistant to colistin and time of the respective bacterial isolation, Marseille, France, 2015–2016 (n = 9 cases)




ST: sequence type; KP: *Klebsiella pneumoniae*; CR: colistin resistant.

Each colour represents a different ward (only the wards of interest are described in the legend). The units in light green and green correspond to two units of the same ward; patients are often transferred between these two units according to the severity of their clinical conditions. The star indicates the first detection of a CRKP.

one colistin-resistance gene compared with the reference strain MGH78578. In nine of these, no mutation predicted or known to be responsible for colistin resistance was detected in any of the genes subjected to PROVEAN analysis.

Five isolates (35.7%) had mutations in the *pmrA*, *phoP*, *phoQ* or *mgrB* genes and these mutations were predicted to modify protein function according to PROVEAN analysis (Table 3). We also noticed five mutations (PmrA A41T, PmrB L213M and T246A, CrrB C68S, AcrS S76R) that were present in all ST307 isolates. These were predicted by PROVEAN to not alter protein function independently. To explore if they might have, together, a synergistic role in colistin resistance and/or if they might be specific for ST307, we investigated the frequency of these mutations in 6,412 genomes of *K. pneumoniae* available on NCBI. Interestingly, simultaneous presence of the five mutations was found in 195 of 6,412 genomes analysed, including all 192 ST307 present in GenBank, one ST2739 (that belongs to CC307), one ST2975 and one genome with an unknown ST (Supplementary Figure S1). The frequency of the five mutations was strongly associated with the ST307 (192/195 vs 3/6,217; $p < 0.001$). Unfortunately, colistin susceptibility data were mostly lacking from the metadata of these genomes, preventing any insight into any potential association with colistin resistance.

Taken one by one, the frequency of each mutation in the 6,412 genomes was as follows: 3.2% (n = 204) of A41T in PmrA, 69.8% (n = 4,474) of C68S in CrrB and 3.3% (n = 209) of S76R in AcrS (Supplementary Table S5). A total of 288 genomes (4.5%) had mutations leading to presence of both L213M and T246A in the PmrB protein.

In terms of the occurrence of each ST or CC among the 6,412 genomes in GenBank, CC258 was most prevalent (n = 1,411; 22%), followed by ST11 (n = 715; 11%), ST15 (n = 336; 5%), ST101 (n = 206; 3%) and ST307 (n = 192; 3%) (Supplementary Figure S1).

## Discussion

In this study, we retrospectively found 22 CRKP cases in four Marseille university hospitals. Univariate analysis identified several characteristics associated with carriage or infection with CRKP including male sex, length of hospital stay, recent bacterial infection, contact with a patient positive for CRKP and previous antibiotic therapy with fluoroquinolones, aminoglycosides and carbapenems. Furthermore, previous isolation of a carbapenemase-producing bacteria in patients was also determined as a risk factor, suggesting that controlling carbapenem resistance may possibly help mitigate emergence of CRKP in this setting.

In multivariate analysis, however, only male sex and contact with another patient carrying a CRKP remained as independent risk factors for CRKP acquisition. Therefore management options for patients carrying or infected by a CRKP strain could include isolation

**Table 3**

Summary of mutations found in the 14 strains of Klebsiella pneumoniae analysed, Marseille, France, 2014–2017 (n = 14 isolates)

| Strain | ST | *wzi* | Colistin MIC (µg/mL) | PmrA | PmrB | PhoP | PhoQ | MgrB | CrrB | CrrA | AcrR | AcrS | *mcr* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| KP1CR | 307 | NF[a] | 16 | A41T [13] | L213M [13]; T246A [26,37] | No[b] | No[b] | No[b] | C68S | No[b] | No[b] | S76R | No[b] |
| KP2CR | 15 | NF[a] | 16 | G53V[c] [26] | No[b] | No[b] | No[b] | No[b] | NF[a] | NF[a] | No[b] | Nob | No[b] |
| KP3CR | 307 | NF[a] | 16 | A41T | L213M; T246A | No[b] | No[b] | No[b] | C68S | No[b] | No[b] | S76R | No[b] |
| KP4CR | 307 | 173 | 8 | A41T | L213M; T246A | No[b] | No[b] | No[b] | C68S | No[b] | No[b] | S76R | No[b] |
| KP5CR | 307 | 173 | 16 | A41T | L213M; T246A | L12Q | No[b] | No[b] | C68S | No[b] | No[b] | S76R | No[b] |
| KP6CR | 15 | NF[a] | 16 | No[b] | No[b] | No[b] | L87P | No[b] | NF[a] | NF[a] | No[b] | No[b] | No[b] |
| KP7CR | 307 | 173 | 16 | A41T | L213M; T246A | No[b] | No[b] | No[b] | C68S | No[b] | No[b] | S76R | No[b] |
| KP8CR | 307 | 173 | 32 | A41T | L213M; T246A | No[b] | No[b] | Stop (13AA)[d] | C68S | No[b] | No[b] | S76R | No[b] |
| KP11CR | 101 | 137 | 16 | A217V | T246A | No[b] | No[b] | No[b] | NF[a] | NF | No[b] | H79Q | No[b] |
| KP12CR | 307 | 173 | 8 | A41T | L213M; T246A | No[b] | No[b] | No[b] | C68S | No[b] | No[b] | S76R | No[b] |
| KP13CR | 307 | 173 | 16 | A41T | L213M; T246A | No[b] | No[b] | No[b] | C68S | No[b] | No[b] | S76R | No[b] |
| KP14CR | 307 | 173 | 4 | A41T | L213M; T246A | No[b] | No[b] | No[b] | C68S | No[b] | No[b] | S76R | No[b] |
| KP15CR | 322 | 50 | >64 | S64T [26] | No[b] | No[b] | No[b] | IS10 in bp 76 [38] | C68S | No[b] | No[b] | P211S | No[b] |
| KP16CR | 101 | 137 | 4 | A217V [13] | T246A | No[b] | N135K | No[b] | NF[a] | NF[a] | No[b] | H79Q | No[b] |

AA: amino acid; CR: colistin resistant; IS: Insertion Sequence; KP: Klebsiella pneumoniae; MIC: minimum inhibitory concentration; NF: not founda; PROVEAN: Protein Variation Effect Analyzer; ST: sequence type.

[a] The gene was not found.

[b] No mutation was found in the gene compared with the reference strain MGH78578.

[c] A mutation at this position was previously reported to alter protein function, but the substitution was G53C.

[d] Stop codon at the codon for the 13th AA, leading to a truncated protein.

Mutations in bold were predicted to modify protein function by PROVEAN analysis. Studies that have previously reported some of the mutations listed in the Table are respectively cited next to the mutation.

by trained staff, decolonisation and/or possibly faecal microbiota transplantation. Interestingly, in terms of patient care, a large proportion of the strains from the CRKP cases analysed here, were susceptible to common antibiotics that have been used in the past (e.g. mecillinam, nitrofurantoin, fosfomycin) [2]. Such antibiotics could represent valuable treatment alternatives in cases of CRKP infection.

The results of our case–control analysis confirmed those of other studies (Supplementary Table S6), though we did not identify colistin administration as a risk factor for CRKP acquisition. It should be nevertheless noted, that in our study, only five patients received colistin (two in the CSKP group vs three in the CRKP group), resulting in relatively weak statistical power. Comparison between groups of cases and controls showed no difference in mortality. This finding differs from a few other previous investigations, which uncovered an excess mortality rate in cases of CRKP infections [7,24]; however, these studies focused on colistin-resistant carbapenemase-producing *K. pneumoniae* strains. Moreover, in our study, patients in both groups had various comorbidities that could have been confounding.

The *K. pneumoniae* isolates retrospectively considered for this study were selected based on colistin resistance only, without prior knowledge of their type. Of the 14 CRKP cases' isolates, where sequencing was possible, nine were ST307, with seven of these belonging to the same clone. The two CRKP cases respectively bearing separate ST307 clones, never met. Among the seven cases affected by the same ST307 clone, five were hospitalised in the same ICUs at different – but sometimes overlapping – times between 2015 and 2016. As has already been demonstrated in colistin-resistance events [25], this might suggest cross-transmission. In this event, cross-transmission could have occurred in the ICU(s) via healthcare workers and/or environmental contamination. For the two remaining single-clone-affected CRKP cases, one had stayed in the same ICU as the other five, but at a completely different time, never meeting them. The other was never hospitalised in ICU, but was admitted to an infectious disease ward after transfer from another hospital near Marseille. This might indicate that the ST307 strain could present as an epidemic colistin-resistant clone, which has spread in our area, especially in the hospital environment [9,10]. In this regard, a national survey in France in 2014 detected three isolates with ST307 CRKP originating from the region Provence-Alpes-Côte d'Azur, to which Marseille belongs [8]. A French study in 2018 showed ST307 emerging in other areas of France, reinforcing its relevance [12].

Resistance to colistin in *K. pneumoniae* has previously been linked to several mechanisms including capsule overexpression, modifications of lipid A by addition of aminoarabinose or phosphoethanolamine due to mutations of target chromosomal genes or acquisition of plasmid-borne colistin resistance *mcr* genes [1]. All strains in our study were negative for *mcr* genes. However, among the 14 isolates analysed by WGS, substitutions, deletions and insertions in genes known to play a role colistin resistance, and likely to result in functional change at the protein level according to PROVEAN, were identified in five strains (two ST15, two ST307 and one ST322). In two of these (ST322 and ST15), the *mgrB* gene was inactivated by an insertion sequence (IS10) and by a mutation leading to a stop codon respectively, while in another (ST307), a substitution occurred at position 53 of PmrA (Table 3). Remarkably, mutations in MgrB have previously been reported to lead to a high MIC of colistin [1,26] as well as a mutation at position 53 in PmrA [26]. The two other mutations (PhoP L12Q and PhoQ P87L), which were respectively found in one ST307 and one ST15 strain, have never been described before and in vitro tests should be performed to confirm their role in colistin resistance.

Unfortunately, for the moment, we were not able to clearly decipher the molecular mechanism of colistin resistance for the remaining nine strains, two of which were ST101 and seven ST307. Of the latter, it can nevertheless be noted that five (i.e. KP4CR, KP7CR, KP12CR, KP13CR and KP14CR) had identical mutations compared to the reference strain MGH78578, but exhibited various colistin MICs. A different level of gene expression might explain these MIC differences, and this could be explored by transcriptomic analysis in further work. The mutations present in these five ST307 isolates (A41T in PmrA, L213M and T246A in PmrB, S76R in AcrS and C68S in CrrB) were also present in all nine ST307 sequences recovered from our CRKP cases. Subsequent finding that all 192 ST307 bacterial genomes in NCBI, as at April 2019, also shared these mutations was surprising. Unfortunately, colistin susceptibility data are mostly lacking from the metadata of these genomes, preventing insight into the relation of these mutations with colistin resistance. Further work is needed to study any possible association (Supplementary Table S7).

Interestingly, seven of the nine ST307 from our CRKP cases had the *wzi*-173 allele which seems to be associated with this ST [11] and which confers an additional capsular locus that may distinguish this particular phenotype [1].

During the 29th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) in Amsterdam, the Netherlands (13–16 April 2019), ST307 was reported as an emerging clone in several countries including Italy [27,28], Russia [29], Qatar [30], France [31], South Africa [32], Spain [33] and the Netherlands [34]. From these reports, colistin resistance was found in 15 of 30 ST307 isolates detected in the Italian study and 24 of 35 isolates in the Russian one. Moreover, among a collection of 27 colistin-resistant *K. pneumoniae* from Kentucky, US [35], three were ST307. This raises the question as to whether our ST307 isolates from Marseille, France, are intrinsically resistant to colistin or if there is a propensity of ST307 clone to be or to become resistant to colistin everywhere in the world.

We previously reported a case of an osteitis caused by a ST307 CSKP [36] treated with colistin, highlighting that this lineage was already present in our hospital centre in 2015. This isolate (Genbank accession number: NJGM01) had the same mutations in PmrA (A41T), PmrB (L213M and T216A) and AcrR (S76R) but the CrrAB proteins were absent. A SNP analysis between this colistin susceptible isolate and the colistin-resistant ST307 isolates from the current study (used as references) found between 995 and 1,546 SNPs (mean = 1,333) on an average of 5,540,016 bp analysed. The ST307 CRKP could have evolved from a CSKP clone in our setting. An exhaustive analysis of a larger sample collected from different sources would confirm this hypothesis.
This study has some limitations. The first is related to the retrospective nature of the study, which limited access to some clinical and laboratory data, such as the results from screening the patient for multidrug-resistant bacteria upon admission to hospital. The restrospective nature also impacted the availability of strains for sequencing from patients included in the study. Moreover as our initial aim was to molecularly characterise CRKP, no strains from patients in the CSKP control group for the case–control analysis were sequenced. The ST characterisation in this group might have provided more information on any relation between ST307 and the 'colistin-resistant' phenotype. The previous description of a carbapenemase-producing but colistin-susceptible *K. pneumoniae* ST307 strain suggests, however, that different clones of ST307 have diffused in our hospital, at least one susceptible and some resistant to colistin. Finally, as seven patients carried the same clone, this could have influenced the results of risk-factor analysis if there had been a clone–host relationship, such as a propensity of the clone to infect the same type of tissue or patient, however we did not observe this in our study.

In conclusion, this study concurs with previous ones, on risk factors for acquisition of CRKP and documents a CRKP ST307 epidemic clone in hospitals in Marseille, France, which is a country with an overall low prevalence of colistin resistance. Further work is warranted to understand why some clones have an ability to become increasingly resistant to antibiotics, by plasmid or chromosomal mechanism, as appears to be the case for CC258 [10] and ST307.

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## Conflict of interest

None declared.

## Authors' contributions

SB, GD, NC and JMR designed the study and drafted and revised the manuscript.

SB, NS and NC performed medical examinations and/or examined medical records.

SB and LH performed microbiology analyses.

SB and MH performed genomic analyses.

All authors have read and approved the final manuscript.

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**Conclusion**

Nos travaux font état de différentes situations concernant l'émergence et la dissémination de la résistance à la colistine (**Article 2, 3**). Ainsi, dans un pays où la consommation de colistine est importante comme en Grèce, la dissémination de la résistance à la colistine est favorisée par cette pression de sélection. Les mécanismes médiant cette résistance chez les isolats de *K. pneumoniae* d'origine humaine ne sont cependant pas d'origine plasmidiques mais chromosomiques. Ils sont majoritairement dus à l'inactivation du gène de régulation *mgrB* par des séquences d'insertion. Ces séquences d'insertion étant présentes généralement dans les génomes de *K. pneumoniae,* elles sont facilement mobilisables pour acquérir la résistance à la colistine. Nous avons cependant observé que les mécanismes de résistance à la colistine pouvaient être plus complexes qu'il n'y paraissait. En effet, 31 % des souches contenaient un gène *mgrB* de type sauvage et nous avons notamment identifié plusieurs souches de *K. pneumoniae* qui présentaient un gène *mgrB* tronqué par une IS tout en restant sensibles à la colistine. Ce phénomène sera analysé dans la 3ème partie de cette thèse.

Dans les pays où la consommation de colistine reste faible comme en France, la résistance à la colistine ne semble pas liée à l'utilisation de colistine. Là encore, les mécanismes responsables de l'émergence de la résistance à la colistine ne sont pas d'origine plasmidique chez les souches de *K. pneumoniae* isolées en clinique, mais plutôt d'origine chromosomique. De plus, l'inactivation du gène *mgrB,* bien que restant un mécanisme rencontré, n'apparait pas comme majoritaire. La plupart de ces souches étaient également résistantes aux carbapénèmes. En effet, des études montrent le lien entre la résistance à la colistine et l'utilisation des carbapénèmes (Galani *et al.*, 2018).

D’autres mécanismes semblent intervenir dans l’émergence de la résistance. Ainsi, nous avons notamment mis en évidence la présence du clone ST307, déjà connu pour être associé à la multirésistance, comme étant un clone pouvant être associé à la résistance à la colistine. Ainsi, il semble que la sélection de ce clone peut favoriser l’émergence de la résistance à la colistine, indépendamment de l’utilisation de colistine. Des études supplémentaires sont nécessaires pour comprendre les facteurs spécifiques de sélection de ces clones qui sont associés à l’acquisition de cette multirésistance.

Durant cette étude rétrospective, nous avons également isolée une souche d’*E. coli* qui présentait la particularité de n’être résistante qu’à la colistine. L’analyse de ce clone a également fait l’objet d’une étude dans la 3ème partie de cette thèse.

# Chapitre III

# Utilisation d'outils génétiques innovants pour la caractérisation de la résistance à la colistine, applications et perspectives.

**Article 4 : Signature tagged mutagenesis to decipher colistin resistance in an *Escherichia coli* clinical isolates, Marseille, France.**

Mouna Hamel, Sophie Alexandra Baron, Jean-Marc Rolain.
Draft manuscript.

**Article 5: Whole genome sequence analysis of truncated *mgrB* phoP/phoQ regulator genes in colistin sensitive *Klebsiella pneumoniae* isolates.**

Mouna Hamel, Mariem Ben khedher, Andriamiharimamy Rajaonison, Sophie Alexandra Baron, Jean-Marc Rolain.
Draft manuscript.

**Avant-propos**

Comme introduit précédemment, l'analyse des mécanismes de résistance à la colistine a été principalement étudié chez les entérobactéries ces dernières années. La plupart des études ont porté sur la détection des gènes *mcr* et sur les mécanismes de résistance liés aux systèmes à deux composants PmrAB et PhoPQ. (Baron *et al.*, 2016). Or, ces mécanismes ne permettent pas d'expliquer la résistance à la colistine chez certaines souches d'entérobactéries (Olaitan *et al.*, 2014), suggérant d'autres voies impliquées dans cette résistance. Les technologies innovantes précédemment développées dans le chapitre I de cette thèse sont de nouveaux outils utiles pour caractériser de nouveaux mécanismes de résistance. Nous avons en effet montré dans notre revue que la découverte de nouveaux mécanismes de résistance à la colistine a évolué simultanément avec la mise en place de nouvelles technologies, notamment pour la caractérisation des gènes *mcr* par la démocratisation du séquençage génomique et des analyses bio-informatiques.

Parmi ces technologies d'intérêt, la mutagenèse aléatoire est une méthode qui n'est pas nouvelle mais qui a été peu utilisée dans la recherche des mécanismes de résistance à la colistine. Cette méthode, longue et fastidieuse, peut être réalisée aujourd'hui à plus haut débit grâce à la diminution des coûts du séquençage. Elle représente un excellent outil pour l'étude des mécanismes de résistance aux antibiotiques et notamment à la colistine, son efficacité ayant déjà été démontrée dans des études antérieures. Elle permet également de décrire des mécanismes de résistance impliquant des gènes de régulation multiples. Au sein de notre laboratoire, elle a été récemment employée et a permis de mettre en évidence le rôle de certaines pompes d'efflux (*AcrAB-tolC)* dans les mécanismes de résistance à la colistine chez *Enterobacter sp*. grâce au criblage de clones mutants générés par mutagenèse

aléatoire (Telke et al., 2017). La mutagenèse des génomes bactériens basée sur les transposons est une méthode puissante pour identifier indirectement les éléments génétiques associés à des fonctions et des phénotypes spécifiques, par la création de mutations aléatoires dans les génomes (Kulasekara, 2014). Les Tn5 génèrent des bibliothèques de mutants, et sont parmi les outils les plus fréquemment utilisées pour ces analyses.

D'autres approches, telle que la génomique fonctionnelle, peuvent présenter un intérêt pour la description de potentiels mécanismes de résistance à la colistine. Cette technique permet d'attribuer un rôle dans la résistance à des gènes qui n'ont pas été identifiés précédemment comme tels et de découvrir de nouveaux gènes de résistance totalement inconnus, de type enzymatique. La résistance à la colistine étant majoritairement médiée par des modifications de la régulation de voies métaboliques, cette technologie a été peu employée pour décrire de nouveaux mécanismes. Cependant, elle a déjà été utilisée avec succès pour déchiffrer la base moléculaire de la résistance à la colistine chez une souche de *Shewanella algae* qui était associée à la modification de la structure du LPS de la membrane externe par l'ajout de phosphoéthanolamine (PEA) via l'activité EptA *(Telke and Rolain, 2015)*. Elle pourrait donc être utilisée chez des entérobactéries ne présentant pas de mutations dans les gènes de régulation connus comme responsables de la résistance à la colistine.

Dans ce chapitre, nous avons utilisé ces technologies pour caractériser les mécanismes de résistance à la colistine de souches d'entérobactéries présentant un profil de résistance atypique. Dans notre premier travail, intitulé « Signature tagged mutagenesis to decipher colistin resistance in an atypical *Escherichia coli* clinical isolate, Marseille, France » nous avons utilisé la mutagenèse aléatoire pour caractériser la résistance chez une souche clinique d'*E. coli* présentant une résistance

isolée à la colistine (Article 4). Cette technologie, associée à du séquençage génomique, une analyse bio-informatique des génomes et des gènes identifiés et à des techniques de Knock-in a permis d'identifier un nombre important de gènes impliqués dans la résistance à la colistine chez cette souche.

Dans un deuxième travail, nous avons effectué une analyse pangénomique et transcriptomique pour élucider le mécanisme moléculaire de resistance à la colistine de 13 souches cliniques de *K. pneumoniae* isolées lors du travail réalisé dans l'article 2 (chapitre II), et qui présentent une sensibilité à la colistine malgré un gène *mgrB* tronqué par des séquences d'insertion. Ce travail, intitulé « Whole genome sequence analysis of truncated *mgrB* phoP/phoQ regulator genes in colistin sensitive *Klebsiella pneumoniae* isolates » (Article 5) nécessite encore de plus amples investigations mais nos premiers résultats suggèrent que d'autres régulateurs pourraient agir sur les systèmes de régulation PmrAB et PhoPQ.

**Article 4 :** Signature tagged mutagenesis to decipher colistin resistance in an *Escherichia coli* clinical isolates, Marseille, France.

Mouna Hamel, Sophie Alexandra Baron, Jean-Marc Rolain.
Draft manuscript.

**Résumé**

La résistance à la colistine chez *E. coli* est principalement médiée par l'acquisition de mutations chromosomiques dans des gènes de régulation et des gènes de biosynthèse des lipides A, conduisant à une augmentation de la synthèse de phosphoéthanolamine (pEtN) et/ou de 4-amino-4-désoxy-L-arabinose (LAra4N) (El-Sayed Ahmed *et al.*, 2020), ou encore par l'acquisition d'un gène transmissible, *mcr*, codant pour une phosphoéthanolamine transférase, qui modifie également le LPS en ajoutant de la pEtN au lipide A (Liu *et al.*, 2016). Lors de la réalisation de notre étude épidémiologique sur la résistance à la colistine chez des souches de *K. pneumoniae* isolées dans des prélèvements cliniques à Marseille, nous avions également isolé trois souches d'*E. coli* résistantes à la colistine. Parmi ces souches, l'une d'elle (R62) présentait une résistance isolée à la colistine (CMI=16µg/mL). Cet isolat était issu de la culture de l'urine d'une patiente de 24 ans hospitalisée au sein de l'Assistance Publique des Hôpitaux de Marseille en 2018.

Le séquençage génomique de cette bactérie a permis d'identifier que cette souche appartenait au sérotype O175 :H15 et était prédite comme bactérie pathogène pour l'homme. En revanche, l'analyse des gènes principalement impliqués dans la résistance à la colistine n'ont pas permis d'identifier de mécanisme de résistance à la colistine. Nous avons donc choisi d'utiliser l'approche de séquençage génomique combiné à de la mutagenèse aléatoire pour tenter de comprendre cette résistance isolée. Nous avons ainsi créé une librairie de clones en introduisant un transposon de type Tn5 qui s'insère aléatoirement dans le chromosome, interrompant ainsi des gènes putativement associés au profil recherché. Les clones redevenus sensibles à la colistine ont ensuite été sélectionné sur un milieu contenant de la colistine.

Ainsi, à partir d'une librairie de 2142 clones, 397 clones ayant une sensibilité réduite à la colistine ont été isolés (CMI<2µg/mL). Le site d'insertion du transposon a été exploré soit par PCR Genome Walker, soit par séquençage génomique, cette dernière technique s'étant révélée plus rapide, moins couteuse et moins fastidieuse que le Genome Walker. En raison de la crise sanitaire, nous n'avons pas pu séquencer la totalité des clones ; néanmoins, 202 clones sensibles à la colistine ont été analysés dont 98 identifiés par PCR genome Walker et 104 par séquençage génomique. Nous avons également désigné des couples d'amorces des gènes retrouvés plusieurs fois par séquençage, afin de screener les 195 clones restants. Au total, nous avons identifié 163 gènes différents interrompus que nous avons classés dans cinq différents clusters selon leur fonction métabolique.

Le premier cluster comprenait 52 (32%) gènes impliqués dans les différents métabolismes (glucides, glycérophospholipides, nucléotides, pyruvate et métabolisme du carbone). Le deuxième cluster était représenté par 28 gènes (17%) impliqués dans le transport membranaire, les systèmes de sécrétion et des protéines de motilité bactérienne. Dans ce cluster, cinq systèmes à deux composants ont été caractérisés. 18 gènes (11%) étaient regroupés dans un troisième groupe impliqué dans la signalisation et les processus cellulaires. Dans un quatrième groupe, 16 gènes (10 %) étaient associés à l'information génétique. Enfin, un dernier cluster représentait 2 gènes (1,5%) impliqués dans le métabolisme des cofacteurs et des vitamines, précisément dans les voies de biosynthèse de la thiamine. Les 47 gènes restants (28%) avaient une fonction inconnue.

De manière intéressante, cette souche d'*E. coli* appartient à un sérotype rarement décrit dans la littérature et 7 sur 131 gènes impliqués dans sa pathogénicité ont été interrompus par le transposon et ont permis le rétablissement de la sensibilité

à la colistine. Face au grand nombre de gènes identifiés, nous avons focalisé notre analyse sur les gènes redondants, les gènes appartenant à des systèmes à deux composants et sur les gènes présentant des homologies de séquences avec des gènes connus pour avoir un rôle dans la résistance à la colistine. Tout d'abord, l'identification des gènes connus comme phosphoéthanolamine transférase *EptC*, *TolC* et *AcrA* dans un clone chacun a validé. Puis, nous nous sommes intéressés à cinq gènes d'intérêt. Puis, nous nous sommes intéressés à une phosphoéthanolamine transférase, *YhbX*, que nous avons retrouvé tronquée dans 15 clones par PCR. Cette phosphoéthanolamine transférase présente une similitude de séquence avec *EptB* mais son rôle n'est pas connu dans la littérature (Kim *et al.*, 2006; Aquilini *et al.*, 2014).

Nous nous sommes ensuite intéressés à une glucosamine N-acetyl-transférase (GNAT). En effet, une protéine présentant cette fonction a été décrite dans la littérature comme étant une enzyme capable d'acétyler la polymyxine B et la colistine (Czub *et al.*, 2018) chez *Pseudomonas aeruginosa*. Enfin, nous avons étudié le rôle de trois serine protéases, suite à la description d'une alkaline protease capable de dégrader la colistine chez *Bacillus licheniformis* (Yin *et al.*, 2019). Ces protéines présentant une activité enzymatique, nous avons d'abord étudié la capacité de dégradation de la colistine utilisée à des concentrations croissantes chez la souche *E. coli* R62 et ses mutants par mesure à l'Autoflex® (Bruker Daltonics, Bremen, Germany). Nous avons observé la disparition du pic de la colistine à 1177 Da pour des concentrations inférieures à 8 µg/mL chez les mutants d'*E. coli* R62 interrompus sur le gène GNAT et interrompus sur les gènes de sérine protéase, suggérant ainsi une inactivation enzymatique. Nous sommes actuellement en train de mettre au point de la génomique fonctionnelle sur la souche de départ afin d'identifier un potentiel mécanisme enzymatique.

**Draft Manuscript**

# Title: Signature tagged mutagenesis to decipher colistin resistance in an atypical *Escherichia coli* clinical isolate, Marseille, France

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## Abstract

**Background:** Colistin resistance mechanisms has been widely studied since its reintroduction as a last line antibiotic for multidrug-resistant Gram-negative bacterial infections. The discovery of the *mcr* gene was the biggest breakthrough in this field in recent years. However, colistin resistance remains complex and many isolates have yet unidentified mechanisms of resistance. Here we used whole genome sequencing (WGS) and signature tagged mutagenesis (STM) to look for putative genes associated with resistance to colistin in an atypical *Escherichia coli* clinical isolate from Marseille, France.

**Materials and methods:** An atypical colistin-resistant clinical isolate *E. coli* R62 (MIC=32µg/mL) was isolated from urine of a patient in Marseille, France. This isolate was resistant only to colistin. WGS and STM were carried out to decipher colistin resistance pathways.

**Results:** After genomic analysis of 2,152 mutants obtained from strain R62 using STM, 202 colistin-sensitive clones (MIC≤2mg/L) were analysed, and the disrupted genes associated with colistin susceptibility were identified. Overall, 163 different interrupted genes were identified and classified into five different clusters according to their metabolic function. The first cluster comprised 53 (32%) genes involved in different metabolisms. The second cluster was represented by 28 genes (17%) involved in membrane transport, secretion systems and bacterial motility proteins. 20 genes (12.3%) were grouped in a third cluster involved in signalling and cellular processes. In a fourth cluster, 16 genes (10%) were associated with genetic information. Finally, a last cluster represented two genes (1.5%) involved in thiamine biosynthetic pathways. The remaining 44 genes (27%) had an unknown function.

**Conclusion:** This study revealed complex mechanism demonstrating that resistance to antimicrobial peptide is an ancient phenomenon associated with survival in harsh

environments. Further works are warranted to understand the link between these pathways and the exact mechanism of resistance to colistin.

## 1. Introduction

Colistin is an antimicrobial agent of last resort used to treat severe infections caused by Gram-negative bacteria (GNB). Given its toxicity, colistin is not used as a first-line antimicrobial agent, but rather used in the treatment of infections caused by multidrug-resistant (MDR) GNB[1]. Colistin targets the negatively charged membrane of GNB, thereby disrupting it and causing cell lysis and death[2]. Unfortunately, colistin resistance has emerged worldwide, threatening it's effectiveness[3,4].

In *Enterobacteriaceae* such as *Escherichia coli*, colistin resistance is mediated by chromosomal mutations in the regulatory genes of lipid A biosynthesis, leading to a modification of the lipid A moiety by the addition of residues of phosphoethanolamine (pEtN) and/ or 4-amino-4-deoxy-L-arabinose (LAra4N)[2]. This LPS modification is due to the activation of the two-component systems PmrAB and PhoPQ, inducing the overexpression of the *pmrC*, *pmrE* and *arnBCADTEF* genes[5]. A transmissible mobile colistin resistance gene *mcr* encoding for a phosphoethanolamine transferase can also modify LPS by adding pEtN to lipid A[6]. In subsequent years, ten additional variants of the *mcr* gene, *mcr*-1 to *mcr*-10, have been described[7]. The mechanisms that convey resistance to colistin are complex and so far, they are not completely understood in GNB[8]. Studies on the mechanisms of colistin resistance are increasing and many mechanisms of colistin resistance have been identified. However, there are still colistin resistant bacterial strains with unknown mechanisms that remain to be elucidated[2].

Lately, proteomics has emerged as an efficient tool for the characterization of colistin resistance at a large scale by the description of new potential targets, through the comparison of protein profiles[9]. In a recent study, Lang Sun et al. report several metabolic pathways related to response to colistin therapy in *Klebsiella pneumoniae*, notably gluconeogenesis, biosynthesis of the arginine cycle, porphyrin, chlorophyll metabolism, and enterobactin

biosynthesis[10]. Fernández-Reyes et al. found that membrane proteins, chaperones, protein biosynthesis factors and metabolic enzymes were downregulated in colistin-resistant strains *Acinetobacter baumannii*[11]. Also, Hui Li et al. showed that several metabolic processes mainly related to glycerophospholipid metabolism, thiamine metabolism and lipopolysaccharide were related with *mcr-1* colistin resistant *E. coli*[12]. These studies suggest that bacteria modify their metabolism to adapt to the action of colistin[12].

Through signature tagged mutagenesis (STM) approach, this study aims to identify the molecular support and the putative genes associated with colistin resistance in an atypical colistin-resistant clinical isolate of *E. coli* with an unknown mechanism of resistance.

## 2. Materials and methods

### 2.1 Bacterial Strain and microbiological procedures

The isolate used in this study is a colistin-resistant *E. coli* R62 that was recovered from the urine of a 24-year-old female patient with a recurrent pyelonephritis history at IHU Mediterranee Infection in Marseille, France, in 2018. Species identification was confirmed by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry (Bruker Daltonik, Bremen, Germany). The *E. coli* R62 was resistant to colistin with a minimal inhibitory concentration (MIC) at 16 mg/L determined by UMIC microdilution method (Biocentric, Bandol, France). A *mcr-1* producing *E. coli* strain (4 mg/L) was used as control for MIC[13]. This resistance to colistin was not associated with a multi-drug resistance phenotype since the strain was susceptible to all other antimicrobials tested with disk diffusion method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) rules. Kanamycin MIC was performed by E-tests method (BioMérieux, Marcy-l'Etoile, France).

### 2.2 Genome sequencing and analysis of the reference isolate

The colistin-resistant *E. coli* isolate R62 DNA was extracted using a QIAamp DNA Mini Kit (Qiagen) and quantified using the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific) and then subjected to whole genome sequencing (WGS) using MiSeq sequencer (Illumina Inc., San Diego, CA, USA) with pairs ends strategy. The reads were *de novo* assembled into contigs using SPAdes v3.14.1 software[14]. Assembled contigs were rearranged by mapping to the *E. coli* reference genome ATCC25922 (accession number CP009072) using MAUVE v2.4.0 software[15]. PROKKA (https://github.com/tseemann/prokka) was used to annotate the genome sequences[16]. The resistome analysis was carried out with Abricate (https://github.com/tseemann/abricate) using the Resfinder database[17], mainly to look for the *mcr-1* to *mcr-10* genes. Genetic alterations potentially associated with colistin resistance were identified in *pmrA/B*, *phoP/Q*, *pmrD*, *eptB*, *eptC* and *arn* operon genes by comparing them to the *E. coli* ATCC25922 reference strain (GenBank accession no. CP009072) carrying wild-type genes. The PROVEAN software (http://provean.jcvi.org/index.php) was used to investigate whether changes in amino acid sequences could induce an alteration in protein function. The identification of serotypes, virulence-associated genes, bacteria's pathogenicity towards human hosts prediction and multi-locus sequence types was performed using respective online tools available in the Center for Genomic Epidemiology (CGE) website, (http://www.genomicepidemiology.org/). The identification of prophage sequences within the R62 bacterial genome was done using the web server PHASTER[18]. We generated a pangenome using Roary v3.12.0 from the annotation files of the *E. coli* R62 colistin resistant strain, the reference strains *E. coli* K12 (accession number PRJNA225) and ATCC 25922, and the *E. coli* 1190 (accession number ASM231061v1) which has the most closely related genome to the R62 strain.

**2.3 Random mutagenesis library generation, and analysis of transposon mutants**

The construction of the transposon mutagenesis library of the R62 *E. coli* isolate was performed by electroporation of the EZ-Tn5™ <R6Kγori/KAN-2> Tnp kit Transposome™ (Lucigen, Tebu-bio, Le Perray-en-Yvelines, France) as described[19]. The bacteria were plated on Luria Bertani (LB, Becton Dickinson, Sparks, MD) agar medium containing 50 mg/L of kanamycin. Thereafter, the transposon library was plated onto two LB agar media, one containing kanamycin (50 mg/L) and colistin (2 mg/L) and the other containing only kanamycin (50 mg/L). After 24 h incubation, clones that did not grown on kanamycin + colistin agar were stored at 30% glycerol stocks at -80°C for further analysis. Colistin MIC was performed using the E-test method (BioMérieux, Marcy-l'Etoile, France). Identification of clones and antibiotic susceptibility testing (AST) of the colistin-susceptible (CS) mutants were performed as described above.

The location of the tn5 transposon insertion site in the genome of CS mutants was identified by one of the following methods: genome walker PCR as previously described[19], using the primers listed in the table S1, or WGS using either Miseq Technology (as described above) or MinION technology (Oxford Nanopore Technologies Inc., United Kingdom). The amplicons obtained after genome walker PCR were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, United-States), and a BlastN search against the colistin-resistant *E. coli* R62 was performed to identify the disrupted genes. For mutants sequenced by Miseq or MinION, identification of the aminoglycoside resistant gene (Aminoglycoside 3'-phosphotransferase) was used to locate the tn5 insertion site (figure S1). Also, standard PCR primers have been designed to screen for redundant genes (Table S1).

### 2.4 Data analysis of the truncated genes

Different tools were used to characterize the protein function and identify the pathways in which they are involved. Kegg software (https://www.genome.jp/kegg/) and uniprot were

used to annotate the genes of interest, determine the function of the protein and to analyse the different metabolic pathways to facilitates biological interpretation. Protein modelling was also carried out using PHYRE2[20] and PSI-BLAST[21] We also used the STRING database[22] to construct a network of interaction between the different disrupted genes and genes involved in the cascade of colistin regulation were also integrated. Finally, we performed a review of literature of every interrupted gene using the following keywords: “interrupted genes” AND “polymyxin”.

### 2.5 GCN5-related N-acetyltransferases family (GNAT) expression

GNAT sequence was synthesized by GenScript (Genscript USA Inc, Piscataway, United States) and inserted in a plasmid pet expression vector which contain a kanamycin resistance gene. Briefly, 1 µL of plasmidic DNA was electroporated into an electrocompetent *E. coli* BL21 DE3 and then recovered by adding 950 µL of SOC medium (ThermoFisher, Waltham, MA, USA), followed by incubation at 37°C for 1 hour. Clones’ selection was done by plating 100 µL on LB agar medium containing 50 mg/L of kanamycin. The clones that grown were incubated up to DO=0.6, and 1 µM of IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added. A co-selection using 50 mg/L of kanamycin and 2mg/L of colistin was made. A colistin E-test was performed to confirm the colistin resistant phenotype.

## 3. Results

### 3.1 Genome analysis of the colistin-resistant R62 reference isolate

The genome of this R62 *E. coli* was assembled into a draft genome of 4.8 Mb with 53 contigs and a 55.68% GC content. The strain did not contain any plasmid. The isolate belonged to the sequence type (ST)349, serotype O175:H15, and carried 6 genes related to virulence (*chuA, eilA, kpsE, kpsMII_K5, sitA, terC*). This bacterium has been predicted as human pathogenic with 131 pathogenic genes using PathogenFinder 1.1 software. Three intact prophages were

characterized two belonging to the Entero_mEp460 phage family with a size of 34.3 kb, 46.4 kb both and one phage belonging to Entero_P4 phage with a size of 11.5 kb.

Missense mutations were identified in PmrB (D126E, I354V), PmrA (S31T, N128I) and PhoQ (H6R, A482T), by comparing with the reference strain ATCC25922 sequences. None of these mutations matched with previously described mutations and were predicted as neutral by PROVEAN. PmrD, EptB, EptC and Arn operon amino-acid sequences showed variations in their sequences that were also predicted to be neutral (Table 1). No *mcr* genes were detected.

### 3.2 Identification of colistin susceptible mutants and characterization of truncated genes

We screened 2,152 colonies from the Tn5 insertion mutagenesis library of *E. coli* R-62 colistin-resistant strain and analysed 202 colistin-sensitive *E. coli* clones with a score ≥ 1.9 confirmed by MALDI-TOF and a colistin MIC ≤2mg/L. The genomic DNA of these mutants was extracted, and the transposon insertion sites were identified by Genome Walker PCR for 98 clones or WGS strategy for 104 clones. A total of 163 genes have been characterised from these 202 clones. The different interrupted genes are represented in Table 2. Analysis of the different metabolic pathways using KEGG allowed us to classify the different knock-outed genes into 5 different clusters (Figure 1).

The first cluster includes 53 (32.5 %) genes associated with the general cell metabolism. Many interrupted genes were associated with the tricarboxylic acid cycle (TCA), carbohydrate and energy metabolism (N=23), fatty acid biosynthesis (N=4), amino acids biosynthesis (N=11), and nucleotide metabolism (N=3). The second cluster was represented by 28 genes (17 %) implicated in the environmental information processing (membrane transport, secretion system and bacterial motility proteins). In this cluster, five members of two-component systems were characterized, which correspond to the *NarX*, *AtoC*, *TorA*,

194 *CheW*, and *OmpF* genes. The analysis revealed that 20 (12.3 %) genes grouped in a third
195 cluster associated with the signalling and cellular processes. In the fourth cluster, 16 genes (10
196 %) were implicated in the genetic information processing. The last cluster represents two
197 genes (1.5 %) involved in thiamine biosynthesis pathways. The 44 remaining genes (27 %)
198 had an unknown function. Interestingly, the transposon interrupted three intergenic spaces
199 between an inner membrane protein *ybcI* and an uncharacterized protein *ybcJ* in two clones,
200 between a penicillin-binding protein 1C *pbpC* and a nucleoside diphosphate kinase *rnk* in one
201 clone and between a 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase *dapD*
202 and a DUF3461 family protein in a last clone.

203 Our method identified genes already known to be involved in colistin resistance such as the
204 phosphoethanolamine transferase *eptC* or *tolC* and *acrA* genes that constitute the efflux pump
205 AcrAB-TolC. Finding known genes validated our method. Twenty-six genes were found in
206 more than one mutant. Among them, 10 belong to the metabolism group, four to the
207 membrane transport group, three to the genetic information processing group, four to the
208 cellular processes group and five to the unknown function group (Table 4) was found in five
209 clones, which include metal-dependent hydrolases (N=5), intergenic spacers (N=5), GNAT
210 family N-acetyltransferase (N=4), ferredoxin reductase family protein (N=2), and LysR
211 family transcriptional regulator (N=2). These different genes have been dispersed through the
212 bacterial genomes and have no particular clusters except for one, which constitutes the serine
213 proteases genes (Figure 2).

214 **3.3 Pangenome analysis**

215 We performed a pangenome of the *E. coli* R62, its closest genome *E. coli* 1190 and two
216 reference genome *E. coli* K-12 and ATCC25922 in order to identify if disrupted genes were
217 specific to our isolate or not. A total of 6,319 genes constituted this pangenome including
218 3,385 core genes and 2,934 shell genes Figure 3). The *E. coli* R62 had 180 unique genes that

were absent in the three other genomes. We looked for the presence of the 163 different disrupted genes in the genomes of these three *E. coli.* Among them, 95 were found in the four genomes whereas 58 genes belonged to the accessory genomes and ten were only present in the R62 isolate. These ten genes were mainly involved in virulence, they were represented by the serine protease operon (N=5), phage-derived genes (N=2) and the remaining genes (N=3) were of unknown function. Taken one by one, 39, 36 and 10 interrupted genes were unique to *E. coli* R62 compared to *E. coli* ATCC25922, *E. coli* K-12 and *E. coli* 1190, respectively.

### 3.4 Transposon mutant's antibiotic susceptibility testing

Interestingly, 16 transposon mutants demonstrated a co-resistance to amoxicillin. Those mutants had truncated genes involved for nine genes in metabolisms, two genes in genetic information processing, two genes in membrane transport, one gene in thiamine metabolism and two had an unknown function including the GNAT enzyme.

### 3.5 GNAT expression

In order to control the role of the disrupted genes in colistin resistance, we selected one gene that we expressed and inserted in a *E. coli* BL21 and in the R62 transposon mutants that had the knock-out gene. A Gcn5-Related N-Acetyltransferase (GNAT) gene was chosen to this complementation assay, because it was a small gene (N= 549 bp) characterized by a low GC % corresponding to 38%, that was found in four mutants and seem to also have a role in amoxicillin co-resistance. The complementation assays in *E. coli* BL21-DE3 did not result in an increase in colistin MIC (MIC=0.125), however, incorporation of the transposon into the GNAT gene disrupted clone by the Tn5 transposon led to an increase of colistin MIC from 0. 125 μg/mL to 16 μg/mL, thus confirming the role of GNAT in colistin resistance and the need for a whole machinery for this gene to function.

## 4. Discussion

It is well known that disruption of the outer membrane of the bacteria is the main antimicrobial action of colistin[2]. The two acquired mechanisms conferring colistin resistance to *Enterobacteriaceae*, chromosomal mutations that constitutively activate two-component systems and plasmid-mobilised *mcr* genes, thereby promoting opposing signalling on the expression of mRNAs of the *arnBCADTEF*, *eptA* and *pmrAB* operons, encoding key LPS-modifying enzymes[23]. Colistin resistance mechanisms are multifactorial and could involve bacterial metabolism pathways in cellular response to antibiotic treatment[10,24]. Here, we demonstrate the diversity of pathways involved in colistin resistance in *E. coli* and highlights the complexity of bacterial adaptation.

The current study shows that colistin resistance may be associated with different genes contributing to the general metabolism processes of the bacteria. Analysis of metabolic pathways has also shown that regulation of these pathways may contribute to the resistant profile of *E. coli*. These results are consistent with recent reports that show that reduced metabolism has a negative feedback on antibiotic-resistant bacteria[25]. Reports of Li, Hui et al, that have shown in a proteomic study that the *mcr*-1 gene causes disturbances in the metabolic regulation such as thiamine metabolism, glycerophospholipid metabolism, LPS biosynthesis and CAMP resistance[12]. In parallel, transcriptional analysis of a colistin-resistant *A. baumannii* demonstrated that the colistin-resistant strain regulates its metabolism by down-regulating energy and amino acid metabolism and up-regulating carbohydrate metabolism[26]. Cheng et al, indicates that the decrease in energy metabolism is a characteristic of *Vibrio alginolyticus* in response to levofloxacin, and is associated with the development of levofloxacin resistance[25]. Among genes involved in metabolism, 2 types of phosphoethanolamine transferase were randomly interrupted by the transposon and induced the recovery of susceptibility, *eptC* largely described, and *yhbX*, reported in a single study

which demonstrated the non-involvement of the latter in the development of colistin resistance, and show a significant similarity with *eptC* in amino acid sequence[27].

Membrane lipids play a vital role in cellular functions, including the regulation of transport processes, protein function and signal transduction[28]. Our results show that the *E. coli* strain altered the glycerophospholipid metabolic pathways to accommodate the colistin selection pressure. In our study, the interruption of the putative undecaprenyl-diphosphatase *ybjG* restore colistin susceptibility, the expression of the latest may be directly regulated by *phoP*[29].

The interruption of pyruvate, thiamine and malate metabolism has restored colistin sensitivity, those genes are implicated in the tricarboxylic acid cycle (TCA cycle), these results are concordant with earlier observations, which showed that the proteins involved in the TCA cycle were over-expressed in bacteria treated with high concentrations of colistin[10]. Also, a pervious study, demonstrated that the increase in energy production may be necessary for the survival of the bacteria in the presence of ampicillin[30]. Our results showed that *E. coli* regulates energy metabolism in response to colistin pressure. It was suggested that colistin stimulates the tricarboxylic acid cycle (TCA) by increasing the production of isocitrate (*icdA*), -ketoglutaric ( *sucB* ) and malate ( *mdh* ) dehydrogenases, in turn leading to an increase in NADH production and an improvement in the respiration rate[31]. Genes implicated in amino acid biosynthesis were also interrupted, the increase in amino acid levels could be, at least in part, the result of increased protein degradation, which may be caused by the need to eliminate abnormal proteins formed as a result of stress, or may be interpreted as a means of increasing the availability of amino acids needed to synthesise new proteins important for survival under new, less favourable conditions[32].

Furthermore, several interrupted genes involved in membrane transport allowed restoration of colistin sensitivity. Efflux pumps cover the inner and outer membrane of gram-negative bacteria and confer resistance to a wide range of antibiotics[33]. Baron et al, reported that the

sensitivity of plasmid-mediated colistin-resistant strains *mcr*-1 to colistin was recovered by the efflux pump inhibitor, m-chlorophenyl hydrazone carbonyl cyanide (CCCP)[34]. *torA* and *atoC*, two genes randomly interrupted by the transposon belonged respectively to the two-component system, *torCAD* and *ato* operon, involved in the regulation of the respiratory system of *E. coli*[35,36]. The serine protease found was mainly described in Enteropathogenic *E. coli* (EPEC) strains and that strains carrying *espC* were associated with a high level of virulence[37]. In the cluster of genes with unknown function, an uncharacterized protein GNAT was identified, and which according to Czub et al; seems to be involved in the enzymatic acylation of polymyxins in *Pseudomonas aeruginosa*[37].

This study demonstrated that random mutagenesis approach was an interesting strategy to identify pathways that could be associated with colistin resistance but also identify new targets to develop antimicrobial agents. This study revealed a membrane potential-dependent resistance mechanism of colistin, affirming that the membrane plays an essential role in colistin resistance. Further work is needed to understand the exact role of these pathways and resistance to colistin.

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## Figure legends

**Figure 1.** Overview of the different disrupted genes by the Tn5 transposon of *E. coli* R62 clones (red: general cell metabolism, blue: environmental information processing, yellow: signalling and cellular processes, orange: genetic information processing, green: metabolism of cofactors and vitamins).

**Figure 2.** Circular representation of the *E. coli* R62 genome, demonstrating the location of the different genes interrupted by the Tn5 transposon compared to the *E. coli* R62 and *E. coli* K12 genomes, figure constructed using CGView software.

**Figure 3.** Visualization of pan-genome analysis by Roary of the *E. coli* R62, the two-reference genome *E. coli* K-12, *E. coli* ATCC29522 and the closest genome *E. coli* 1190.

## Table legends

**Table 1.** Genetic characterisation of the *E. coli* R62 colistin-resistant clinical strain.

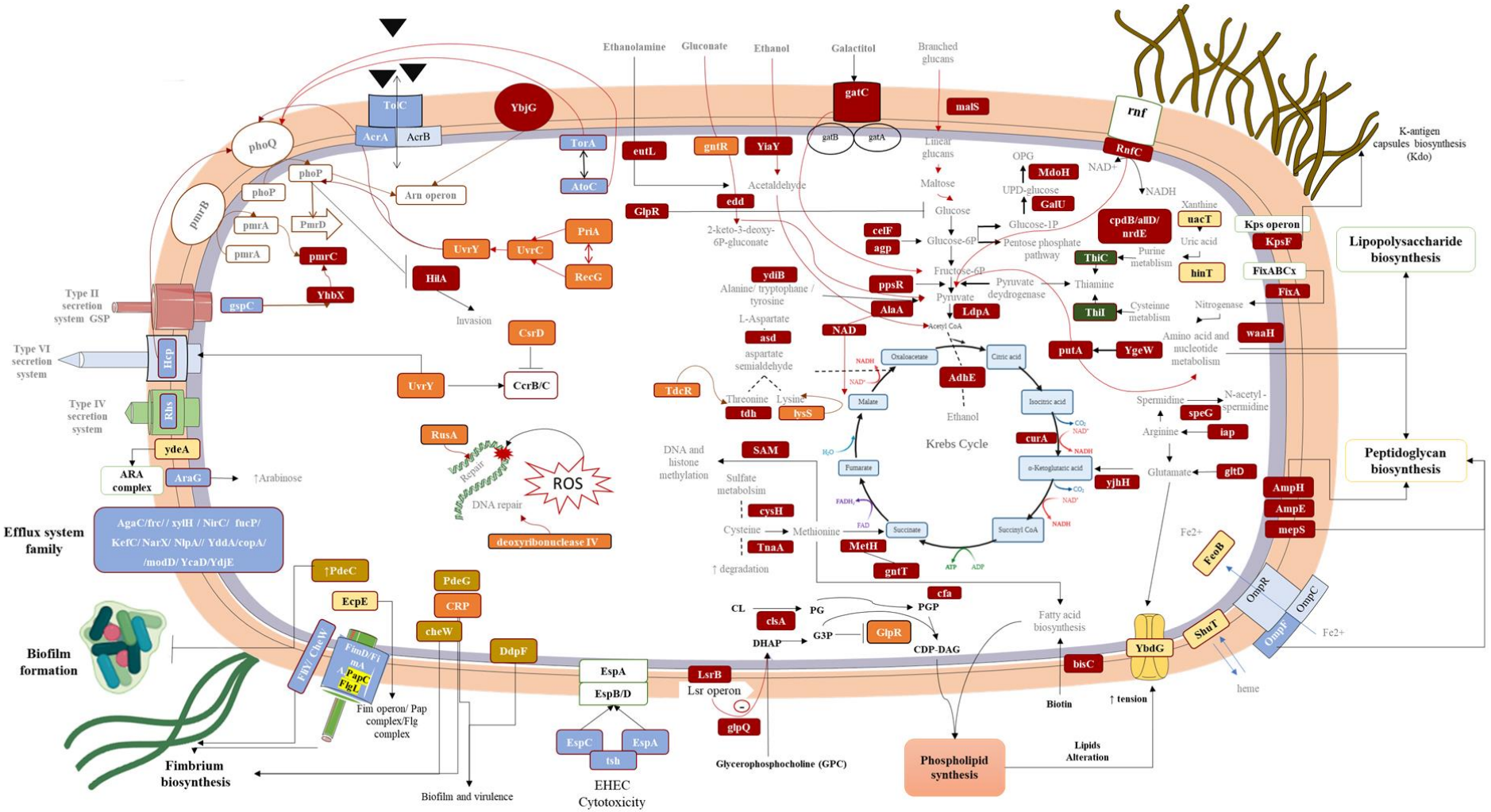
**Table 2.** Functions and characteristics of disturbed genes involved in the recovery of colistin susceptibility.

**Table 3.** Characteristics of disrupted redundant genes involved in the restoration of colistin susceptibility.

## Supplementary data

**Figure S1.** Schematic demonstration of the methodologies and technologies used for the identification of the Tn5 insertion site in the genomic sequence of *E. coli* mutants.

**Table S1.** Oligonucleotide primers of the Genome walker PCR amplification and redundant genes used in this study.

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458 **Figure 1.** Overview of the different disrupted genes by the Tn5 transposon of *E. coli* R62 clones (red: general cell metabolism, blue: environmental information processing, yellow: signalling and cellular processes, orange: genetic information processing, green: metabolism of cofactors and vitamins).

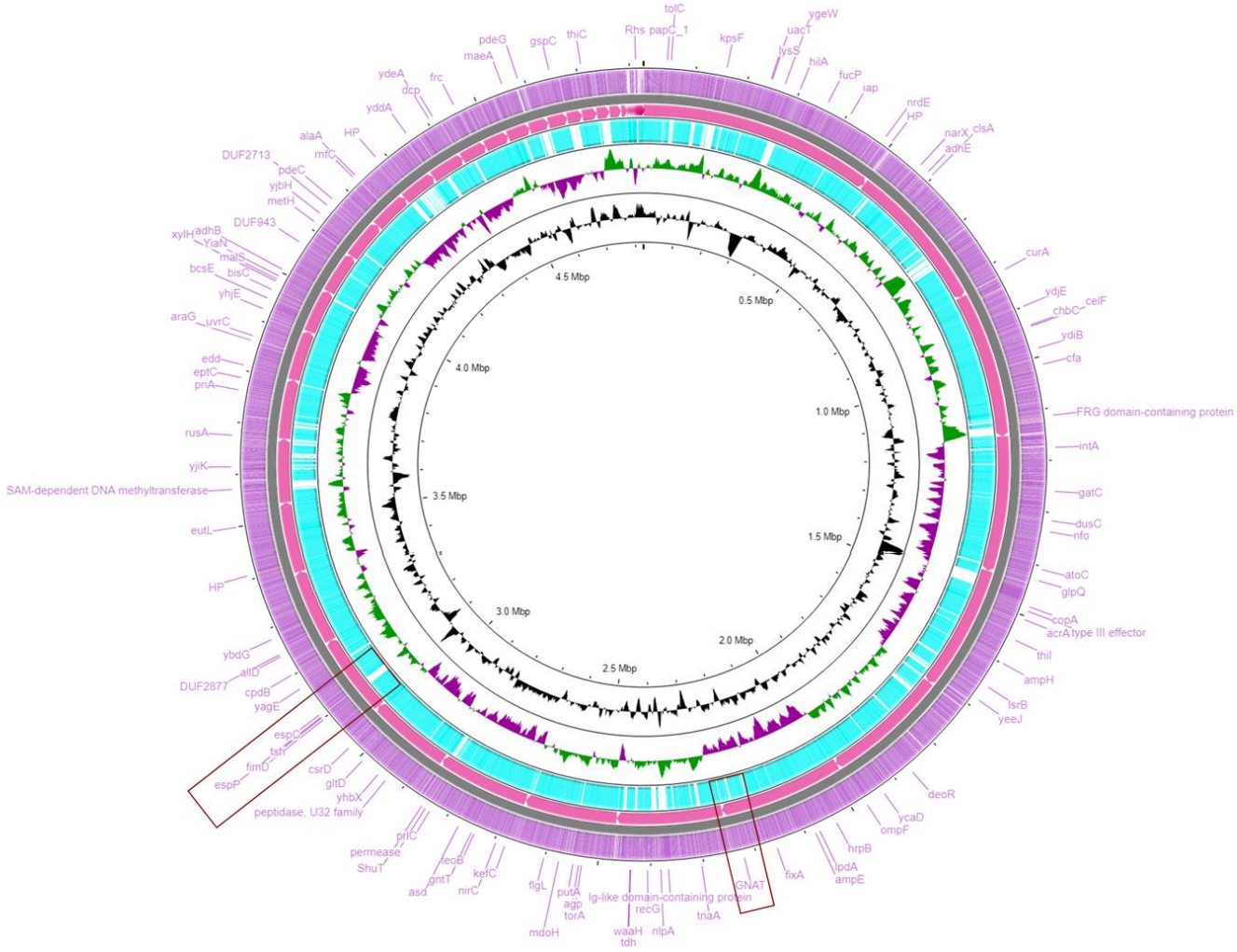


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**Figure 2.** Circular representation of the *E. coli* R62 genome, demonstrating the location of the different genes interrupted by the Tn5 transposon compared to the *E. coli* R62 and *E. coli* K12 genomes, figure constructed using CGView software.

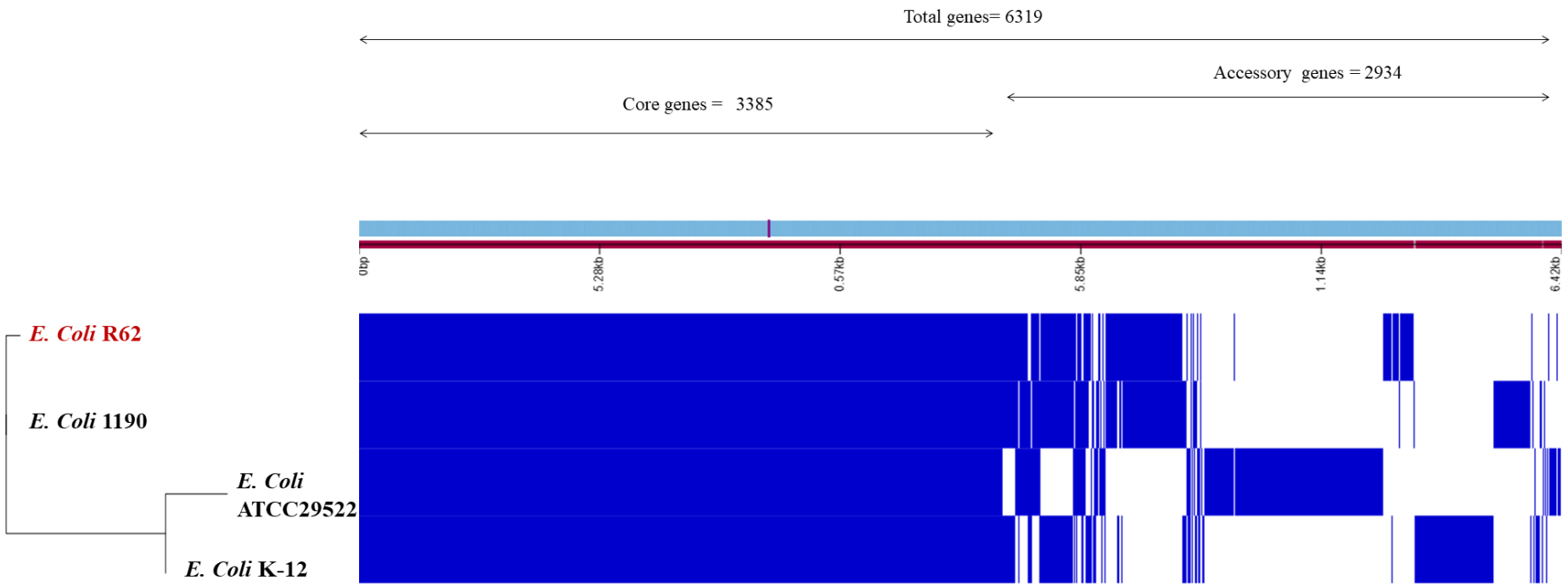


**Figure 3.** Visualization of pan-genome analysis by Roary of the *E. coli* R62, the two-reference genome *E. coli* K-12, *E. coli* ATCC29522 and the closest genome *E. coli* 1190.

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462 **Table 1.** Genetic characterisation of the *E. coli* R62 colistin-resistant clinical strain

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| **Strain** | **Genome size (Mb)** | **GC%** | **Contigs** | **Mutations in colistin-resistant genes** | | | | | | | | | | | | | | **Other's ARGs** | **Plasmid** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *E. coli* (R62) | 4.8 | 55.68 | 53 | *pmrA* | *pmrB* | *phoP* | *phoQ* | *pmrD* | *EptB* | *EptC* | *arnB* | *arnC* | *arnA* | *arnD* | *arnT* | *arnE* | *arnF* | No | No |
| | | | | **S31T N128I** | **D126E I354V** | **H6R A482T** | **Intact** | **I19L C71S K82T** | **Intact** | **V90I D403N E412D N419S T450A** | **G3E E69K A116S H219Q R368H** | **A32T G66N** | **R98A V150I D204E S213P P249S S252P K253A A311T M357L E569D Y593H** | **Q56R T187N Q222L V257M T269S** | **T116A P408S V535A** | **A27V T28A V29I V57I L72M** | **V93I G96S** | | |

464 **Table 2.** Functions and characteristics of disturbed genes involved in the recovery of colistin susceptibility.

| Interrupted genes | MIC mg/mL | Gene no | Group | Functions | Kegg No | N |
|---|---|---|---|---|---|---|
| Phosphoethanolamine transferase *eptC* (*cptA*) | 0.50 | R62_03656 | Metabolism | Lipopolysaccharide biosynthesis<br>Add a PEtN residue on the HeptI of the inner core of lipopolysaccharide | K19353 | 1 |
| Phosphoethanolamine transferase *yhbX* | 0.25 | R62_02829 | | Lipopolysaccharide core region biosynthetic process – exact function unknown | | 1 |
| UDP-glucuronate: LPS(HepIII) glycosyltransferase *waaH* | 1 | R62_04058 | | Incorporation of glucuronic acid (GlcUA) by the PhoB/R-inducibleWaaH glycosyltransferase with a concomitant loss of phosphate residue on HepII. Involved in inner core modification like eptC<br>Carbohydrate metabolism/Cationic antimicrobial peptide (CAMP) resistance | K19354 | 2 |
| Cardiolipin synthase A (*cls*) | 0.25 | R62_00584 | | Glycerophospholipid metabolism<br>Essential for lipopolysaccharide biosynthesis | K06131 | 2 |
| Putative undecaprenyl-diphosphatase *YbjG* | 0.25 | R62_01789 | | Regulated by PmrAB and PhoPQ, member of PAP2 family. Recycle C55-P Active the synthesis of aminoarabinose | K19302 | 1 |
| PTS system sorbose specific EIIA component *sorF* | 0.50 | R62_03989 | | Carbohydrate uptake and control of carbon metabolism<br>Fructose and mannose metabolism/ Phosphotransferase system | K02812 | 1 |
| PTS N, N'-diacetylchitobiose transporter subunit IIC *chbC* | 0.38 | R62_00931 | | Carbohydrate uptake and control of carbon metabolism<br>Starch and sucrose metabolism/ Phosphotransferase system<br>Chitosugars inner membrane system<br>Involved in chitinase and chitobiase metabolism in serratia | K02761 | 1 |

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|---|---|---|---|---|---|
| PTS system galactitol-specific EIIC component *gatC* | 0.25 | R62_01248 | Carbohydrate uptake and control of carbon metabolism<br>Galactose metabolism/ Phosphotransferase system | K02775 | 1 |
| NAD-dependent malic enzyme | 0.38 | R62_04406 | Carbon metabolism<br>Pyruvate metabolism/Carbon metabolism/ Two-component system | K00027 | 1 |
| Bifunctional glucose-1-phosphatase/inositol phosphatase (*agp*) | 0.50 | R62_02401 | Under the regulation of Cpx operon (involved in CAMP resistance and fimA expression)<br>Glycolysis / Gluconeogenesis | K01085 | 1 |
| Alpha-amylase (*malS*) | 0.25 | R62_03858 | Carbohydrate metabolism<br>Endohydrolysis of (1->4)-alpha-D-glucosidic linkages in polysaccharides containing three or more (1->4)-alpha-linked D-glucose units<br>Starch and sucrose metabolism | K01176 | 1 |
| L-threonine dehydrogenase (*yiaY/adhB*) | 0.38 | R62_03879 | Glycolysis / Gluconeogenesis/ Fatty acid degradation/ Amino acid metabolism/ Pyruvate metabolism/ Xenobiotics biodegradation and metabolism | K13954 | 1 |
| Glycerophosphodiester phosphodiesterase (*glpQ*) | 0.25 | R62_01398 | Anaerobic catabolism of glycerol. Repressed by exposure to polymyxin. intracellular uptake and production of sn-glycerol-3- phosphate, a carbon and energy source and a precursor for novel phospholipid synthesis and recycling of membrane phospholipids. Tn5 mutant in yersinia decrease polymyxin resistance<br>Glycerophospholipid metabolism | K01126 | 1 |
| Bifunctional protein *putA* | 0.125 | R62_02413 | mgrR overexpression (dependant of the PhoPQ system) increase the expression of putA<br>Oxidizes proline to glutamate for use as a carbon and nitrogen source Amino acid | K13821 | 1 |

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|---|---|---|---|---|---|
| | | | metabolism/Arginine and proline metabolism/Biosynthesis of secondary metabolites | | |
| 2',3'-cyclic-nucleotide 2'-phosphodiesterase/3'-nucleotidase (*cpdB*) | 0.125 | R62_03079 | Degrading nontransportable organophosphates, which is regulated by carbon source availability. Regulated by the phosphorus stress response (PhoPQ) Nucleotide metabolism (purine/pyrimidine) | K01119 | 2 |
| Cobalamin-dependent methionine synthase (*metH*) | 0.125 | R62_03982 | Amino acid metabolism (Cysteine and methionine) | K00548 | 1 |
| Peptidyl-dipeptidase *dcp* | 0.125 | R62_04281 | Peptidases and inhibitors | K01284 | 1 |
| Dihydrodipicolinate synthase family protein *yjhH/yagE* | 0.50 | R62_03041 | Carbohydrate metabolism | K22397 | 1 |
| Phosphoadenosine phosphosulfate reductase family protein (*cysH*) | 0.25 | R62_00380 | Energy metabolism (sulfur) | K00390 | 1 |
| Dihydrolipoamide dehydrogenase (*lpdA*) | 0.50 | R62_01974 | Glycolysis / Gluconeogenesis/Citrate cycle (TCA cycle) /Pyruvate metabolism | K00382 | 1 |
| UTP--glucose-1-phosphate uridylyltransferase *galU* | 0.75 | R62_00571 | Carbohydrate metabolism | K00963 | 1 |
| Glucans biosynthesis glucosyltransferase *mdoH* | 0.38 | R62_02442 | Glycosyltransferases/ osmoregulated periplasmic glucan (OPG) synthesis | K03669 | 1 |
| Aspartate-semialdehyde dehydrogenase (*asd*) | 0.38 | R62_02644 | Amino acid metabolism (lysine, methionine, and threonine) | K00133 | 2 |
| L-threonine 3-dehydrogenase (*tdh*) | 0.75 | R62_02308 | Amino acid metabolism | K00060 | 2 |
| Spermidine N (1) -acetyltransferase (*speG*) | 0.50 | R62_01091 | Amino acid metabolism/ optimal cell growth | K00657 | 2 |
| Glutamate-pyruvate aminotransferase (*alaA)* | 1 | R62_04093 | Amino acid metabolism: major alanine-synthesizing transaminases | K14260 | 2 |
| Putative electron transfer flavoprotein *fixA* | 0.38 | R62_02054 | Energy metabolism: Anaerobic carnitine reduction | K03521 | 3 |

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|---|---|---|---|---|---|
| Protein *AmpE* | 0.75 | R62_01979 | Signaling proteins/ *AmpR* mediated regulation of *AmpC* | K03807 | 1 |
| DD-endopeptidase / DD-carboxypeptidase *ampH* | 0.38 | R62_01543 | Peptidases and inhibitors/Remodeling or recycling process of peptidoglycan. | K18988 | 1 |
| Tryptophanase *tnaA* | 0.125 | R62_02187 | Tryptophan metabolism/ responsible for the production of indole, an important factor in biofilm formation and antibiotic resistance. | K01667 | 1 |
| Thermosensitive gluconokinase (*gntT*) | 0.38 | R62_03435 | Carbohydrate metabolism: gluconate transporter | K00851 | 1 |
| Alcohol dehydrogenase *adhE* | 0.38 | R62_03879 | Carbohydrate metabolism: catalyzes the sequential reduction of acetyl-CoA to acetaldehyde and then to ethanol/Lipid metabolism/ Amino acid metabolism/ Xenobiotics biodegradation and metabolism | K13954 | 1 |
| Biotin sulfoxide reductase *bisC* | 0.75 | R62_03836 | Biotin metabolism: protect the cell from oxidation damage | K08351 | 1 |
| 6-phospho-beta-glucosidase *celF* | 0.25 | R62_00934 | Carbohydrate metabolism | K01222 | 1 |
| Arabinose-5-phosphate isomerase *kpsF* | 0.25 | R62_00145 | Lipopolysaccharide biosynthesis : KDO biosynthesis | K06041 | 1 |
| Murein DD-endopeptidase MepS/Murein LD-carboxypeptidase *mepS* | 0.125 | R62_00961 | Peptidases and inhibitors | K13694 | 1 |
| Ethanolamine utilization protein *EutL* | 0.75 | R62_03366 | Amino acid metabolism/ ethanolamine catabolic process | K04026 | 1 |
| NADPH-dependent curcumin/dihydrocurcumin reductase *curA* | 0.125 | R62_00814 | Oxidoreductase activity | K23256 | 1 |
| Alkaline phosphatase isozyme conversion aminopeptidase *iap* | 0.75 | R62_00383 | Peptidases and inhibitors | K09612 | 1 |
| Cyclopropane-fatty-acyl-phospholipid synthase *cfa* | 0.25 | R62_01011 | Lipid metabolism | K00574 | 1 |
| Phosphogluconate dehydratase *edd* | 0.75 | R62_03680 | Carbohydrate metabolism | K01690 | 1 |
| Cold shock protein *ydfK* | 0.125 | R62_04287 | Response to cold stress | | 1 |

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|---|---|---|---|---|---|---|
| Putative cyclic di-GMP phosphodiesterase *pdeG* | 0.75 | R62_04433 | | Hydrolase/ Polyamine signal transposition in the periplasm for biofilm formation. | | 1 |
| Hydrogenase maturation protease *hyaD, hybD* | 0.75 | R62_02381 | | Peptidases and inhibitors | K03605 | 1 |
| Ribonucleoside-diphosphate reductase alpha chain *nrdE* | 0.125 | R62_00469 | | Nucleotide metabolism (purine/pyrimidine) | K00525 | 1 |
| Oligopeptidase A, *prlC* | 0.75 | R62_02701 | | Peptidases and inhibitors | K01414 | 1 |
| Ureidoglycolate dehydrogenase *allD* | 0.25 | R62_03118 | | Nucleotide metabolism (purine) | K00073 | 1 |
| Putative carbamoyltransferase *ygeW* | 0.25 | R62_00251 | | Arginine and citrulline biosynthetic process | | 2 |
| Glutamate synthase (NADPH) small chain *gltD* | 0.38 | R62_02871 | | Energy metabolism/ Amino acid metabolism | K00266 | 1 |
| ATP-dependent RNA helicase *hrpB* | 0.50 | R62_01940 | | Hydrolase | K03579 | 3 |
| Quinate/shikimate dehydrogenase *ydiB* | 0.75 | R62_00977 | | Amino acid metabolism (Phenylalanine, tyrosine, and tryptophan biosynthesis) | K05887 | 1 |
| Posphoenolpyruvate synthetase regulatory kinase/phosphorylase P*psR* | 0.50 | R62_00966 | | Protein dephosphorylation | K09773 | 1 |
| NAD+ oxidoreductase subunit C *rnfC/nuoC* | 0.25 | R62_04088 | | Energy metabolism | K13378 | 1 |
| tRNA sulfurtransferase *ThiI* | 0.25 | R62_01493 | Metabolism of cofactors and vitamins | Thiamine metabolism/ Sulfur relay system | K03151 | 1 |
| Phosphomethylpyrimidine synthase *ThiC* | 0.50 | R62_04584 | | Thiamine metabolism | K03147 | 1 |
| Type IV secretion protein *Rhs* | 0.38/0.75 | R62_04695 | Environmental Information Processing | Bacterial secretion system | K11904 | 3 |
| Serine protease *EspC* | 0,19/0.50 | R62_02983 | | Serine protease autotransporter | K01347 | 2 |
| Temperature-sensitive hemagglutinin tsh autotransporter | 0.38 | R62_02978 | | Serine protease autotransporter | K12684 | 1 |
| Serine protease *EspP* | 0.38 | R62_02979 | | Serine protease autotransporter | K01347 | 2 |

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|---|---|---|---|---|---|
| Outer membrane usher protein *FimD* | 0.75 | R62_02974 | Bacterial motility proteins | K07347 | 4 |
| Type 1 fimbrial major subunit *FimA* | 0.50 | R62_04237 | Bacterial motility proteins | K07345 | 1 |
| Type VI secretion system tube protein *Hcp* | 0.38 | R62_04539 | Bacterial secretion system | K11903 | 1 |
| Lipoprotein 28 (lipoprotein *NlpA)* | 0.25 | R62_02250 | ABC transporters (D-methionine) | K02073 | 1 |
| Xylose transport system permease protein *XylH* | 0.50 | R62_03855 | ABC transporters (Xylose) | K10544 | 1 |
| Autoinducer 2-binding protein *LsrB* | 0.38 | R62_01589 | Saccharide, polyol, and lipid transporters | K02058 | 1 |
| Glutathione-regulated potassium-efflux system protein *kefC* | 0.125 | R62_02561 | Transporters | K11747 | 1 |
| Nitrite transporter *nirC* | 0.50 | R62_02578 | Nitrite transporter | K02598 | 1 |
| Nitrate/nitrite sensor protein *narX* | 0.50 | R62_00554 | Two-component system | K07673 | 1 |
| PTS mannose/fructose/sorbose/N-acetylgalactosamine transporter subunit IIC (*agaC*) | 0.38 | R62_02788 | Phosphotransferase system (PTS)/ Galactose metabolism | K02746 | 1 |
| L-cystine-binding protein *fliY* | 0.50 | R62_03749 | ABC transporters/ Flagellar assembly | K02424 | 1 |
| Arabinose import ATP-binding protein *araG* | 0.38 | R62_03729 | ABC transporters | K10539 | 1 |
| Multidrug efflux pump subunit *acrA* | 0.50 | R62_01452 | beta-Lactam resistance/ Cationic antimicrobial peptide (CAMP) resistance acrAB-TolC more expressed under acidic pH. | K03585 | 1 |
| Formyl-CoA:oxalate CoA-transferase *frc* | 0.125 | R62_04320 | Transferase | K07749 | 1 |
| Acetoacetate metabolism transcriptional regulator *AtoC* | 0.75 | R62_01381 | Two-component system AtoS/AtoC is associated with cationic iron transporters | K07714 | 1 |
| Trimethylamine-N-oxide reductase *torA* | 0.75 | R62_02396 | Two-component system | K07811 | 1 |

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|---|---|---|---|---|---|---|
| Putative type II secretion system protein C *gspC* | 0.25 | R62_04523 | | Bacterial secretion system/Biofilm formation | K02452 | 1 |
| Inner membrane ABC transporter ATP-binding protein *yddA* | 0.38 | R62_04226 | | ABC transporters | K02471 | 1 |
| Chemotaxis protein *cheW* | 0.125 | R62_03719 | | Two-component system/ Bacterial chemotaxis | K03408 | 1 |
| tolC family outer membrane protein | 0.75 | R62_01425 | | Adhesin transport system | K12543 | 1 |
| ribosomal protein S5-alanine N-acetyltransferase *rimJ* | 0.38 | R62_02460 | | Ribosome biogenesis/Transferase | K09811 | 1 |
| porin *ompF* | 0.38 | R62_01880 | | Two-component system/ beta-Lactam resistance | K09476 | 1 |
| Molybdenum transport protein, *modD* | 0.38 | R62_00520 | | Transport protein | K03813 | 1 |
| Copper-exporting P-type ATPase *copA* | 0.50 | R62_01428 | | Signal transduction | K17686 | 1 |
| Excinuclease ABC subunit *uvrC* | 0.38 | R62_03742 | Genetic Information Processing | Nucleotide excision repair | K03703 | 1 |
| Transcriptional regulator *gntR/glcc* | 0.125 | R62_00112 | | Transcription factors | K11474 | 2 |
| ATP-dependent DNA helicase *RecG* | 0.25 | R62_02274 | | Homologous recombination | K03655 | 1 |
| *uvrY/sirA/gacA* family response regulator transcription factor | 0.125 | R62_03743 | | Two-component system/ Biofilm formation | K07689 | 1 |
| Primosomal protein N'(*priA*) | 0.94 | R62_03636 | | Homologous recombination | K04066 | 1 |
| CRP/FNR family transcriptional regulator, cyclic AMP receptor protein | 0.094 | R62_02568 | | Two-component system/Biofilm formation | K10914 | 1 |
| Crossover junction endodeoxyribonuclease r*usA* | 0.25 | R62_03540 | | DNA repair and recombination proteins | K01160 | 1 |
| HTH-type transcriptional regulator *cynR* | 0.25 | R62_00196 | | Transcription factors | K21755 | 1 |
| Acid stress chaperone *hdeA* | 0.25 | R62_02728 | | Chaperones and folding catalysts/ role in resistance to low pH | K19777 | 1 |
| tRNA-dihydrouridine(16) synthase *dusC* | 0.38 | R62_01299 | | Transfer RNA biogenesis | K05541 | 1 |
| *deoR/glpR* transcriptional regulator | 0.38 | R62_02633 | | Transcription factors/ negative expression of genes related to transport and catabolism of | K02444 | 1 |

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|---|---|---|---|---|---|---|
| | | | | deoxyribonucleoside nucleotides | | |
| Transcriptional regulator *hilA* | 0.38 | R62_00283 | | Transcription factors/ Activates the expression of invasion genes in *salmonella* | K22486 | 1 |
| DNA-binding transcriptional activator *tdcR* | 0.50 | R62_02773 | | Transcription/ participates in the control of genes involved in the transport and metabolism of threonine and serine during anaerobiosis | K07591 | 1 |
| Lysine--tRNA ligase, *lysS* | 0.25 | R62_02987 | | Translation/ necessary for normal growth under aerobic conditions and at growth temperatures below 37 degrees | K04567 | 2 |
| Deoxyribonuclease IV *nfo* | 0.50 | R62_01318 | | Base excision repair | K01151 | 1 |
| RNase E specificity factor *csrD* | 0.25 | R62_02903 | | Messenger RNA biogenesis | K18765 | 2 |
| Ig-like domain-containing protein | 0.38 | R62_02241 | Cellular Processes | Bacterial invasion of epithelial cells | K20276 | 2 |
| Uric acid transporter u*acT* | 0.38 | R62_00234 | | Uric acid transporter | K24206 | 1 |
| Intimin-like adhesin *fedC/ yeeJ* | 0.50 | R62_01613 | | Bacterial invasion of epithelial cells/ pilus assembly/ biofilm formation | K13735 | 1 |
| Putative D, D- dipeptide transport ATP-binding protein *ddpF* | 0.38 | R62_04418 | | D,D-dipeptide transport | K02032 | 2 |
| Purine nucleoside phosphoramidase *hinT* | 0.25 | R62_02498 | | Growth promotion in high salt conditions | K02503 | 1 |
| Putative cyclic di-GMP phosphodiesterase *PdeC* | 0.50 | R62_04034 | | Stress-induced mutagenesis response promotion | | 2 |
| SAM-dependent DNA methyltransferase | 0.25 | R62_03443 | | Prokaryotic defense system/ critical roles in bacterial growth and virulence | K03427 | 1 |
| 2,3-diketo-L-gulonate transporter large permease *yiaN* | 0.125 | R62_03866 | | Carbohydrate transmembrane transport | K21393 | 1 |
| Flagellar hook-associated protein *flgL* | 0.25 | R62_02477 | | Flagellar assembly: junction proteins connect the filament to the hook | K02397 | 1 |

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|---|---|---|---|---|---|---|
| Inner membrane symporter *yicJ* | 0.50 | R62_04697 | | Proton or sugar-driven metabolite absorption system | K07491 | 1 |
| Mechanosensitive ion channel *ybdG* | 0.38 | R62_03149 | | Protection against hypoosmotic shock | K16053 | 1 |
| Inner membrane metabolite transport protein *ydjE* | 0.38 | R62_00894 | | Proton-driven metabolite absorption system | K08369 | 1 |
| Putative MFS-type transporter *ycaD* | 0.38 | R62_01851 | | Proton-driven metabolite absorption system | K08219 | 1 |
| Heme ABC transporter substrate-binding protein *shuT* | 0.50 | R62_02720 | | Iron complex transport system substrate-binding protein | K02016 | 1 |
| L-arabinose MFS transporter *ydeA* | 0.38 | R62_04270 | | L-arabinose/isopropyl-beta-D-thiogalactopyranoside export | K08159 | 1 |
| Fe (2+) transporter *feoB* | 0.50 | R62_02619 | | Ferrous iron absorption system | K04759 | 1 |
| Putative fimbrial chaperone *ecpE* | 0.50 | R62_01628 | | Predicted chaperone of the common pilus expressed in low temperature pathogenic E. coli strains | K21968 | 1 |
| Putative thiamine transport system ATP-binding protein *malK* | 0.25 | R62_00912 | | Transporter : maltose | K05779 | 1 |
| L-fucose:H+ symporter permease (*fucP*) | 0.38 | R62_00340 | | Transporter : arabinose, fucose and galactose transmembrane transport | K02429 | 1 |
| Outer membrane usher protein *papC* | 0.50/0.38 | R62_04296 | | Secretion system/ pilus assembly | K12518 | 2 |
| GNAT family N-acetyltransferase | 0.125 | R62_02115 | | Transferase | K03790 | 4 |
| Metal-dependent hydrolase | 0.125/0.125 | R62_03128 | | Uncharacterized | K07038 | 5 |
| Permease | 0.50 | R62_02713 | Unknown Function | Uncharacterized | K07089 | 1 |
| Putative protein *yjiK* | 0.25 | R62_03475 | | Uncharacterized | | 1 |
| Hypothetical protein: yjbH domain-containing protein | 0.25 | R62_04001 | | Uncharacterized | | 1 |
| Ferredoxin reductase family protein | 0.50 | R62_00202 | | Uncharacterized | | 2 |

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|---|---|---|---|---|---|
| Glycine zipper 2TM domain-containing protein | 0.38 | R62_03307 | Uncharacterized | | 1 |
| Protein of unknown function (DUF2686)/*ydjM* | 0.19 | R62_00940 | Uncharacterized | K07038 | 1 |
| OB fold stress tolerance protein YgiW | 0.38 | R62_00067 | Uncharacterized | | 1 |
| Type III effector | 0.38 | R62_01435 | Transposase | K07486 | 1 |
| Cyclic di-GMP binding protein *bcsE* | 0.50 | R62_03816 | Uncharacterized | | 1 |
| Hypothetical protein | 0.38 | R62_02241 | Uncharacterized | K13735 | 1 |
| *LysR* family transcriptional regulator | 0.38 | R62_01663 | Uncharacterized | | 2 |
| Inner membrane metabolite transport protein y*hjE* | 0.125 | R62_03803 | Uncharacterized | | 1 |
| FRG domain-containing protein | 0.38 | R62_01109 | Uncharacterized | | 1 |
| Tail fiber protein | 0.38 | R62_04445 | Uncharacterized | | 1 |
| Inner membrane protein *yqiJ* | 0.25 | R62_00034 | Uncharacterized | | 1 |
| YdeI family stress tolerance OB fold protein | 0.38 | R62_04280 | Uncharacterized | | 1 |
| TIR domain protein | 0.38 | R62_04474 | Uncharacterized | | 1 |
| Hyphothetical protein | 0.38 | R62_03523 | Uncharacterized | | 1 |
| DUF1479 family protein | 0.50 | | Uncharacterized | | 1 |
| Peptidase, U32 family | 0.19 | R62_02811 | Uncharacterized | | 1 |
| NADP (+) - dependent aldehyde reductase | 0.25 | R62_01410 | Uncharacterized | | 1 |
| Hypothetical protein | 0.50 | R62_04150 | Uncharacterized | | 1 |
| YehE family protein | 0.125 | R62_01269 | Uncharacterized | | 1 |
| Type III effector | 0.38 | R62_02024 | Uncharacterized | | 1 |
| DUF2713 family protein | 0.50 | R62_04020 | Uncharacterized | | 1 |
| Uncharacterized protein YhfL | 0.50 | R62_02580 | Uncharacterized | | 1 |
| Divergent polysaccharide deacetylase family protein | 0.38 | R62_02310 | Uncharacterized | K09798 | 1 |
| Uncharacterized protein YjeI | 0.125 | R62_03002 | Uncharacterized | | 1 |

| | | | | |
|---|---|---|---|---|
| DUF1471 domain-containing protein | 0.50 | R62_03062 | Uncharacterized | 1 |
| KilA-N domain protein | 0.50 | R62_04704 | Uncharacterized | 1 |
| DUF943 family protein | 0.125 | R62_03939 | Uncharacterized | 1 |
| Hypothetical protein | 0.38 | R62_01093 | Uncharacterized | 1 |
| DUF1376 domain-containing protein | 0.125 | | Uncharacterized | 1 |
| Hypothetical protein | 0.38 | R62_00486 | Uncharacterized | 1 |
| Autotransporter outer membrane beta-barrel domain-containing protein | 0.38 | R62_00530 | Uncharacterized | 1 |
| Hypothetical protein | 0.50 | R62_00486 | Uncharacterized | 1 |
| Hypothetical protein | 0.50 | R62_02982 | Uncharacterized | 1 |
| Tyrosine-type recombinase/integrase intA | 0.50 | R62_03522 | Uncharacterized | 1 |
| Hypothetical protein | 0.125 | R62_04150 | Uncharacterized | 1 |
| Hyphothetical protein | 0.38 | R62_03288 | Uncharacterized | 1 |
| DUF2877 domain-containing protein | 0.50 | R62_03121 | Uncharacterized | 1 |
| Intergenic spaces | 0.50 | | | 5 |
| Total | | | | 202 |

465

466

467 **Table 3.** Characteristics of disrupted redundant genes involved in the restoration of colistin susceptibility.

| Interrupted genes | MIC mg/mL | Gene no | Group | Functions | Kegg No | N |
|---|---|---|---|---|---|---|
| Metal-dependent hydrolase | 0.125/0.125/0.25/0.5/0.25 | R62_03128 | Unknown Function | Uncharacterized | K07038 | 5 |
| Intergenic spaces | 0.50/ 0.75/0.5/ 0.75/0.75 | | Unknown Function | | | 5 |
| Outer membrane usher protein *fimD* | 0.75/0.5/0.5/0.5 | R62_02974 | Environmental Information Processing | Bacterial motility proteins | K07347 | 4 |
| GNAT family N-acetyltransferase | 0.125/0.25/0.125/0.25 | R62_02115 | Unknown Function | Transferase | K03790 | 4 |
| Putative electron transfer flavoprotein (*fixA*) | 0.38/0.38/0.25 | R62_02054 | Metabolism | Energy metabolism | K03521 | 3 |
| ATP-dependent RNA helicase (*hrpB*) | 0.50/0.25/0.5 | R62_01940 | Metabolism | Hydrolase | K03579 | 3 |
| Type IV secretion protein Rhs | 0.38/0.75/0.38 | R62_04695 | Environmental Information Processing | Bacterial secretion system | K11904 | 3 |
| Cardiolipin synthase A (*cls*) | 0.25/0.25 | R62_00584 | Metabolism | Glycerophospholipid metabolism | K06131 | 2 |
| 2',3'-cyclic-nucleotide 2'-phosphodiesterase/3'-nucleotidase (*cpdB*) | 0.125/0.25 | R62_03079 | Metabolism | Nucleotide metabolism (purine/pyrimidine) | K01119 | 2 |
| Aspartate-semialdehyde dehydrogenase (*asd*) | 0.38/0.5 | R62_02644 | Metabolism | Amino acid metabolism | K00133 | 2 |
| L-threonine 3-dehydrogenase (*tdh*) | 0.75/0.5 | R62_02308 | Metabolism | Amino acid metabolism | K00060 | 2 |
| Spermidine N (1) -acetyltransferase (*speG*) | 0.50/0.5 | R62_01091 | Metabolism | Amino acid metabolism | K00657 | 2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Glutamate-pyruvate aminotransferase (*alaA*) | 1/0.75 | R62_04093 | Metabolism | Amino acid metabolism | K14260 | 2 |
| UDP-glucuronate:LPS(HepIII) Glycosyltransferase (*waaH*) | 1/0.75 | R62_04058 | Metabolism | Carbohydrate metabolism/Cationic antimicrobial peptide (CAMP) resistance | K19354 | 2 |
| Putative carbamoyltransferase (*ygeW*) | 0.25/0.25 | R62_00251 | Metabolism | Arginine and citrulline biosynthetic process | | 2 |
| Serine protease *espC* | 0,19/0.50 | R62_02983 | Environmental Information Processing | Serine protease autotransporter | K01347 | 2 |
| Serine protease *espP* | 0.38/0.5 | R62_02979 | Environmental Information Processing | Serine protease autotransporter | K01347 | 2 |
| transcriptional regulator GntR/glcc | 0.125/0.25 | R62_00112 | Genetic Information Processing | Transcription factors | K11474 | 2 |
| Lysine--tRNA ligase, lysS | 0.25/0.25 | R62_02987 | Genetic Information Processing | Translation | K04567 | 2 |
| RNase E specificity factor CsrD | 0.25/0.25 | R62_02903 | Genetic Information Processing | Messenger RNA biogenesis | K18765 | 2 |
| Ig-like domain-containing protein | 0.38/0.75 | R62_02241 | Cellular Processes | Bacterial invasion of epithelial cells | K20276 | 2 |
| Putative D, D- dipeptide transport ATP-binding protein DdpF | 0.38/0.50 | R62_04418 | Cellular Processes | Quorum sensing | K02032 | 2 |
| Putative cyclic di-GMP phosphodiesterase PdeC | 0.50/0.50 | R62_04034 | Cellular Processes | Hydrolase | | 2 |
| Outer membrane usher protein PapC | 0.50/0.38 | R62_04296 | Cellular Processes | Secretion system | K12518 | 2 |
| Ferredoxin reductase family protein | 0.50/0.25 | R62_00202 | Unknown Function | Uncharacterized | | 2 |
| LysR family transcriptional regulator | 0.38/0.25 | R62_01663 | Unknown Function | Uncharacterized | | 2 |
| Total | | | | | | 65 |

468

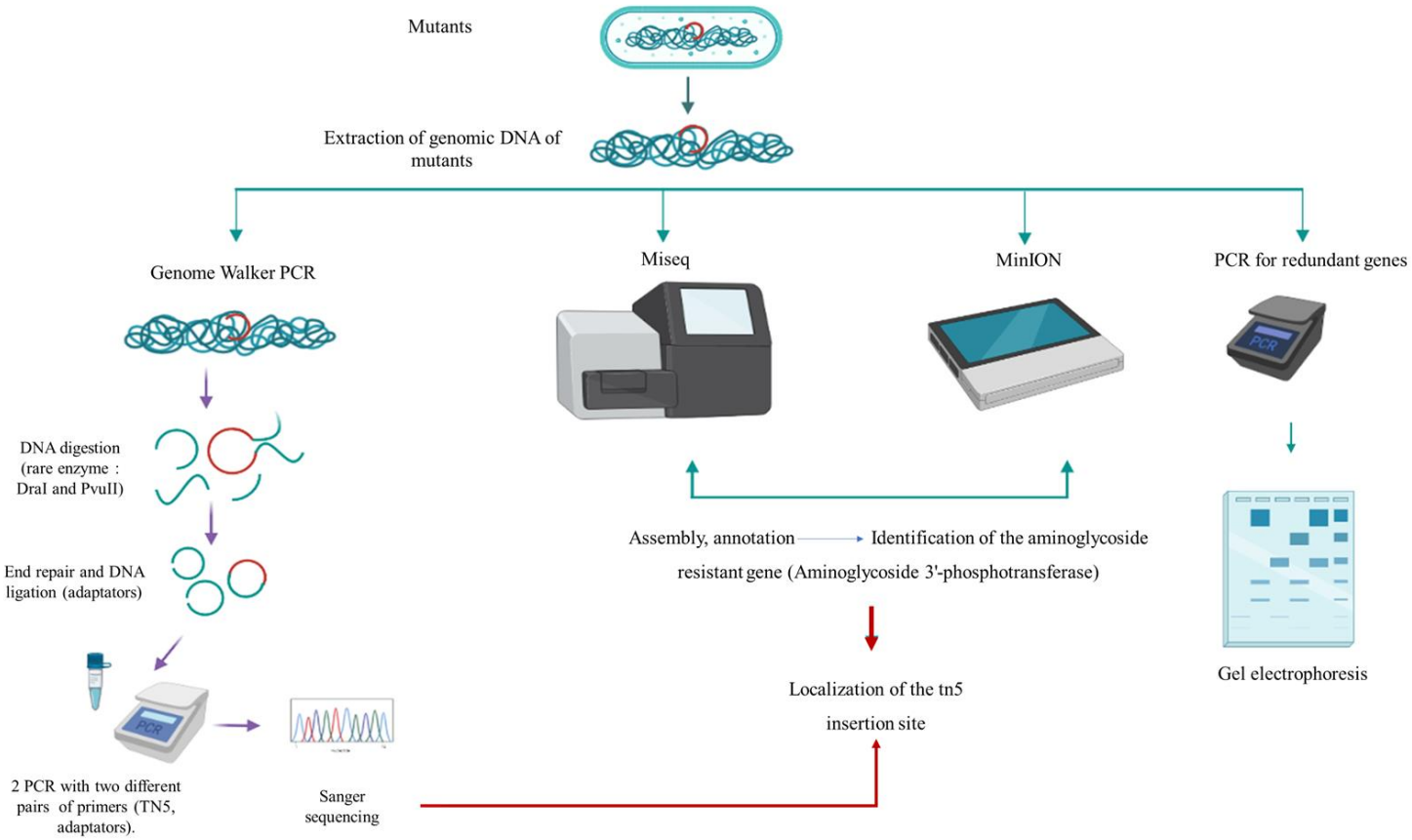


**Figure S1.** Schematic demonstration of the methodologies and technologies used for the identification of the Tn5 insertion site in the genomic sequence of *E. coli* mutants.

**Table S1.** Oligonucleotide primers of the Genome walker PCR amplification and redundant genes used in this study.

| Primer name | Sequence |
|---|---|
| Adaptor primer 1 | 5'-GTAATACGACTCACTATAGGGC-3' |
| Adaptor primer 2 | 5'- ACTATAGGGCACGCGTGGT-3' |
| R6KAN-2 Forward | 5'-ACCTACAACAAAGCTCTCATCAACC -3' |
| R6KAN-2 Reverse | 5'-CTACCCTGTGGAACACCTACATCT-3' |
| VgrG- Forward | TGTCTGCCAAATCCAACTCTC |
| VgrG- Reverse | ATGCACAGTGAGGTATTCCC |
| YhbX- Forward | CGATACCCGCGAGAAACTTT |
| YhbX- Reverse | AACTTAGTCGCCTCTTCCGG |
| EptC- Forward | CCAGACGCGATACAAAGGTC |
| EptC- Reverse | GTTCTAACACCCTCGTTTTCAAT |
| ClsA- Forward | GGCCTGCTCAAGTGTTTCAT |
| ClsA- Reverse | GCTGACCAGTGCTTTTCCC |
| YdjM- Forward | CAGTCATAGTGTTGGCGCAA |
| YdjM- Reverse | GTTACACCGTCACCACTTCC |
| GNAT- Forward | CACACACTAGCGGGCATTTT |
| GNAT- Reverse | GCTTTATAAGACGCTTGCGC |
| EspP- Forward | GGAGGCCACTAACAGCTCAT |
| EspP- Reverse | GAGCAACGGTCATAGCACATATA |
| EspC- Forward | ATACTCTTGGACGGCGCAAT |
| EspC- Reverse | ATCACACAGGCCCCTCTTC |

**Article 5:** Whole genome sequence analysis of truncated *mgrB phoP/phoQ* regulator genes in colistin sensitive *Klebsiella pneumoniae* isolates.

Mouna Hamel, Mariem Ben khedher, Andriamiharimamy Rajaonison, Sophie Alexandra Baron, Jean-Marc Rolain.

Draft manuscript.

**Résumé**

Chez *K. pneumoniae*, l'inactivation du gène *mgrB* se traduit par une résistance à la colistine. La principale altération du gène *mgrB* correspond à une inactivation par des insertions de séquences (Olaitan *et al.*, 2014; Mouna *et al.*, 2020), qui sont dans la plupart des cas associées à des concentrations minimales inhibitrices (CMI) très élevées (Poirel, Jayol and Nordmanna, 2017).

Lors du criblage des souches de *K. pneumoniae* de Grèce (Article 2, chapitre II), nous avons isolé 13 souches sensibles à la colistine présentant des CMI très faibles (< 2 µg/mL) malgré la présence de différentes insertions de séquences dans le gène régulateur *mgrB*. Nous avons ainsi cherché à comprendre l'impact de cette inactivation sur la régulation des systèmes à deux composants et sur l'ajout de sucres sur le lipide A et essayé de comprendre les mécanismes responsables de cette absence de résistance. Pour cela, nous avons réalisé de la microscopie électronique après coloration au rouge de ruthénium (pour visualiser les sucres de la paroi) sur nos souches anormalement sensibles à la colistine ainsi que sur des souches contrôles sensibles et résistantes par inactivation du gène *mgrB*. Nous avons ainsi constaté que l'épaisseur de la membrane externe (composée de sucres) était significativement plus faible chez les souches de *K. pneumoniae* anormalement sensibles à la colistine que chez les souches résistantes à la colistine par inactivation de *mgrB.* Le séquençage des génomes de ces souches atypiques a permis de confirmer l'inactivation des gènes *mgrB* dans les souches sensibles. Ces souches appartenaient à différents séquences types, confirmant que ce phénomène n'était pas spécifique à un clone particulier. Nous avons ensuite voulu mesurer l'impact de l'inactivation du gène *mgrB* sur la régulation des systèmes à deux composants *phoPQ*, *pmrAB* ainsi que sur l'opéron *arnBCADTEF* chez ces souches sensibles comparées aux souches résistantes présentant

également une inactivation du gène *mgrB* et à des souches sauvages. Nous avons donc évalué le niveau d'expression des systèmes à deux composants *phoPQ*, *pmrAB*, ainsi que du gène *arnT.* Nous avons ainsi montré qu'alors que l'inactivation du gène *mgrB* chez la souche résistante à la colistine entraine une surexpression des systèmes à deux composants et de l'opéron *arn* dans les souches résistantes à la colistine*,* ces gènes ont une expression similaire à celle du contrôle sensible dans les souches anormalement sensibles à la colistine. Cette observation est cohérente avec le phénotype observé par microscopie électronique, et explique l'absence d'épaississement de la membrane externe par l'activation de la synthèse de sucres. L'absence de surexpression du système à deux composants *phoPQ* suite à l'inactivation de son répresseur *mgrB* nous conduit à poser l'hypothèse qu'il existe dans le génome un autre système de régulation capable de réguler les gènes *phoPQ* et *pmrAB*. Ces gènes étant impliqués dans de nombreuses voies métaboliques, il est très probable que d'autres gènes interviennent dans la régulation de leur expression. Ces gènes pourraient également s'auto-réguler, mais l'avantage sélectif de ce phénomène est encore inconnu.

Des études de transcriptomique sont nécessaires pour nous permettre d'étudier l'expression des gènes potentiellement impliqués dans ce phénomène. Du fait de la pandémie de COVID-19, cette analyse n'a pour le moment par pu être réalisée. Les résultats préliminaires nous ont cependant permis de montrer que la résistance à la colistine était encore plus complexe que nous le pensions et nécessite encore d'être étudiée. L'isolement de ces souches nous montre également l'importance d'évaluer la sensibilité de la colistine par méthode phénotypique, les mécanismes génomiques étant actuellement trop complexe pour pouvoir prédire de la sensibilité ou de la résistance des souches.

**Title:** Whole genome sequence analysis of truncated *mgrB phoP/phoQ* regulator genes in colistin sensitive *Klebsiella pneumoniae* isolates.

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**Abstract**

**Background:** Colistin is used as a last-line therapy to combat multidrug-resistant (MDR) *Klebsiella pneumoniae*. Unfortunately, resistance to colistin has emerged with different mechanisms involved. Insertion sequence (IS) in the *mgrB* gene, encoding a negative-feedback regulator of the *PhoQ-PhoP* system, lead to colistin resistance in *K. pneumoniae* strains, due to the upregulation of the *Pmr* lipopolysaccharide modification system. Those ISs are often associated with high minimal inhibitory concentrations (MICs) against colistin. In this work we have isolated *K. pneumoniae* clinical strains with truncated *mgrB* gene that were unexpectedly susceptible to colistin. We aim to investigate the molecular mechanism associated with their atypical phenotype using whole genome sequencing and transcriptomic analyses.

**Methods:** A total of 13 colistin-susceptible *K. pneumoniae* strains carrying this particular phenotype were isolated between 2015 and 2018 from a previous work in Greece. Whole genome sequencing of the isolates was carried out, the morphology of bacteria was analysed with electron microscopy and finally explored using transcriptomic analyses and transposon mutagenesis.

**Results:** All isolated strains were sensitive to colistin at very low MIC ranging from 0.25 to 0.5 µg/mL. Genome-analysis confirmed the presence of insertion sequences in the *mgrB* gene, 4 strains harboured IS*Kpn14*-like element at nucleotide position 76 of *mgrB*, 6 with IS*Ec68* at position 7 and 3 IS*Kpn26* type at position 44. All strains were carbapenem-resistant associated with carbapenemase genes (*KPC-2* in five isolates and *NDM-1* in four isolates and four strains co-producing two type of carbapenemases). No change in the levels of expression of known regulatory genes associated with colistin resistance compared to the susceptible strain was observed, suggesting an alternative inactivator of the phoP/Q system.

**Conclusions:** The discovery of IS in the *mgrB* gene of susceptible strains questioned what has already been described on resistance to colistin likely suggesting the possibility of other complex dynamic pathways modulating the two-component systems. Further work are warranted to confirm this hypothesis.

## Introduction

*Klebsiella pneumoniae* is an opportunistic Gram-negative pathogen and one of the major causes of nosocomial infections such as pneumonia, urinary tract infections and bacteraemia, notably in intensive care units[1]. Recently, *K. pneumoniae* strains have rapidly become multidrug-resistant pathogens due to the acquisition of resistance to third generation cephalosporins, fluoroquinolones and aminoglycosides and they are becoming increasingly resistant to carbapenems through the acquisition of carbapenemases[2]. The latter has increased all over the world, especially in Greece, limiting the effectiveness of therapeutic regimens[3]. These constraints led to the revival of the use of older antibiotics such as colistin in the treatment of *K. pneumoniae* complex infections[4]. However, colistin resistance has emerged recently all over the world[5].

Colistin is a cationic lipopeptide antibiotic that acts on Gram-negative bacteria by electrostatic interactions with the anionic phosphate groups of the lipid A fraction of lipopolysaccharides (LPS)[5]. In *K. pneumoniae,* colistin resistance is mediated by modification of lipid A via mutations in the *pmrA*, *pmrB*, *phoP*, *phoQ* genes, which in turn upregulate both PhoP/PhoQ and PmrA/PmrB systems, leading to the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) or phosphoethanolamine (PEtN) to LPS, thus, reducing affinity for colistin[6]. Besides, the disruptions and mutations of the *mgrB* gene, a negative regulator of PhoP/PhoQ, has been associated with an increased alteration of LPS and colistin resistance[7]. Inactivation of the *mgrB* gene, a negative regulator of the PhoP/PhoQ two-component system, by insertion sequences (IS), is a particularly frequently observed colistin resistance mechanism[6], and these ISs are commonly associated with high minimum inhibitory concentrations (CMI) against colistin[8]. Deactivation of the *mgrB* gene seems to be the main mechanism of colistin resistance among those described so far,  we have reported *mgrB* insertional inactivation by various IS in 94 out of 209 colistin-resistant *K. pneumoniae* clinical isolates collected in

Greece[3]. Likewise, Cannatelli *et al.* observed that out of 35 colistin-resistant *K. pneumoniae*, 22 demonstrates an inactivation of *mgrB* gene by sequence insertion[9].

In a previous study, where colistin resistance and its molecular epidemiology were documented in a collection of 288 *K. pneumoniae* carbapenem resistant (CRPK) clinical isolates isolated in Greece, we have isolated 13 *K. pneumoniae* colistin-susceptible clinical strains with truncated *mgrB* gene that were unexpectedly susceptible to colistin. Therefore, the aim of this work was to characterize the colistin molecular mechanism of these *K. pneumoniae* colistin-susceptible clinical strains using whole genome sequencing and transposon mutagenesis.

## Materials and methods

### Bacterial Strains and antimicrobial susceptibility testing

In the period between 2015 and 2018, 13 *K. pneumoniae* colistin-susceptible clinical strains were isolated in a previous work from hospitals in Greece[3]. The strains were identified at the species level using Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry (Bruker Daltonik, Bremen, Germany). Antimicrobial susceptibility testing to 16 antibiotics was performed using the disk diffusion method and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Carbapenem (imipenem) and kanamycin minimum inhibitory concentrations (MIC) were performed by E-tests method (BioMérieux, Marcy-l'Etoile, France), and colistin MIC was determined by UMIC microdilution method (Biocentric, Bandol, France), the results were interpreted according to EUCAST breakpoints.

### Whole Genome sequencing

DNA of the 13 *K. pneumoniae* colistin-susceptible strains was extracted using a QIAamp DNA Mini Kit (Qiagen), DNA was quantified using the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific). Isolates were sequenced using the MiSeq sequencer (Illumina Inc., San Diego, CA, USA) with a 2x250 paired-end approach strategy using the Nextera102 Mate Pair sample prep kit (Illumina). The raw data were assembled denovo using the SPAdes pipeline[10], and then rearranged by mapping to a *K. pneumoniae* reference genome MGH78578 using MAUVE then annotated by PROKKA. The resistome of each strain was identified by abricate using the argannot database[11]. The *phoP/Q, pmrA/B, mgrB,* and *crrA/B* and operon arn genes (arnBCADTEF) were then compared to the reference genome. PROVEAN software was used to predict the impact of the mutations found on protein function. Multilocus sequence typing (MLST) analysis hosted by Centre for Genomic Epidemiology was used for further analysis (http://www.genomicepidemiology.org/). New ST were assigned using the MLST database (www.pasteur.fr/mlst/Kpneumoniae.html). ResFinder 2.1 was used to identify acquired antibiotic resistance genes, plasmid replicons were analysed using abricate with the PlasmidFinder. The pangenome analysis was performed using Roary using the *K. pneumoniae* genome sequences annotated by Prokka and the *K. pneumoniae* reference genome MGH78578. The *K. pneumoniae* genomes (complete and WGS) were retrieved from the NCBI database for further analysis.

**Sequence insertion analysis**

Sequence insertions on the *mgrB* gene in the 13 colistin-sensitive *K. pneumoniae* strains were initially identified by PCR and sanger sequencing. These insertion sequences and the genome assemblies were then analysed using the ISfinder database to determine the type of IS elements present.

## Transmission Electron Microscopy

A staining based on ruthenium red was carried out using the Luft method[12]. The analysis involved five susceptible *K. pneumoniae* strains with sequence insertion in *mgrB* gene, one susceptible *K. pneumoniae* and two resistant *K. pneumoniae* strains were used as controls for manipulation. The bacterial pellet was resuspended in a mixture of 1.2% glutaraldehyde and 0.05% ruthenium red in 0.1 M cacodylate buffer and fixed for 1 hour at 4°C under gentle agitation. Bacteria were ultracentrifuged and then fixed for 3 h at 4°C in a mixture of 1.2% glutaraldehyde/0.05% ruthenium red in 0.1 M cacodylate buffer, followed by three 10 min washes with 0.1 M cacodylate buffer at 4°C. The bacteria were then washed with 0.1 M cacodylate/0.2 M sucrose solution twice for 15 min, and then dehydrated with 50, 70 and 96% ethanol for 15, 30 and 30 min, respectively. Bacteria were placed for 1 h in a mixture of 100% LR-White resin and 96% ethanol in a 2:1 ratio. After 30 min were placed in 100% LR-White resin overnight at room temperature. The bacteria were then immersed in 100% resin for 1 h at room temperature. Finally, 1.5 mL of pure 100% LR-White resin was added to the pellet and polymerisation was performed at 60°C for 3 days. Ultrathin sections were cut on a UC7 ultramicrotome (Leica), and subsequently deposited on 300 mesh copper/rhodium grids (Maxtaform HR25, TAAB). They were then treated with 5% uranyl acetate and lead citrate according to the Reynolds method[13]. Observation was done using the Tecnai G2 transmission electron microscope at a voltage of 200 keV and with an eagle camera (FEI) of 4096 × 4096 pixel resolution, then images were segmented and thresholded using OpenCV libraries, then the density of pixels were evaluated with pixel intensity. The statistical analyses were performed using the Kruskal-Wallis test.

## Transposon mutagenesis library generation

The transposon mutagenesis library of one of the thirteen colistin-sensitive *K. pneumoniae* strains (kanamycin sensitive) was carried by electroporation of the EZ-Tn5™ <R6Kγori/KAN-2> Tnp kit Transposome™ (Epicenter, Tebu-bio, Le Perray-en-Yvelines, France) as described[14]. Briefly, 1 µL of EZ-Tn5 was used for electroporation and the bacteria were placed on LB agar plates containing 50 mg/L of kanamycin. Thereafter, the transposon library was plated onto two different LB agar plates, one containing kanamycin (50 mg/L) and colistin (2 mg/L) and the other containing kanamycin (50 mg/L). 24 h later, clones that had not grown on kanamycin + colistin agar. The transposon library was stored as 30% glycerol stocks at -80°C. The identification of the transposon mutants was performed by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry (Bruker Daltonik, Bremen, Germany). Colistin MIC was performed by the E-test method (BioMérieux, France), the results were interpreted according to EUCAST breakpoints (susceptible, ≤2 µg/ml and resistant, >2 µg/ml). Transposon mutant with increase of colistin resistance was subjected to walker genome PCR to determine the site of insertion of the Tn5 transposon as previously described using the primers listed in the table S1[14].

**RNA extraction and qPCR**

The gene expression levels of *phoP, phoQ, pmrA, pmrB, arnT, eptB,* and *TssF* were determined by quantitative PCR (qPCR) using the primers listed in the table S1. qRT-PCR was performed for each isolate, one susceptible and one colistin-resistant *K. pneumoniae* isolates were also included. Briefly, the isolates were grown in Luria bertani (LB) media with colistin, and total RNA was extracted from bacterial cultures when optical density at 600 nm (OD600=0.19) using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, The RNA was quantified with a NanoDrop, all the RNAs were calibrated at a concentration of 500 ng/µL. Transcription levels were normalized to the

maintenance gene expression level, *rpoB*. The threshold cycle (CT) method was used to calculate the fold changes in expression, qPCR was performed in triplicate.

## Results

### Bacterial Strains and antimicrobial susceptibility testing

The 13 *K. pneumoniae* colistin susceptible strains used in this study were identified as *K. pneumoniae* using MALDI-TOF with a score ≥ 1.9. Among these isolates, eleven were isolated from blood, one from urine and one from inflammatory exudate. All isolates exhibited a multi-drug resistance profile, with a high resistance level (100%) for eleven antibiotics including penicillin, cephalosporin, carbapenems, quinolones, furanes, sulfamides, and phosphonic acids. (77%, 10/13) were resistant to gentamicin and (69, 9/13) to amikacin, (53%, 7/13) were resistant to doxycycline. All the *K. pneumoniae* strains were sensitive to colistin with an MIC ranging from 0.25 to 0.5µg/mL (Figure 1).

### Electron microscopy staining

The first feature observed was the thickness of the outer membrane (polysaccharides), which in contrast to the two resistant strains, was less intense and dispersed in the colistin-sensitive strains with IS in the *mgrB* gene (Figure 2). For this purpose, the microscopic images were segmented, and the intensity of the pixels was evaluated. A total of 5 measurements were made for each strain. The non-parametric test of the mean shows a significant difference per phenotype (Kruskal-Wallis chi-squared = 27.338, df = 2, p-value = 1.158e-06) (Figure 3).

### Whole genome sequence analysis

Genomes sizes ranged from 5.4 Mb to 6.7 Mb with 168 a GC% ranging from 55.8 to 56.3%. Genomes were assembled in 5 to 89 contigs with a raw coverage of 21-42. Out of the 13 *K.*

*pneumoniae* strains, five belonged to the ST39, five ST11, two ST147, and a newly assigned ST corresponding to 5552. The main features of the different genomes are reported in table 1. The pangenome analysis of the 13 *K. pneumoniae* strains shows a total of 8677 cluster genes distributed as follows: (Core genes = 3956), (Shell genes = 2788) and (Cloud genes = 1933), respectively (Figure 4).

Notably, all *K. pneumoniae* strains were carbapenemase-producers, five strains were KPC-2 producers, four were NDM-1, and 4 out of the 13 strains exhibited the coexistence of two carbapenemases (KPC-2, VIM-1 / NDM-1, KPC-11 / NDM-1, VIM-12 /NDM-1, OXA-48) (table 2). Characterisation of in the *mgrB* gene revealed the presence of different insertion elements (IS) in Col-S strains, et different positions. Most strains (n = 6/13) had IS*Ec68* at position 75 with a size of 891 bp. Four isolates had IS*Kpn14*-like element at nucleotide position 76 and with a size of 779pb and the three other strains had an insertion element of IS*Kpn26* type at position 44 and with a size of 1184 bp (Figure 5).

Analysis of the different regulatory associated with colistin resistance genes revealed the presence of one mutation in the *pmrA* gene for one strain, which was predicted to be neutral. The *pmrB* gene carried the same set of mutations, the T246A/R256G mutations that were present in 9 out of 13 strains. Only one strain carried a mutation in the *phoP* gene, the *phoQ* gene was intact in 3 out of 13 strains. The *crrA*, *crrB* and *crrC* genes were absent in 3 strains, *crrA/C* was intact in 10 other strains. However, *crrB* carried the same C68S mutation in the other 10 genomes (table 2). Four of the genes comprising the arn operon carried mutations, the neutral mutation D112A was present in the *arnB* gene in 13 strains, 4 strains carried the same set of mutations (A18S, D205N, L260I) and (V53I, I94L, I300V) on the *arnA* and *arnB* gene, respectively (Figure S1). No *mgrB* gene duplicate was found.

**Random mutagenesis and qPCR**

After the screening of 500 colonies of the Tn5 mutagenesis library of the colistin-sensitive *K. pneumoniae* strain, one colistin resistant clone (MIC=16mg/L) was obtained and identified by MALDI-TOF with a score of ≥ 1.9. After the extraction of the genomic DNA the transposon insertion site was identified by PCR Genome Walker and sequencing. Sequence analysis revealed the disruption of the TssF gene belonging to the type VI secretion system, the genetic environment of this gene is illustrated in Figure 6. To determine the frequency of this gene and its cluster in *K. pneumoniae* genomes, a blastN was performed against the 7365 *K. pneumoniae* genomes in the NCBI database, a total of 3706 genomes with a coverage ≥ 96% and an identity of ≥ 97% carried this cluster. Transcriptomic analysis of *phoP/Q, pmrA/B, arnT, eptB, TssF* was done to evaluate the expression levels of these genes. No change in the levels of expression of the different genes compared to the susceptible strain was observed for the sensitive strain with IS in *mgrB* gene, conversely those genes were upregulated in the control strain resistant to colistin (Figure 7).

## Discussion

A strong association between colistin resistance in *K. pneumoniae* strains and chromosomal genetic alterations of *mgrB* (disruptions and mutations) have been described[7]. It is now well established that inactivation of the *mgrB* gene, encoding a negative regulator of the PhoQ/PhoP system, can explain the acquired resistance to colistin in *K. pneumoniae* strains through the activation of the Pmr system which modulates the lipopolysaccharide polymyxin target[15]. Reports of this mechanism in *K. pneumoniae* strains are increasing, including our previous study in Greece which found that *mgrB* inactivation was the most common mechanism of colistin resistance and considered this phenomenon an emerging epidemic[3,16].

Sequence insertions have been reported to be strongly associated with resistance to colistin in *K. pneumoniae*[17–19]. The inactivation of *mgrB* by insertion sequences, particularly by IS5-like

elements, has emerged as the most common mechanism of colistin resistance in *K. pneumoniae* strains[9]. In this work, we investigated 13 colistin-sensitive *K. pneumoniae* strains with an *mgrB* gene truncated by sequence insertions, 6 has an IS*Ec68* element, 3 strains had an insertion element of IS*Kpn26* type, both belonging to the IS5 family and 4 isolates had IS*Kpn14* element, belonging to the IS1 family. These *K. pneumoniae* strains belonged to different sequence types, confirming that this phenomenon was not specific to a particular clone. The random mutagenesis approach demonstrated that inactivation of the *TssF* gene belonging to the type VI secretion system results in colistin-resistant mutant of *K. pneumoniae.* Indeed, the two-component PhoPQ system regulates the activation of *K. pneumoniae* T6SS in the context of bacterial competition. Storey et al, demonstrated that PhoPQ activation results in the upregulation of the T6SS system in *K. pneumoniae* and also revealed that the *mgrB* gene is a negative regulator of T6SS[20], which is particularly critical since the great majority of *K. pneumoniae* isolates resistant to the last-line antibiotic colistin display loss of function in *mgrB*[20]. The inactivation of the *mgrB* gene in the colistin-resistant strains leads to overexpression of the two-component systems (PhoPQ, PmrAB) and the arn operon[4], assessment of the expression levels of these two components and arn operon in the abnormally colistin-susceptible strains revealed similar expression levels to those in the colistin-sensitive strains. The existence of this paradoxical situation in our study suggest the presence of another negative regulatory system for outer membrane polysaccharides synthesis.

The investigation of other negative regulatory genes responsible for the maintenance of colistin sensitivity is necessary. he complexity of colistin resistance mechanisms in *K. pneumoniae* has been highlighted in our research and pointed towards potential problems arising from the use of genome-based interrogation alone for the detection of colistin resistance, and the importance of assessing colistin sensitivity by phenotypic methods.

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## Figure Legends

**Figure 1.** Hierarchical clustering of antibiotic susceptibility profiles representing the activity of the 16 antibiotics tested against the 13 colistin sensitive *K. pneumoniae* strains isolated in this study. AMX: Amoxicillin; AMC: Amoxicillin + clavulanic acid; FEP: Cefepime; TPZ: Piperacillin + Tazobactam; CRO: Ceftriaxone; CEF: Cefalotin; ERT: Ertapenem; IMP: Imipenem; CIP: Ciprofloxacin; AMK: Amikacin; GEN: Gentamicin; DOX: Doxycycline; CST: Colistin; FOF: Fosfomycin; SXT: Trimethoprim / sulfamethoxazole; NIT: Nitrofurantoin.

**Figure 2.** Electronic microscopy of the colistin susceptible *K. pneumoniae* strains with IS in *mgrB* gene, susceptible and resistant to colistin. (A): colistin resistant strains with IS in *mgrB* gene (control), (B): colistin sensitive strain, (C and D): colistin sensitive strain with IS in *mgrB* gene.

**Figure 3.** Box plot showing the median distribution of the proportion of white pixels per phenotype, the bold horizontal lines represent the median. The analysis shows a statistically significant difference (P = 1.158e-06) between the different phenotypes studied (resistant *K. pneumoniae*, susceptible *K. pneumoniae*, susceptible *K. pneumoniae* with insertion sequences in the *mgrB* gene), R: resistant, S: sensitive, SIS: sensitive with IS, proportion: number of white pixels/total number of pixels.

**Figure 4.** Visualization of pan-genome analysis by Roary of 13 colistin-susceptible *K. pneumoniae* strains, the reference genome of *K. pneumoniae* MGH78578 is added to analysis. Year of isolation is indicated.

**Figure 5.** Chromosomal location and genetic environment of the truncated *mgrB* gene and the three different types of IS found in the colistin susceptible *K. pneumoniae* strains, using the EasyFig software. The arrows indicate the positions and directions of the ORFs, direction indicate the gene orientation.

**Figure 6.** Genetic environment of the TssF gene belonging to the type VI secretion system, disrupted by the Tn5 transposon, effectively restore the resistance profile of colistin-sensitive *K. pneumoniae* strains.

**Figure 7.** Transcriptional analysis and expression level of the different regulatory genes of colistin resistance, calculated by the ΔΔCT, the result is considered as significant when the ΔΔCT is > 2.

**Table Legends**

**Table 1.** Main features of the different genomes of the 13 *K. pneumoniae* colistin susceptible strains after assembly.

**Table 2.** Resistome, and mutations found in the different colistin resistance regulatory genes in the 13 *K. pneumoniae* colistin susceptible strains.

**Supplementary Data**

**Table S1.** Oligonucleotide primers of the Genome walker PCR amplification and qPCR for gene expression levels used in this study.

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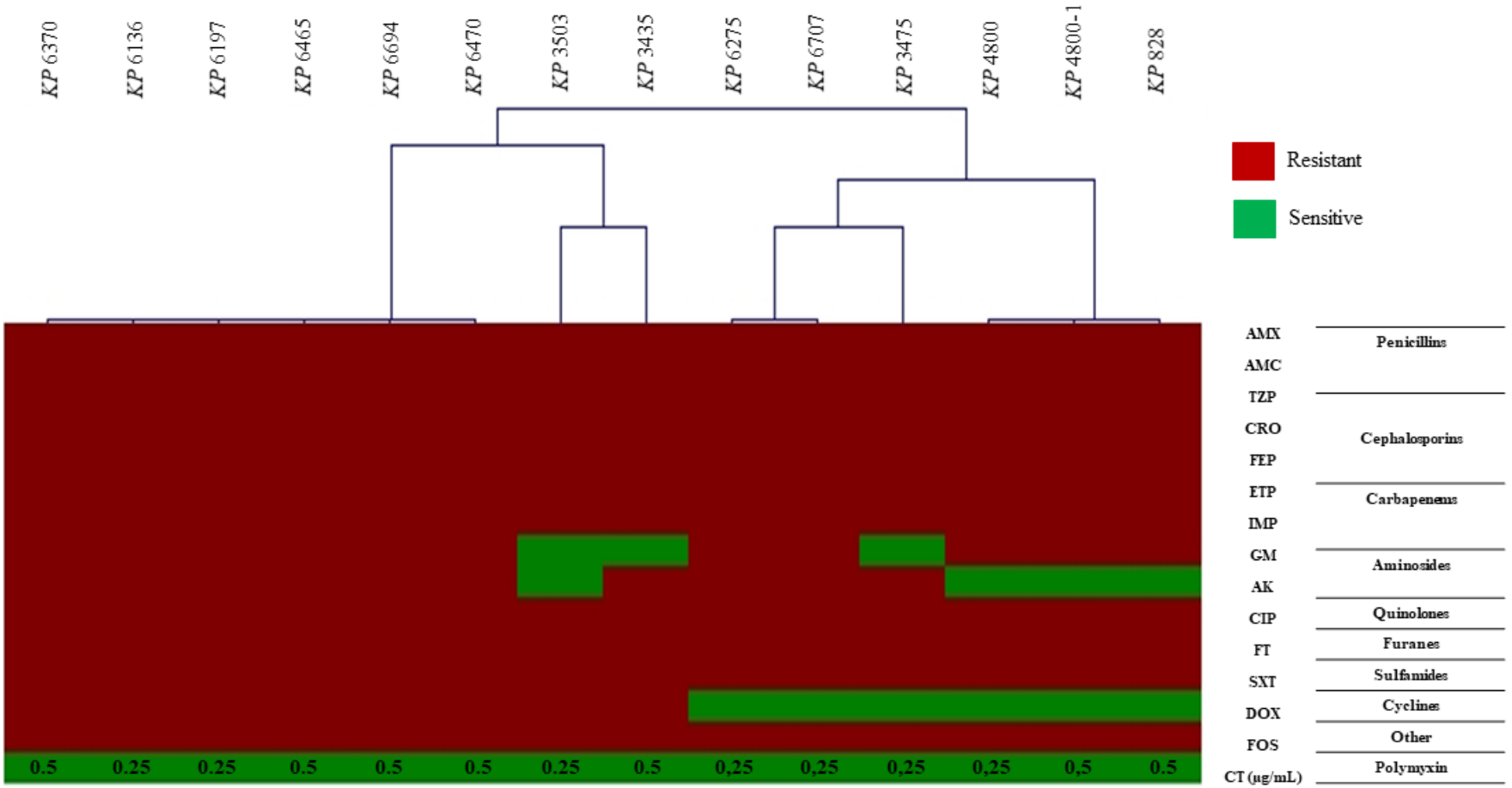


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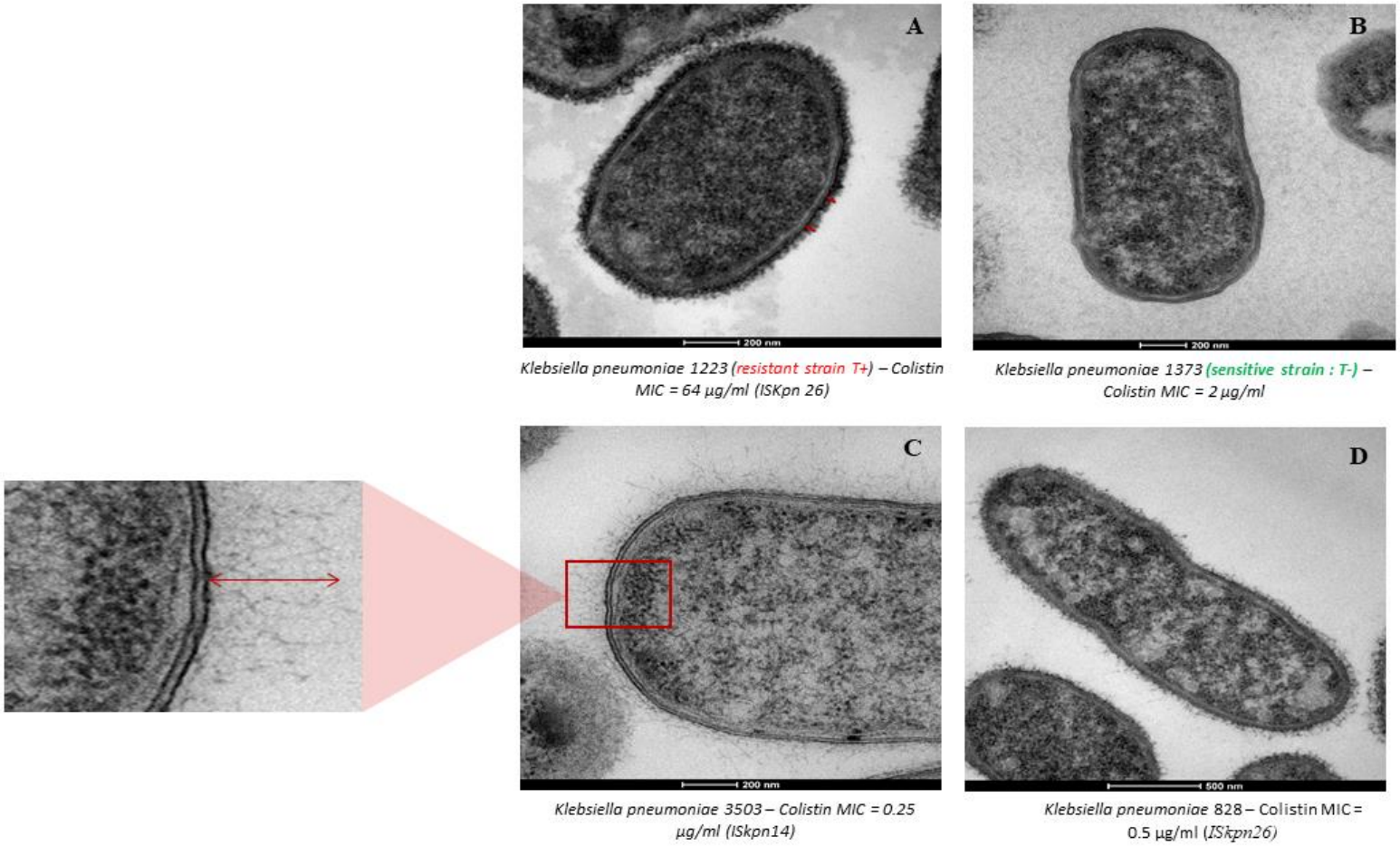


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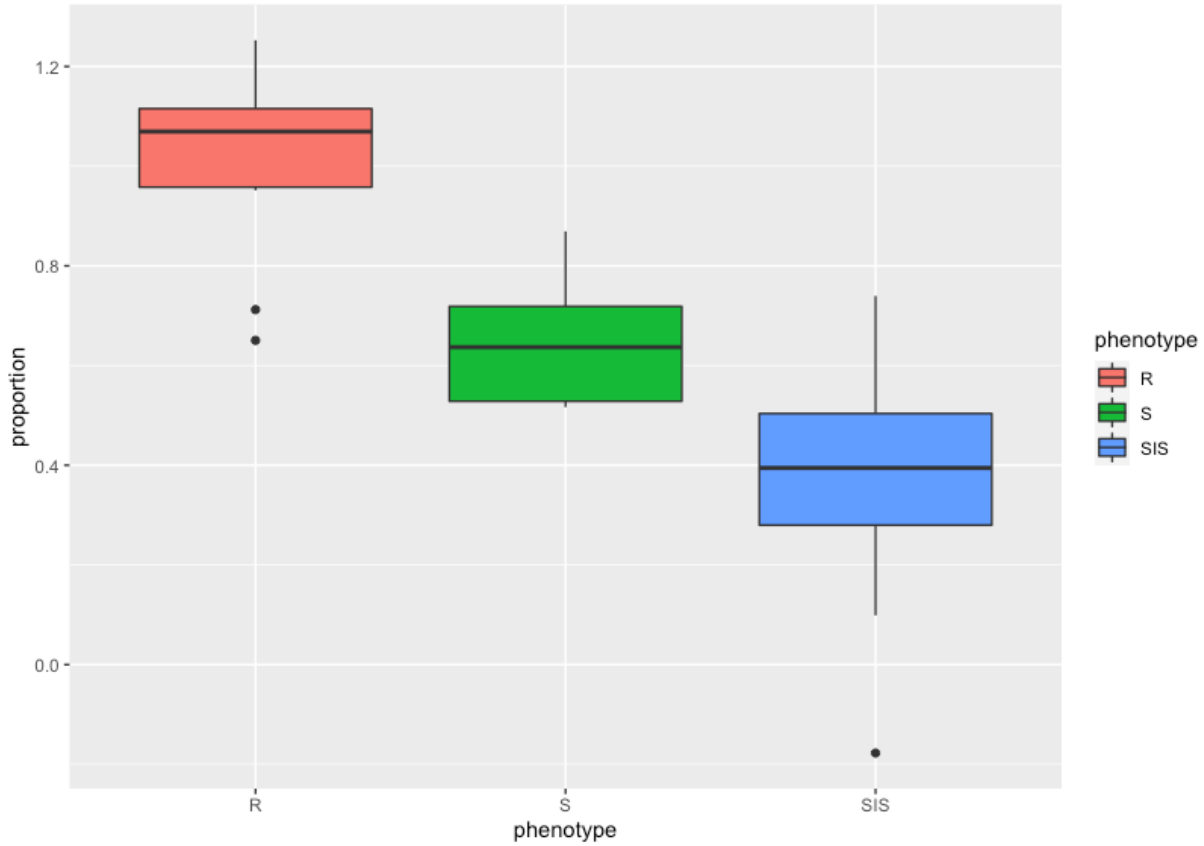


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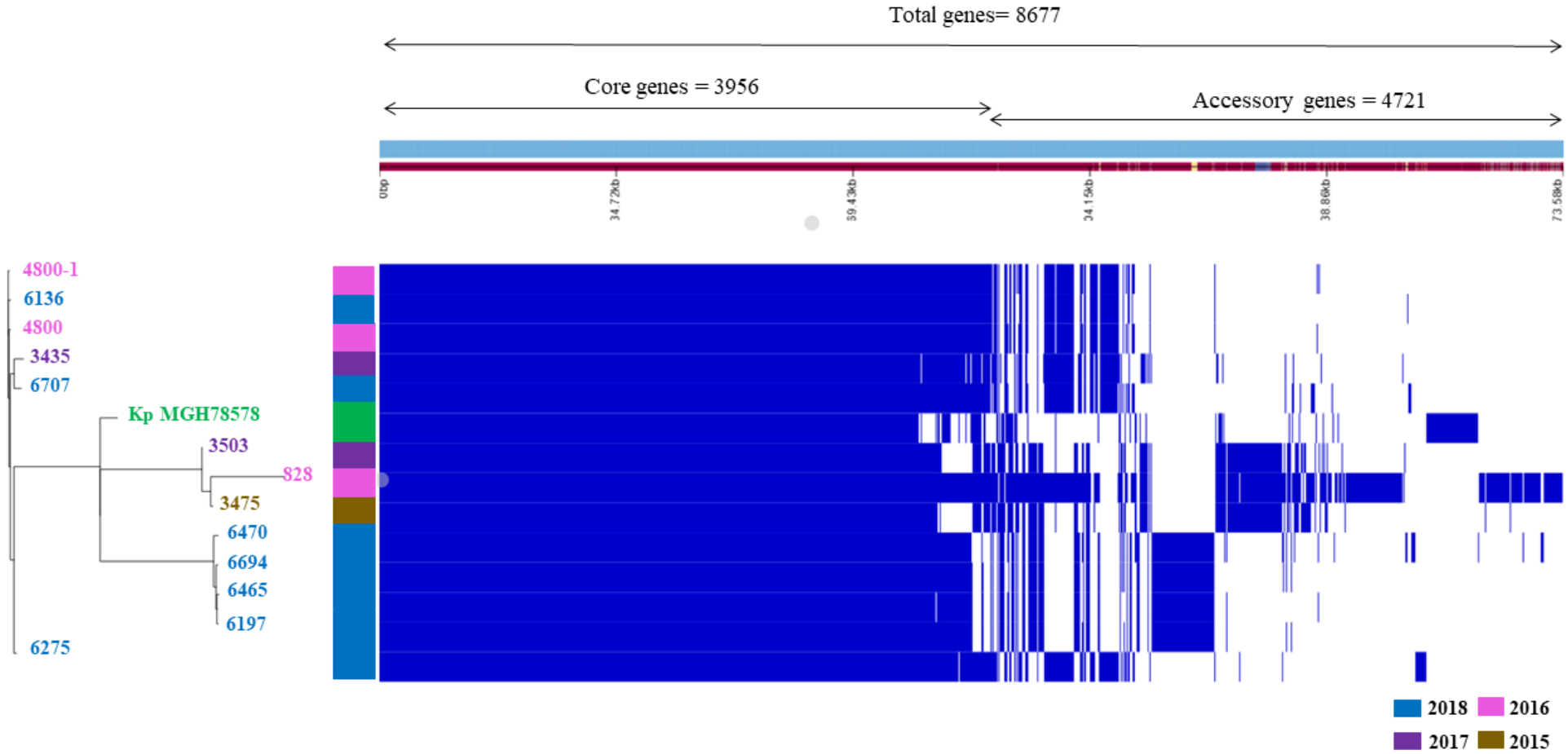


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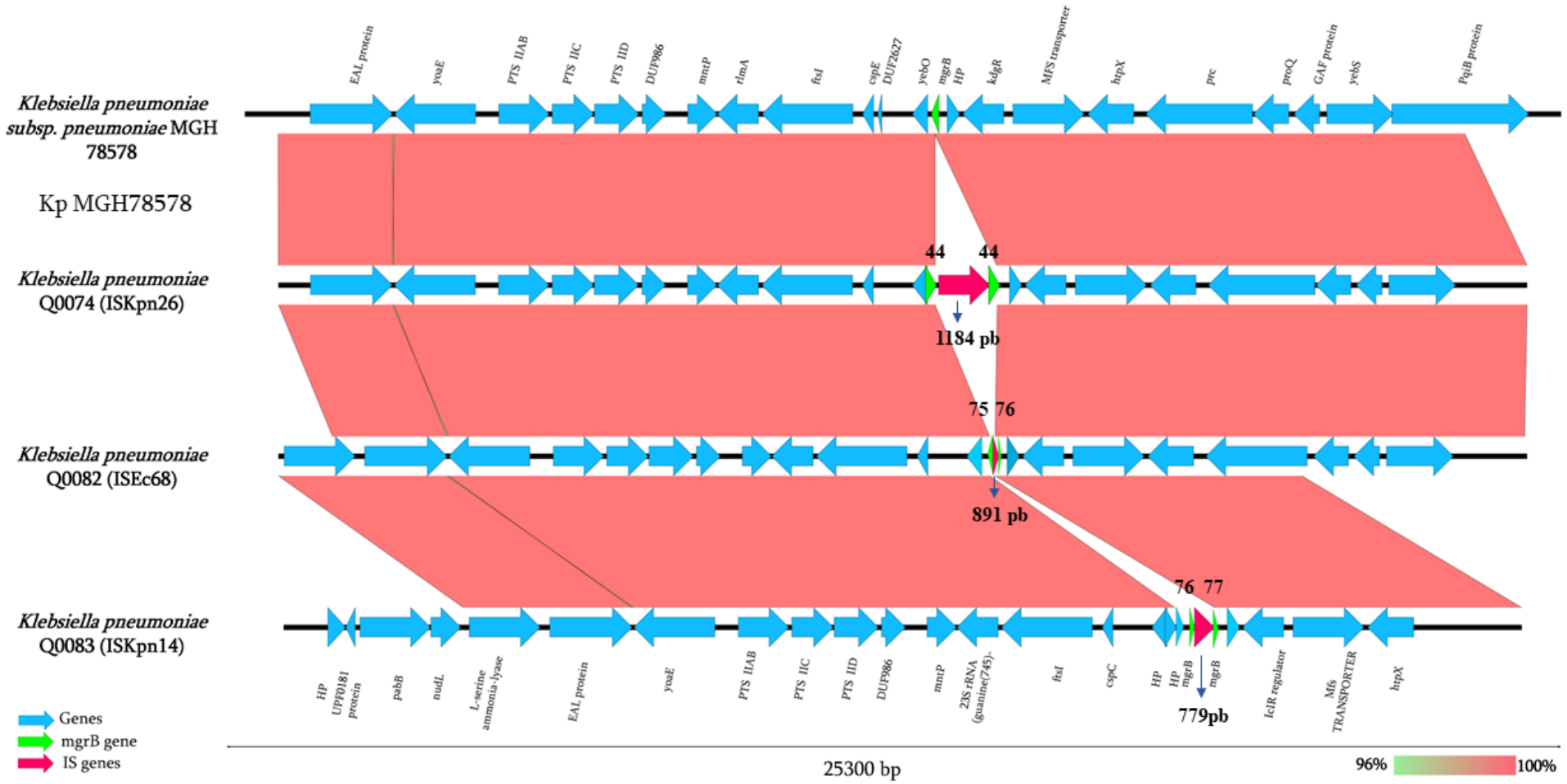


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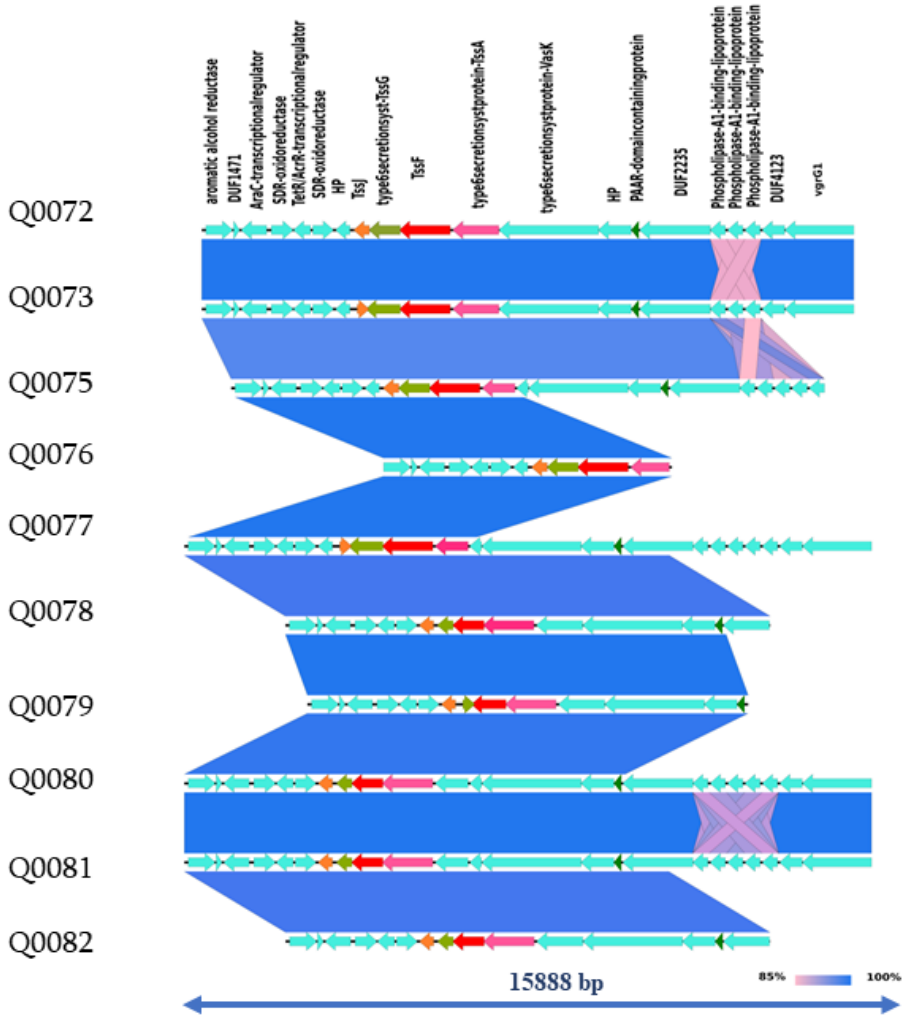


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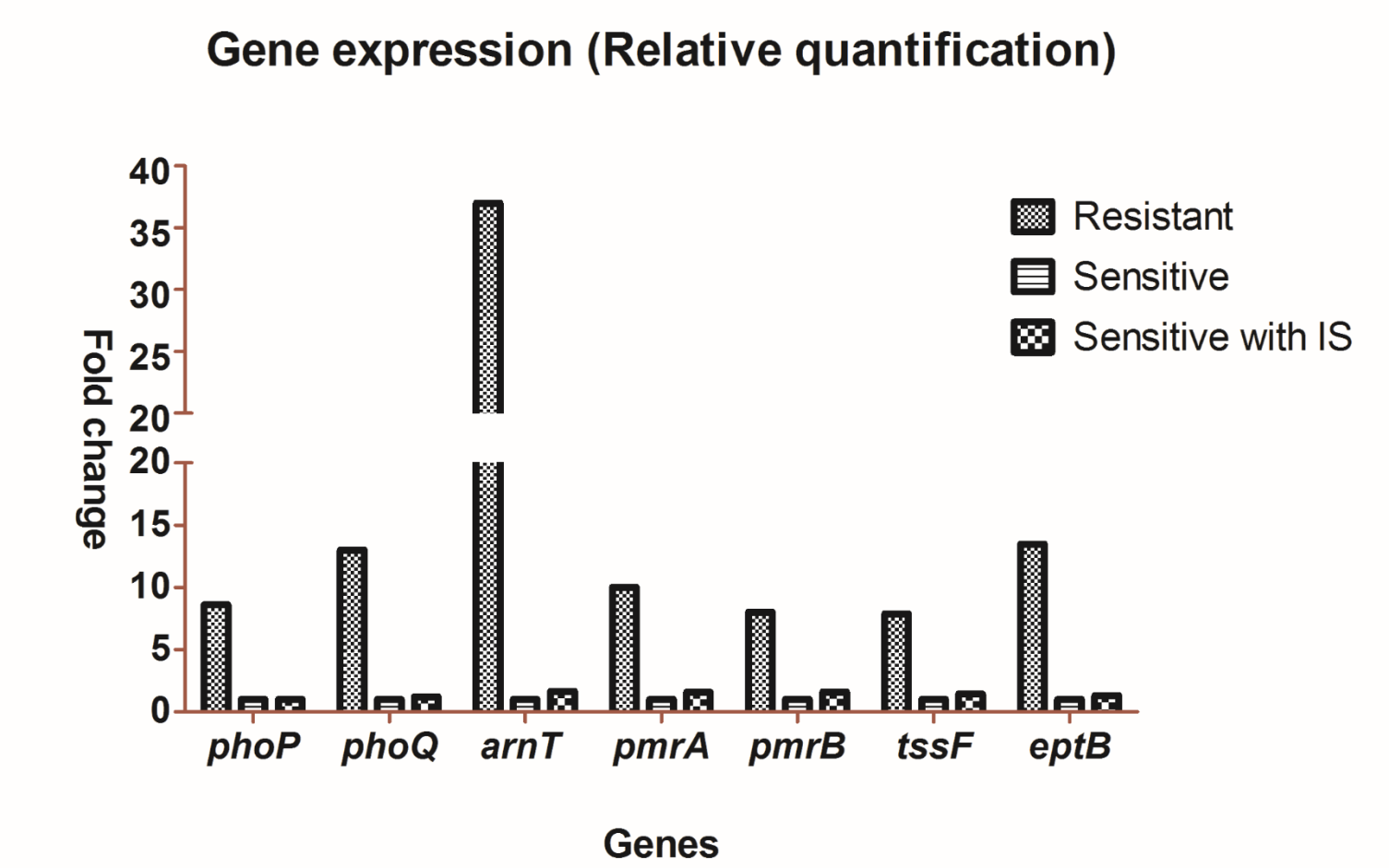


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**Figure 7.** Transcriptional analysis and expression level of the different regulatory genes of colistin resistance, calculated by the ΔΔCT, the result is considered as significant when the ΔΔCT is > 2.

**Table 1.** Main features of the different genomes of the 13 *K. pneumoniae* colistin susceptible strains after assembly.

| Strains | Species | Q Number | Source | Year of isolation | Contigs Number | Genome size | GC% | Cover | Raw Reads (Illumina) | ST |
|---|---|---|---|---|---|---|---|---|---|---|
| 3503 | *K. pneumonaie* | Q0072 | Inflammatory exudate | 2017 | 47 | 5.6 | 56.9 | 37 | 458859 | 147 |
| 3475 | *K. pneumonaie* | Q0073 | Blood | 2015 | 5 | 5.8 | 56.7 | 21 | 176279 | 147 |
| 828 | *K. pneumonaie* | Q0074 | Blood | 2017 | 44 | 6.7 | 56.3 | 30 | 35029 | 39 |
| 4800 | *K. pneumonaie* | Q0075 | Blood | 2016 | 75 | 5.4 | 57.3 | 43 | 647522 | 11 |
| 3435 | *K. pneumonaie* | Q0076 | Urine | 2017 | 89 | 6.3 | 56.4 | 29 | 779988 | 11 |
| 4800-1 | *K. pneumonaie* | Q0077 | Blood | 2016 | 77 | 5.4 | 57.3 | 29 | 352704 | 11 |
| 6465 | *K. pneumonaie* | Q0078 | Blood | 2018 | 81 | 5.6 | 57.1 | 42 | 552281 | 39 |
| 6694 | *K. pneumonaie* | Q0079 | Blood | 2018 | 85 | 5.6 | 57.1 | 31 | 399248 | 39 |
| 6136 | *K. pneumonaie* | Q0080 | Blood | 2018 | 78 | 5.4 | 57.3 | 32 | 380345 | 11 |
| 6197 | *K. pneumonaie* | Q0081 | Blood | 2018 | 86 | 5.6 | 57.4 | 46 | 462231 | 39 |
| 6707 | *K. pneumonaie* | Q0082 | Blood | 2018 | 73 | 5.4 | 57.2 | 27 | 331872 | 11 |
| 6470 | *K. pneumonaie* | Q0083 | Blood | 2018 | 5 | 6 | 55.8 | 24 | 661406 | 39 |
| 6275 | *K. pneumonaie* | Q0084 | Blood | 2018 | 87 | 5.6 | 57.0 | 21 | 135394 | 5552 (new ST) |

402 **Table 2.** Resistome, and mutations found in the different colistin resistance regulatory genes in the 13 *K. pneumoniae* colistin susceptible strains.

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| | | Mutations in regulator genes | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Strains** | **Antibiotic resistance profile** | ***pmrA*** | ***pmrB*** | ***phoP*** | ***phoQ*** | ***CrrA*** | ***CrrB*** | ***Crrc*** | **Plasmid** |
| 3503 | **KPC-2,** OXA-9, SHV-11, TEM-1A, fosA5, oqxA, oqxB | Intact | T246A / R256G | Intact | N378S | Intact | C68S | Intact | IncFIB(pKPHS1) / IncFIB(pQil) |
| 3475 | aac(6')-Il, ant(3'')-Ia, aph(3')-Ia, aph(6)-Id, OXA-9, **KPC-2**, SHV-11, TEM-1A, **VIM-1**, dfrA1, fosA5, mph(A), oqxA, oqxB, strA, sul1 | Intact | T246A / R256G | V90E | Intact | Intact | C68S | Intact | IncFIB(pKPHS1), IncFIB(pQil), IncR |
| 828 | fosA6, fosA5, **KPC-2**, oqxB, oqxA, aph(3'')-Ib, aph(6)-Id, aph(3')-Ia, sul1, dfrA1, aac(6')-Il, **VIM-1**, mph(A), SHV-182, aac(6')-Ib, aph(3')-Ia, mph(A), aadA2, dfrA12, catA1, aac(6')-Ib, TEM-1A, OXA-9. | Intact | T246A / R256G/T140P | Intact | G39S | Intact | C68S | Intact | ColRNAI, IncFIB(K), IncFIB(pKPHS1), IncFIB(pQil), IncR, IncX3 |
| 4800 | SHV-182, oqxB, oqxA, **NDM-1**, blaTEM-1B, aph(6)-Id, aph(3'')-Ib, sul2, dfrA14, blaCTX-M-15, aac(3)-IIa, OXA-1, fosA6. | Intact | T246A / R256G/T140P/ G347R | Intact | Intact | Intact | C68S | Intact | IncFIA(HI1) |
| 3435 | oqxA, oqxB, **NDM-1**, fosA, blaSHV-182, sul2, aph(3'')-Ib, aph(6)-Id, TEM-1B, OXA-1, aac(6')-Ib-cr, dfrA14. | Intact | T246A / R256G/T140P/ G347R | Intact | Intact | Intact | C68S | Intact | IncFIA(HI1), IncFIB(pKPHS1), IncFIB(pQil), IncR |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 4800-1 | OqxB, OqxA, **NDM-1**, TEM-1B, aph(6)-Id, aph(3")-Ib, sul2, dfrA14, blaCTX-M-15, aac(3)-Iia, fosA6, aac(6')-Ib, blaOXA-1, blaSHV-182. | Intact | T246A / R256G/T140P/ G347R | Intact | G39S | Intact | C68S | Intact | IncFIA(HI1) |
| 6465 | OqxA, OqxB, **KPC-2**, fosA3, sul2, dfrA12, aadA2, cmlA1, ant(3")-Ia, tet(M), sul3, aac(3)-IId, SHV-187, aph(3')-Ia, aac(6')-Ib. | M66I | T246A | Intact | P369T/Q 405L | - | - | - | ColRNAI, IncFIB(pQil) |
| 6694 | OqxA, OqxB, SHV-187, **KPC-2**, TetM, fosA3, ant(3")-Ia, cmlA1, aadA2, dfrA12, sul2, sul3, aac(3)-IId, aph(3')-Ia, aac(6')-Ib. | Intact | T246A | Intact | P369T/Q 405L | - | - | - | ColRNAI, IncFIB(pQil) |
| 6136 | OqxB, OqxA, AmpH, SHV-11, **NDM-1**, Aac6-Ib, TEM-105, StrB, StrA, SulII, CTX-M-15, Aac3-Iia, Aac6Ib-cr, OXA-1, CatB4, fosA6 | Intact | T246A / R256G/T140P/ G347R | Intact | G39S | Intact | C68S | Intact | IncFIA(HI1) |
| 6197 | oqxA, oqxB, **KPC-2**, fosA3, sul2, dfrA12, aadA2, cmlA1, ant(3")-Ia, tet(M)_8, sul3, aac(3)-Iid, aph(3')-Ia, aac(6')-Ib, TEM-1B, SHV-187 | Intact | T246A | Intact | P369T/Q 405L | - | - | - | ColRNAI, IncFIB(pQil) |
| 6707 | OqxB, OqxA, AmpH, SHV-11, **NDM-1**, Aac6-Ib, TEM-105, StrB, StrA, SulII, CTX-M-15, Aac3-Iia, Aac6Ib-cr, OXA-1, CatB4, fosA6. | Intact | T246A / R256G/T140P/ G347R | Intact | G39S | Intact | C68S | Intact | IncFIA(HI1) |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 6470 | OqxA, OqxB, SHV-40, AmpH, **KPC-2,** SulII, DfrA12, AadA2, CmlA1, AadA1-pm, TetM, SulIII, Aac3-Iid, Aph3-Ia, Aac6-Ib, Aac6-Iy, TEM-105, fosA3 | M66I | T246A | Intact | P369T | - | - | - | ColRNAI, IncFIB(S), IncFIB(pQil) |
| 6275 | aph(3')-Ia, aac(6')-Ib, oqxB, oqxA, SHV-182, **OXA-48, NDM-1,** dfrA14, sul2, aph(3")-Ib, aph(6)-Id, aac(3)-Iia, aac(6')-Ib, OXA-1, CTX-M-15, fosA6, TEM-1B | Intact | T246A / R256G/T140P/ G347R | Intact | G39S | Intact | C68S | Intact | ColRNAI, IncFIA(HI1), IncL/M(pOXA-48) |

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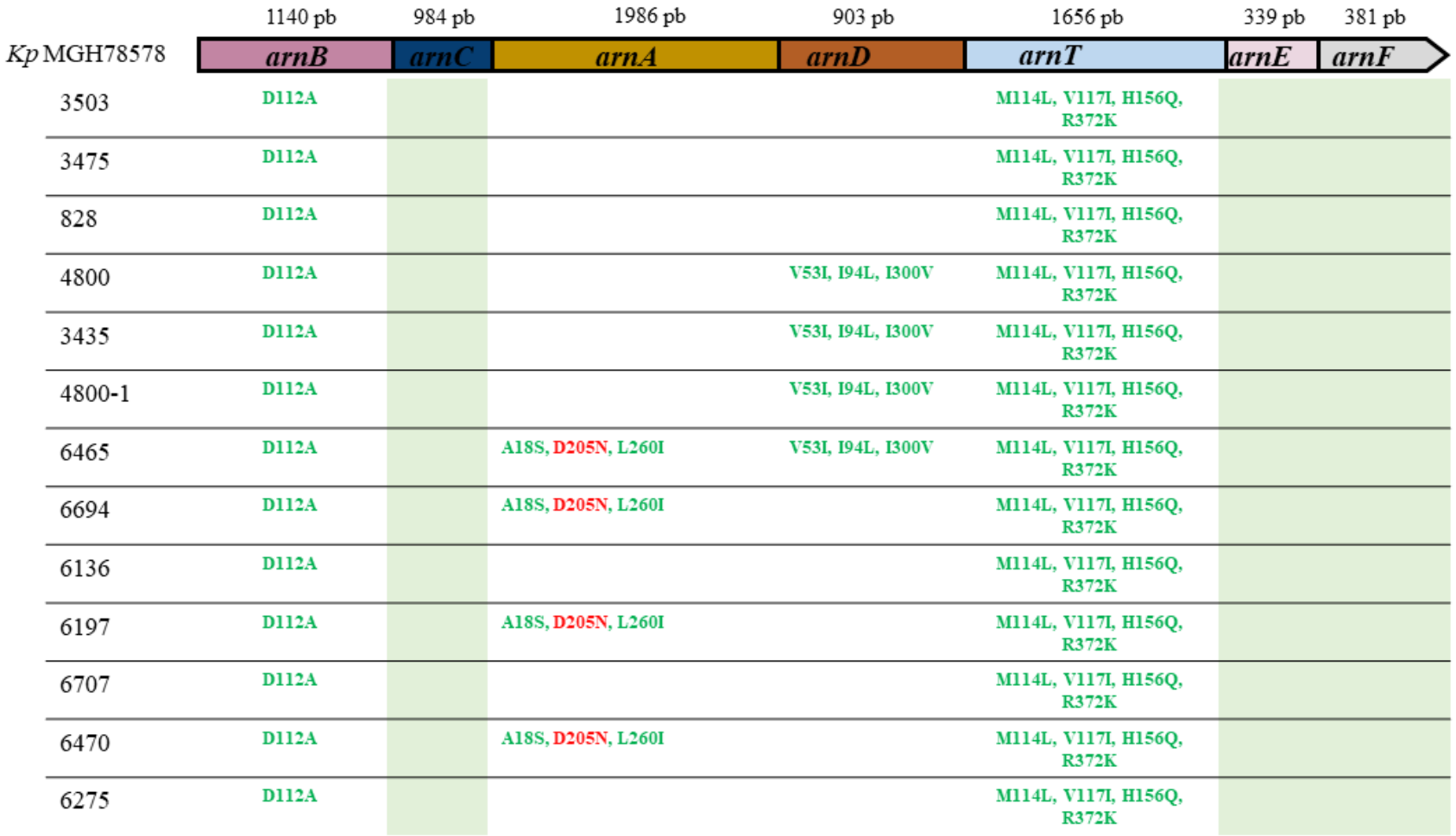


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**Table S1.** Oligonucleotide primers of the Genome walker PCR amplification and qPCR for gene expression levels used in this study.

| Primer and probes name | Sequence |
|---|---|
| *tssF*_F | 5'-CCAGCTGGCCACCGATAC-3' |
| *tssF*_R | 5'-ACATCCGGGCCACATTGAG-3' |
| *tssF*_P | 5'-GCCGGTCGGTGATCACGCTG-3' |
| *arnT*_F | 5'-GCCGGTTACTGGATCAACAG-3' |
| *arnT*_R | 5'-CAGCATCGCGGTGAGGAAG-3' |
| *arnT*_P | 5'-GGTCAGTGGCTGTTTGGCCATAA-3' |
| *phoP*_F | 5'-GAATGACCAGCCGATCAAGC-3' |
| *phoP*_R | 5'-GTGATGACGTCCTGTGGGTA-3' |
| *phoP*_P | 5'-TGCGCAAGAAAATTCAGGCT-3' |
| *phoQ*_F | 5'-CTACGATGAGCAGGGCAAAC-3' |
| *phoQ*_R | 5'-GTGGGTCATCTCAGAGTCGT-3' |
| *phoQ*_P | 5'-TGCTGTTGCGCAATAACCAT-3' |
| *pmrA*_F | 5'-GGCGGATGACTACCTGGTAA-3' |
| *pmrA*_R | 5'-CAGCAGGGCATACTCTTTCG-3' |
| *pmrA* _P | 5'-GCATTCGCGCCCTGCTCCGC-3' |
| *pmrB*_F | 5'-TCGATCAGATGACCACCAGC-3' |
| *pmrB*_R | 5'-GTCATCCAGCAACAGCACC-3' |
| *pmrB*_P | 5'-GCTGCTGCAGCTGGCGCGCG-3' |
| *rpoB*_F | 5'-GAAGGGCAGTATGGTCTGGA-3' |
| *rpoB*_R | 5'-CAGCGGTGCAGAATAAGTCA-3' |
| *rpoB*_P | 5'-TCCGTATTCCCGATTCAG-3' |
| *eptB*_F | 5'-CTATAGCCGCAATACCACGC-3' |
| *eptB*_R | 5'-AGCACGGCAAAGACATTCTG-3' |
| *eptB*_P | 5'-TACCGCTACCAAACTGTCGT-3' |
| Adaptor primer 1 | 5'-GTAATACGACTCACTATAGGGC-3' |
| Adaptor primer 2 | 5'- ACTATAGGGCACGCGTGGT-3' |
| R6KAN-2 Forward | 5'-ACCTACAACAAAGCTCTCATCAACC -3' |
| R6KAN-2 Reverse | 5'-CTACCCTGTGGAACACCTACATCT-3' |

## Conclusion

Les mécanismes de la résistance à la colistine ne sont pas encore définitivement résolus, mais des stratégies adaptées peuvent être développées pour traiter cette problématique. Ces stratégies devraient être basées sur une connaissance approfondie du développement de la résistance à la colistine et des mécanismes associés, et cela par l'amélioration des méthodologies actuelles en développant des technologies qui surmontent les limites des méthodes actuellement disponibles.

Les études de protéomique et de transcriptomique permettent d'explorer l'expression de gènes ou de protéines afin d'aider à comprendre les différents mécanismes de résistance des bactéries. Dans ce chapitre nous avons utilisé des approches différentes basées sur la génomique, pour étudier les mécanismes de résistances à la colistine de souches ayant des profils atypiques. En effet, nous avons démontré dans l'article 4, grâce à la technique de mutagénèse aléatoire, la complexité des mécanismes de résistance à la colistine. Nous avons également mis en évidence les voies importantes associées à la réponse à la colistine chez *E. coli*, impliquées notamment dans le métabolisme général de la bactérie. Un grand nombre de protéines membranaires, de protéines d'efflux, de protéines liées à la réponse au stress et de protéines impliquées dans la régulation de l'expression des protéines sont également associées à cette résistance. L'utilisation de méthodes complémentaires telle que la transcriptomique permettrait d'évaluer la sur- ou la sous-expression des différents gènes identifiés. La modulation des voies métaboliques peut être une stratégie développée par les bactéries pour contrer l'action de la colistine et pour s'adapter aux environnements difficiles.

Nos travaux ont également souligné dans l'article 5 l'importance de l'évaluation de la sensibilité à la colistine par méthode phénotypique. Enfin, des études

supplémentaires sont nécessaires pour comprendre la manière dont l'expression de la résistance à la colistine peut être modulée dans les souches porteuses de mécanismes de résistance similaires ou différents, et les voies dynamiques complexes modulant les systèmes à deux composants.

# CHAPITRE IV

# Travaux annexes

**Article 6: *fosM*, a New Family of Fosfomycin Resistance Genes Identified in Bacterial Species Isolated from Human Microbiota.**

Khabthani, S. et al. (2021) '*fosM,* a New family of fosfomycin resistance genes identified in bacterial species isolated from human microbiota', Antimicrobial Agents and Chemotherapy. American Society for Microbiology, 65(2). doi: 10.1128/AAC.01712-20.

Impact factor: 4.9

**Article 7: Glyphosate** (Draft manuscript, en cours de rédaction)

**Avant-propos**

Dans le cadre de l'élargissement de mes champs d'activité au cours de cette thèse, certains projets annexes ont été réalisés. J'ai participé d'une part à la description de trois nouveaux gènes de résistance à la fosfomycine, *FosM1, FosM2 et FosM3*, provenant de nouvelles espèces bactériennes isolées à partir du microbiote humain, en nous appuyant sur des analyses *in silico* et sur la méthode d'expression génétique (Article 6), et d'autre part à l'identification de gènes et de mécanismes intervenant dans le processus de la résistance au glyphosate dans les souches d'*E. coli* et *K. pneumoniae*, par l'utilisation de la méthode de la mutagenèse aléatoire et de la génomique (Article 7).

Le premier Article intitulé "*fosM*, a New Family of Fosfomycin Resistance Genes Identified in Bacterial Species Isolated from Human Microbiota." faisait suite à une étude visant à évaluer la prévalence des gènes de résistance à la fosfomycine de 350 nouvelles espèces isolées du microbiome humain isolées par culturomics à l'institut hospitalo-Universitaire méditerranée infection, Marseille, France, par une approche *in silico*, en utilisant la base de données ARG-ANNOT (Gupta *et al.*, 2014) avec une identité minimale de 70% et une couverture minimale de 50%. Au total, 25 génomes de nouvelles espèces bactériennes semblaient contenir des gènes de résistance à la fosfomycine. Les résultats du test *in vitro* relatif à l'activité antimicrobienne ont montré que trois espèces *Bacillus massiliogabonensis*, *Bacillus phoceensis* et *Gracibacillus timonensis* avaient une CMI élevée supérieure à 1 024 µg/ml de fosfomycine. L'analyse des séquences protéiques a montré que ces nouvelles séquences putatives nommé FosMs partagent les meilleures correspondances avec les membres de la famille FosB avec une similarité de séquence allant de 64 à 68%. Une différence au

niveau de l'environnement génétique a également été observée : les gènes fosMs sont chromosomiques, aucun phage ni insertion de séquence dans l'environnement de ces 3 gènes de résistance n'est présent, ce qui exclut leur origine commune par transfert latéral. Enfin, l'expression de ces différents gènes par complémentation dans une souche *d'E. coli* Bl21 DE3 *nous* a permis de confirmer le rôle de ces trois gènes putatifs identifiée *in silico* dans la résistance à la fosfomycine. Nous avons observé une augmentation de la CMI de 1 µg/ml à 1024 µg/ml pour les protéines Fos de *B. massiliogabonensis*, *B. phoceensis* et *G. timonensis*. L'analyse phylogénétique basée sur les séquences protéiques démontre que nos trois gènes sont plus proches les uns des autres que des autres membres de la famille FosB. Ils semblent donc constituer une nouvelle famille de gènes Fos conférant une très haute résistance à la fosfomycine, ces gènes ont été nommés fosM1, fosM2 et fosM3.

Mon deuxième projet annexe, intitulé « glyphosate », a porté sur l'étude des gènes associés à la résistance au glyphosate par une approche de mutagénèse aléatoire en utilisant les librairies de clones d'*E. coli* et de *K. pneumoniae* qui ont fait l'objet des précédentes études (chapitre III). Nous avons ainsi isolé trois clones d'*E. coli* présentant des CMI réduites au glyphosate par rapport à la souche mère. Les gènes *acrA* et *acrB,* constituants la pompe à efflux *acrAB-tolC* ont été identifiées par séquençage génomique dans les souches mutantes. Nous avons ainsi démontré que les pompes à efflux jouent un rôle dans la résistance au glyphosate chez *E. coli*. Ce résultat est en accord avec ceux de Randall et al. (Randall *et al.*, 2007). Chez *K. pneumoniae* en revanche, l'augmentation de la sensibilité au glyphosate a été causée par l'interruption du gène *waaE* participant à la voie de biosynthèse des LPS, qui fait partie de la biogénèse de la membrane externe bactérienne.

Mon troisième projet annexe, intitulé « First co-occurrence of chromosomic *mcr-1* and plasmidic *mcr-3* in producing *Escherichia coli* isolated from pig in France » Cette étude a permis d'élucider le mécanisme de résistance à la colistine dans une souche d'*E. coli* isolée des fèces d'un porc grâce aux analyses génomiques par séquençage MINION et a révélé pour la première fois en France la localisation chromosomique du gène *mcr-1.1* et en parallèle la détection d'un plasmide hébergeant le gène *mcr-3.5*.

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MECHANISMS OF RESISTANCE

# *fosM*, a New Family of Fosfomycin Resistance Genes Identified in Bacterial Species Isolated from Human Microbiota

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**ABSTRACT** Fosfomycin is a decades-old antibiotic, currently reused because of its activity against multidrug-resistant bacteria. Here, we used a combined *in vitro/in silico* approach to search for fosfomycin resistance determinants in 25 new bacterial species isolated from the human microbiota. Putative resistance genes were cloned into a susceptible *Escherichia coli* strain. MIC values increased from 1 $\mu$g/ml to 1,024 $\mu$g/ml. Here, we report a new family of potential chromosomal fosfomycin resistance genes, named *fosM*.

**KEYWORDS** FosM, fosfomycin resistance, human microbiota, genomes, bacillithiol transferase, phylogenetics

Antibiotics are one of the main solutions for the treatment of bacterial infections and have been shown to reduce mortality from infectious diseases and contribute to increased life expectancy (1). The spread of multidrug-resistant (MDR) bacteria now has been reported worldwide. Epidemiological surveillance networks report an increase in the isolation of carbapenemase-producing *Enterobacteriaceae* in some European countries (2, 3). One of the solutions to overcome the problem of MDR bacteria is to reuse "old" antibiotics (4), such as fosfomycin. This natural antibiotic product has a broad-spectrum activity and a bactericidal effect (5) against both Gram-positive and Gram-negative bacteria. Although the current level of resistance to fosfomycin is low, therapeutic failures have been reported, revealing bacterial populations resistant to fosfomycin (6). Different fosfomycin-inactivating enzymes have been described, including metalloenzymes (FosA, FosB, FosX, etc.) and fosfomycin kinases (FomA, FomB, and FosC) (7). The aim of this study was to describe fosfomycin resistance determinants in the human microbiota using *in vitro* testing and a genomic search for putative genes conferring resistance to fosfomycin (*fosRG*). Hence, we identified three novel *fos* genes that belong to a new family of *fosRG* that we have named *fosM*.

Bacterial strains of 25 new species isolated from human microbiota by culturomic methods at the Institut Hospitalo-Universitaire Méditerranée Infection, Marseille, France, were retrieved from our bacterial collection, CSUR (Collection de Souche de l'Unité des Rickettsies). They were grown on Columbia agar medium and sheep blood agar (bioMérieux, France) and then reidentified using matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonik, Bremen, Germany). Bacterial colonies were tested *in vitro* with fosfomycin Etest strips (bioMérieux, France). Given the MIC values (≥1,024 $\mu$g/ml) and despite the lack of CLSI/EUCAST interpretation criteria for new species, *Bacillus massiliogabonensis* (8), *Gracilibacillus timonensis* (9), and *Bacillus phoceensis* (10) can be considered highly resistant bacteria (Table 1). The corresponding genomes were downloaded from the internal database IHU and annotated using PROKKA (11). Fos protein detection using protein BLAST (BLASTp) against the ARG-ANNOT (12) database identified three amino acid sequences from *B. massiliogabonensis*, *G. timonensis*, and *B. phoceensis* having

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**TABLE 1** Characteristics and MICs for fosfomycin for the 25 new bacterial species isolated from the human microbiota used in this study[a]

| Bacterium | Phylum | MIC (μg/ml) |
|---|---|---|
| *Cellulomonas massiliensis* | *Actinobacteria* | 192 |
| *Citricoccus massiliensis* | *Actinobacteria* | 192 |
| *Flaviflexus massiliensis* | *Actinobacteria* | 2 |
| *Actinomyces ihuae* | *Actinobacteria* | 24 |
| *Bacillus andreraoultii* | *Firmicutes* | 24 |
| *Bacillus massiliogabonensis* | *Firmicutes* | 1,024 |
| *Bacillus mediterraneensis* | *Firmicutes* | 32 |
| *Bacillus phocaeensis* | *Firmicutes* | 1,024 |
| *Clostridium amazonitimonense* | *Firmicutes* | 24 |
| *Clostridium nigeriense* | *Firmicutes* | 24 |
| *Enterococcus massiliensis* | *Firmicutes* | 8 |
| *Gracilibacillus timonensis* | *Firmicutes* | 1,024 |
| *Niameyia massiliensis* | *Firmicutes* | 8 |
| *Paenibacillus antibioticophila* | *Firmicutes* | 16 |
| *Paenibacillus bouchesdurhonensis* | *Firmicutes* | 16 |
| *Paenibacillus senegalimassiliensis* | *Firmicutes* | 16 |
| *Paenibacillus tuaregi* | *Firmicutes* | 128 |
| *Planococcus massiliensis* | *Firmicutes* | 96 |
| *Romboutsia massiliensis* | *Firmicutes* | 2 |
| *Streptococcus timonensis* | *Firmicutes* | 16 |
| *Urmitella massiliensis* | *Firmicutes* | 24 |
| *Virgibacillus dakarensis* | *Firmicutes* | 192 |
| *Massilibacillus massiliensis* | *Firmicutes* | 1 |
| *Vitreoscella massiliensis* | *Proteobacteria* | 48 |
| *Xanthomonas massiliensis* | *Proteobacteria* | 96 |

[a]MIC determinations were repeated at least three times. All studied species have been isolated from the human gut, except for *Citricoccus massiliensis*, which was isolated from the skin. *B. massiliogabonensis*, *G. timonensis*, and *B. phoceensis* were isolated from the stools of a healthy 16-year-old Gabonese boy, a healthy 10-year-old Senegalese boy, and a Senegalese boy with kwashiorkor, respectively.

FosBx1, FosB2, and FosB as best matches, with a sequence similarity ranging from 64% to 68%. These putative *fosRG* sequences were synthesized by GenScript (GenScript USA, Inc., Piscataway, NJ) and inserted in a plasmid pET-11 expression vector between NdeI and BamHI. This vector contains an ampicillin resistance gene to select transformed strains. Briefly, 1 μl of plasmidic DNA was electroporated into electrocompetent *E. coli* BL21(DE3) and then recovered by adding 1 ml SOC medium, followed by incubation at 37°C for 1 h. One hundred microliters was plated on LB agar medium containing 100 mg/liter ampicillin. The clones that were grown were inoculated in Luria-Bertani (LB) broth and incubated up to a DO of 0.6. Next, 1 μM IPTG (isopropyl β-D-1-thiogalactopyranoside) was inoculated into the broth for 1 h, and 150 μl was taken from LB broth and plated on selective LB agar containing 100 μg/ml ampicillin and 32 μg/ml fosfomycin. Complementation assays on *E. coli* led to an increase in fosfomycin MICs from 1 μg/ml to 1,024 μg/ml, confirming the role of *fosRG* in fosfomycin resistance (Fig. 1).

The phylogenetic tree obtained for all the known Fos enzymes showed that the newly identified proteins were related to the FosB/D sequences with high bootstrap values (97%) (Fig. 2). However, these Fos enzymes form a cluster rather distinct from those of all existing FosB, as suggested by the high bootstrap value of the node (86%). Genes coding for these proteins can be considered a new family of *fosRG* genes called *fosM*. Their discovery follows the recent identification of *fosL1* by Kieffer et al. (13). They were given the names *fosM1* (GenBank accession no. BK012111), *fosM2* (BK012112), and *fosM3* (BK012113) according to the current nomenclature. Analysis of the functional domain of FosMs against the Conserved Domain Database (CDD) from NCBI showed that they consist of a functional domain conferring a metallothiol transferase function in the superfamily of the vicinal oxygen chelate (VOC) superfamily.



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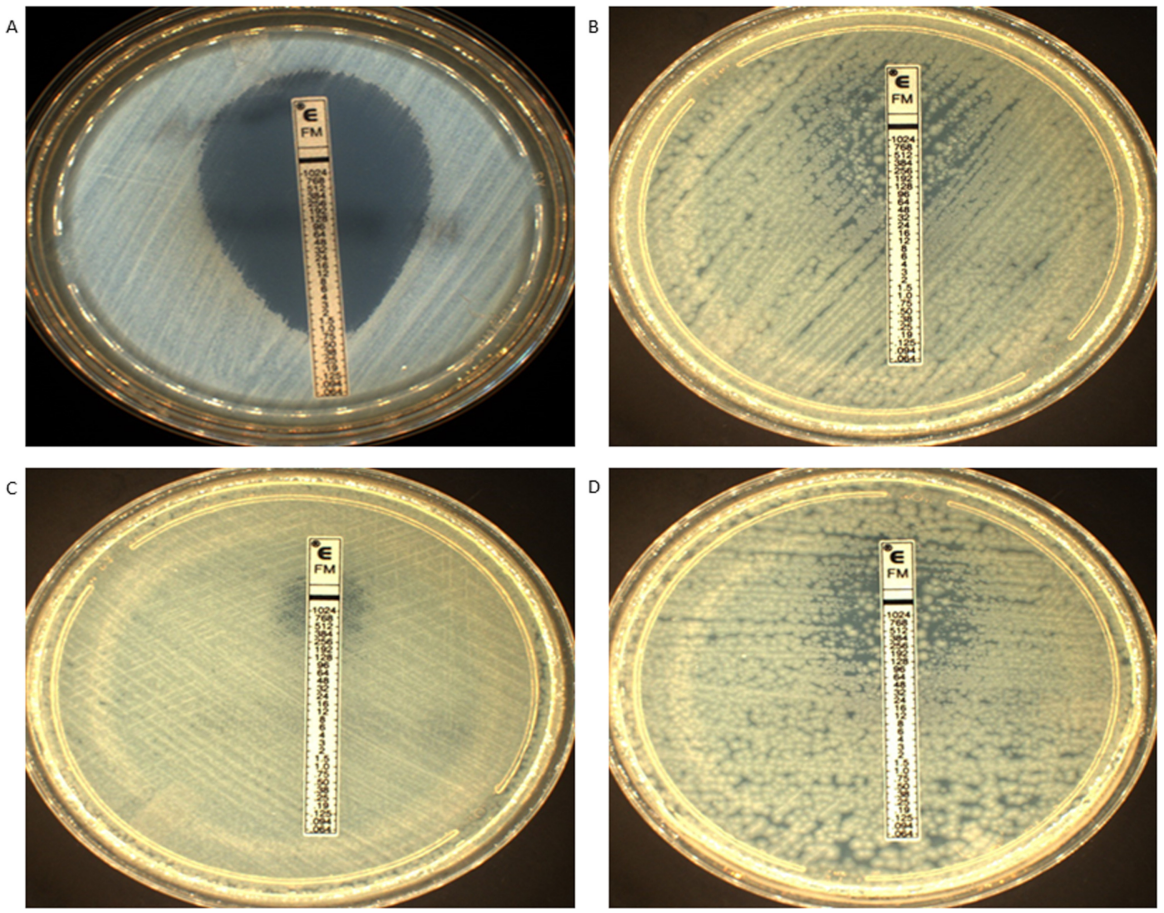


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**FIG 1** Fosfomycin Etest results of *Escherichia coli* BL21 (MIC of 1 $\mu$g/ml) (A), *Escherichia coli* BL21 expressing the *fosM1* gene (MIC of 1,024 $\mu$g/ml) (B), *Escherichia coli* BL21 expressing the *fosM2* gene (MIC of 1,024 $\mu$g/ml) (C), and *Escherichia coli* BL21 expressing the *fosM3* gene (MIC of 1,024 $\mu$g/ml) (D).

The amino acid identity shared between FosM and FosB enzymes suggests that the fosfomycin resistance pattern was related to the production of a bacillithiol transferase, such as those belonging to the FosB family. The alignment of the FosM with the sequence of FosB2 of *Bacillus cereus* showed that the amino acids at positions 39, 46, 48, 64, 100, 105, and 124 were retained. This indicates that the amino acids that build the cage structure of fosfomycin and the hydrogen bonds are identical to those found in all bacillithiol transferases (14). However, the cysteine 9 residue in FosB2 is replaced in FosMs by a leucine (Fig. 3).

F3

The *fosM* genes were found on the chromosomes of three distinct bacterial species. Genome analysis revealed that these *fosM* genes had no phage or insertion sequence in their nearby environments, and the GC content of the genes was close to that of the bacterial genome (see Fig. S1 in the supplemental material). The search for FosM homologs, using a BLASTP algorithm against 4,175 proteomes of *Bacillus* spp. that are available from NCBI, revealed that *fosM* genes were frequently found in *Bacillus* species on the chromosome. Phylogenetic analysis confirmed the great variability of FosB/D protein sequences previously described in the *Bacillus* genus (15). It showed that FosM sequences were distinct from the reference FosB sequences that have been reported in the literature, and some already known fosfomycin resistance genes, annotated as *fosB-fosD* sequences, might be *fosM* genes (Fig. S2). This is the case of *Bacillus solani* and *Bacillus praedii* (bootstrap value, 78%). Similar to the Gram-negative bacteria encoding



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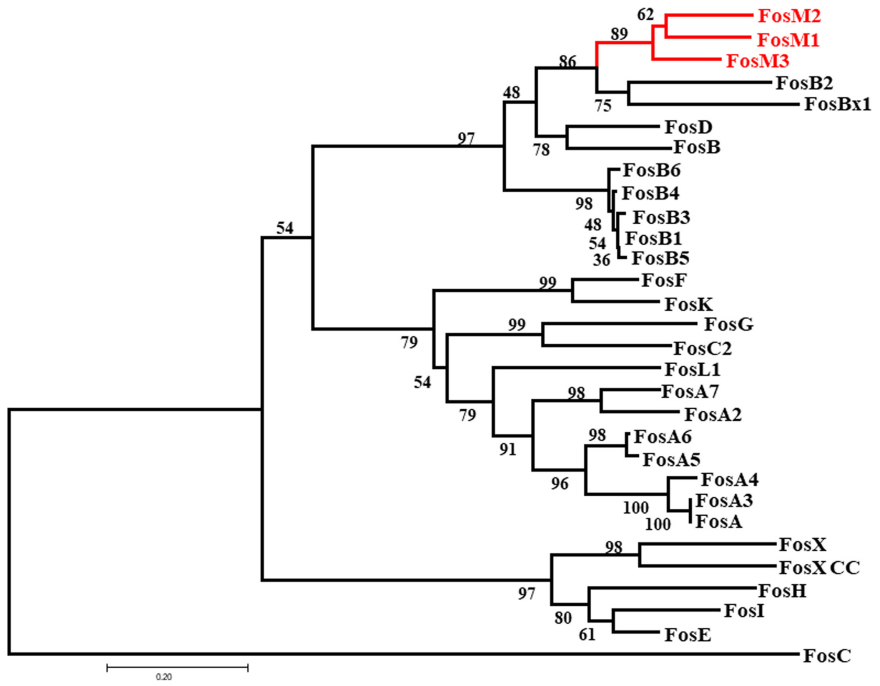


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**FIG 2** Phylogenetic tree obtained for the newly identified Fos enzymes and principal known Fos proteins by the distance method using the sequence alignment tool Muscle software (20) and using the neighbor-joining method within MEGA v7 software (21).

*fosA* genes on their chromosomes (16), our analysis suggests that human gut bacteria harboring *fosM*-type genes represent reservoirs of this *fosRG*. These *fosM* genes are fosfomycin-resistant determinants that can be mobilized into the microbial community, including pathogenic bacteria (17–19). Further studies are needed to determine *fosM* frequency and evaluate their ability to spread through horizontal transfer.

In summary, we characterized three new fosfomycin resistance genes, known as *fosM1*, *fosM2*, and *fosM3*, from new bacterial species of the human gut, all conferring a high level of resistance to fosfomycin. We showed that bacteria in the human microbiota might be a potent reservoir of fosfomycin resistance genes.

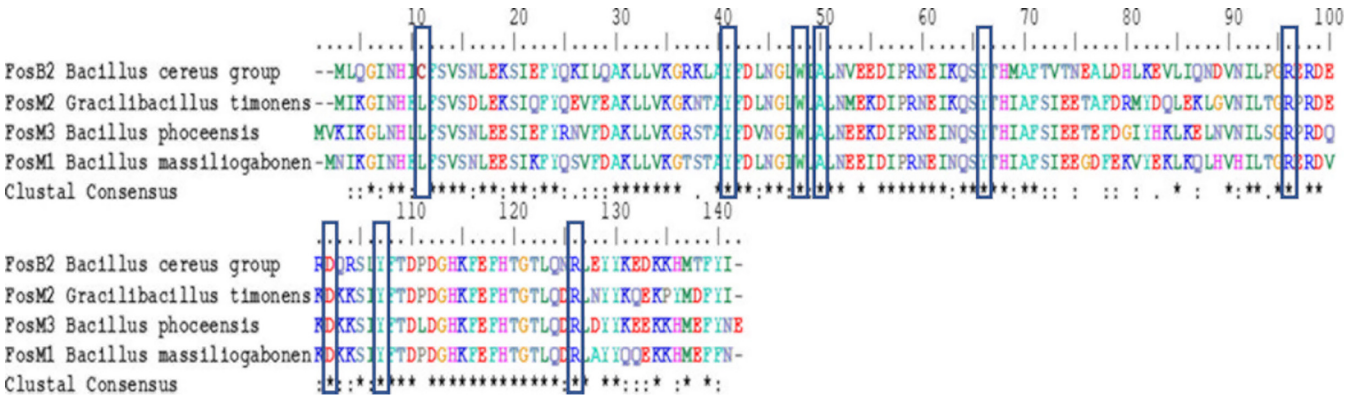


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**FIG 3** Multiple-sequence alignment of the new fosfomycin resistance enzymes FosM1, FosM2, and FosM3 with FosB2 from *Bacillus cereus*.

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**Data availability.** Nucleotide sequence data reported are available in the Third-Party Annotation Section of the DDBJ/ENA/GenBank databases under the following accession numbers: *fosM1*, BK012111; *fosM2*, BK012112; and *fosM3*, BK012113.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.4 MB.

## ACKNOWLEDGMENT

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# First co-occurrence of chromosomic *mcr-1* and plasmidic *mcr*-3 in producing *Escherichia coli* isolated from pig in France.

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**Abstract**

**Objective:** Phenotypic and genotypic characterization of an atypic *Escherichia coli* multidrug resistant strain hosting two colistin resistance genes, for a first time in France.

**Methods:** Fecal samples were withdrawn from pigs in 2020. These samples were collected from a pigsty in Avignon department of Vaucluse, France. The whole DNA from stools were extracted to detect *mcr* genes enabling selection of positive samples. Then carry out selective culture on LBJMR medium followed by a series of isolation and identification by MALDI TOF. On the other side, antibiogram and E-test test were done evincing the antibioresistance phenotype. The co-occurrence of *mcr* was confirmed by biomolecular methods PCR. Although, the genome sequencing using Miseq and MinION technologies were performed on the isolate's strain. A series of bioinformatics analysis has been carried out to concretely analyze the genome.

**Results:** Among 80 pigs' faeces, one of them was positive to *mcr* genes. Going from the selected stool the MALDI-TOF identified an *E. coli* named Q4552. The phenotypic analysis divulges its multi-resistant phenotype to antibiotics. Performed qPCR, from extracted DNA of *E. coli* reveals the co-occurrence of two colistin reistance genes both of *mcr-1* and *mcr-3*. This strain has a genome size Miseq: (4'702'528-pb) with GC 50.8% and cov 34.13. Further genomic analysis through MINION sequencing highlighted the chromosomic localisation of *mcr-1.1* and the detection of a plasmid harboring *mcr-3.5*.

**Conclusion:** This study elucidates a unique phenotype and genotype pattern in France. Co-existence of *mcr* genes need more studies to understand the new colistin resistance mechanism.

**Keywords:** *mcr* genes, *mcr-1*, *mcr-3*, colistin resistance, whole-genome sequencing.

## Introduction

Colistin (polymyxin E) is a polycationic antibiotic that interacts with the lipid A of (LPS) lipopolysaccharide in the outer membrane of Gram-negative bacteria GNB leading to cell lysis [1]. This antibiotic is considered as one of the last-line antimicrobial agent in many areas used to treat infections due to multidrug Gram-negative bacteria, including Enterobacteriaceae such as *Escherichia coli* and *Klebsiella pneumoniae* [2]. Furthermore, the use of polymyxins in veterinary medicine becomes very common within the European union and European economic area (EU/EEA). It is the only effective antibiotic agent against bacteria, particularly carbapenemases-producing bacteria [3]. This wide use of colistin caused a significant increase in colistin resistance, especially in *Escherichia coli* from pigs [3]. Moreover, pigs are in a close contact with humans. The emergence of colistin resistant bacteria in animals and humans is consequently higher than before [4], as well as the lack of new antibiotics.

Previously, it has been shown that colistin resistance is mediated by chromosomal mutations [5,6]. Modification in bacterial DNA leading replacement of amino acids in an important target leads to colistin resistance [7]. Gram-negative bacteria developed several mechanisms against polymyxin E including a variety of (LPS) modification of lipid A with phosphoethanolamine and 4-amino-4-deoxy-L-arabinose. These modifications reduce affinity binding of colistin and LPS and it decreased bacterial susceptibility [8], overexpression of the outer membrane protein OprH, which are all regulated by molecular mechanisms. Other resistance mechanisms are associated with efflux pumps [9].

Liu et *al*. report the first *E. coli* harboring the plasmid bearing mobile colistin resistance gene *mcr-1*. This strain SHP45 was isolated from pigs in China [10]. After this 9 other plasmid colistin resistance genes were discovered : *mcr-2* on an IncX4 plasmid in *E.coli* Belgium porcine and bovine [11], *mcr-3* contains (261-kb) IncHI2-type plasmid pWJ1 from porcine *E. coli* in Malaysia [12], *mcr-4* in *Salmonella* and *E. coli* from Italy 2013 [13], *mcr-5* in *Salmonella enterica*

subsp, *Enterica serovar paratyphi B* in Germany [14], *mcr-6* variant genes identified in *Moraxella pluranimalium* isolated from pigs in Britain [15], *mcr-7* in *Klebsiella pneumoniae* from chicken in China this gene was found in an IncI2-type plasmid (pSC20141012) that co-harbored the $bla_{\text{CTX-M-55}}$ gene in one isolate [16], *mcr-8* in *NDM*-producing *Klebsiella pneumoniae* was found in human patient with pneumonia syndrome in the respiratory intensive care unit of a Chinese hospital. In 2019 a novel *mcr-9* is identified in *Salmonella enterica* and *Serotype typhiurium* isolated from human patient in Washington. Recently in 2020 a new *mcr* variant has been reported *mcr-10* on an IncFIA of an *Enterobacter roggenkampii* clinical strain [17]. Protein resolution structure of all nine *mcr* homologues revealed that *mcr-9*, *mcr-3*, *mcr-4* and *mcr-7* have a high degree of similarity [18]. All most of novel variant and colistin resistance genes are abundant in pigs and poultry farming because of polymyxin use to prevent infections caused by pathogens [19].

Here we report the first co-occurrence of two colistin resistance genes *mcr-1* and *mcr-3* harboring *E. coli* Q4552 isolated from pigs. His phenotypic pattern is unique in France and here is its first genomic description, more than this the isolate show a multidrug resistance phenotype.

## Materials and methods:

**Decree and law authorizing the use of animal products for research and diagnosis:** Prefectorial authorization (Bouches-du-Rhône) n°13 205 107 of 04 September 2014 authorizing the IHU Mediterranee Infection to use unprocessed animal by products of categories 1, 2 and 3 for research and diagnostic purposes. Order of 8 December 2011 laying down health rules concerning animal by products and derived products in application of Regulation (EC) No 1069/2009 and Regulation (EU) No 142/2011.

In 2020, 80 fecal pig's samples were collected from a pig fattening area in Avignon department of Vaucluse, France. The pigs were sampled in several batches. After fattening, these animals are intended for human food consumption in Corsica, France.

**DNA extraction:** DNA extraction from pig's feces samples was done according to a protocol used in previous studies [20]. 1g of feces, Buffer G2 and proteinase K are incubated at 56°C overnight and extracted with the EZ1 DNeasy Blood Tissue Kit (Qiagen GmbH, Hilden, Germany) using the protocol of the manufacturer.

**Molecular analysis:** Real-time PCR screening of colistin resistance genes. The presence of genes encoding for colistin resistance (*mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4, *mcr*-5, *mcr*-8) were investigated by Real-Time Polymerase Chain Reaction [21,22]. qPCR reactions were performed using CFX96 TM Real time System / C1000 TM Touch thermal Cycler (Bio-Rad, Singapore). Genes encoding for colistin resistance genes were detected using specific primers and probes (suppl. Table S1) [23]. Positive control template (DNA extracted from *E. coli* and *K. pneumoniae*) were included in each qPCR experimental run. Despite negative control which contains the mix of primers and probes without bacterial DNA. Results were considered positive when the cycle threshold value of real-time PCR was ≤30. Then a standard PCR multiplex was performed to confirm positive qPCR results and sequencing.

**Screening of colistin resistant Gram-negative bacteria:** Fecal samples were enriched in TSB for 72 hours. The suspension is than cultured on a selective medium called LBJMR (Lucie Bardet Jean Marc Rolain) containing colistin sulfate salt at (4µg/ml) concentration and vancomycin (50µg/ml), with glucose as fermentative substrate, to a Purple Agar Bas. LBJMR was developed deprived of any ions in its original composition and was also deprived of any electrolytes to avoid growth of *proteus sp*. This medium is used to screen colistin / vancomycin resistant bacteria: the Enterobacteriaceae have a yellow color, contrasting with the purple agar and have a different sizes 2-3 mm and enterococci has a round little shape 0.1-1 mm [24]

**MALDI-TOF MS Identification:** Colonies with different morphologies have been picked up from LBJMR agar plate. Those colonies were spotted and identified using Microflex LS spectrometer (Bruker Daltonics, Bremen, Germany) [25]. The strain is properly identified only when the score is 2.

**Antimicrobial susceptibility test:** The current method DD test (Kirby-Bauer procedure) is a routine susceptibility testing method according to the CLSI and EUCAST guidelines [26]. The antibiotic disk diffusion leads the diffusion of a given concentration from disks into Muller Hinton Agar (MHE agar) that has been seeded with a bacterial inoculum corresponding to 0.5 McFarland standard (approximately $10^8$ CFU / ml) suspended in a NaCl solution 0.85%, into all MHE agar surface using a sterile cotton swab. Sixteen antibiotics were used: Ampicillin, Amoxicillin-Clavulanic Acid, Aztreonam, Ceftazidime, Cefotaxime, Cefepime, Cefoxitin, Piperacillin-Tazobactam, Colistin, Meropenem, Ertapenem, Imipenem, Tigecycline, Ciprofloxacin, Gentamicin, Trimethoprim-Sulfamethoxazole (Bio-Rad, Marne-la-Coquette, France). The minimal inhibition concentration is determined by the diameter of the inhibition zone. E-test and microdilution method are performed to confirm some AST results as previously reported [26]. Minimum inhibitory concentration (MIC) was determined by E-test

132 method (Biomérieux), whereas colistin MIC was determined using UMIC microdilution
133 method (Biocentric, Bandol, France).

134 **Plasmid transferability conjugation assay:** The purpose of this experiment is to check
135 if conjugative plasmid can be detected using *E. coli* J53 as a receptor strain which resistant to
136 sodium azide and colistin. The *E. coli* Q4552 were inoculated with the *E. coli* J53 in the
137 selective medium already prepared [27].

138 **Genome sequencing and Bioinformatic analysis**: *E. coli Q4552* strain was sequenced
139 using Hitachi HIGH TECHNOLOGY Sanger sequencing to confirm the presence of colistin
140 resistance genes. The sequences were analyzed using ARG-ANNOT, BLAST-N in NCBI and
141 aligning sequences using ClustalW Omega. The whole genome was sequenced by Miseq
142 (Illumina Inc., San Diego CA, United states) and Nanopore MINION. Spades and canu were
143 used to assemble the generated long read sequencing data. Genomes was annotated with
144 Prokka. The detection of resistance genes was performed with databases: Resfinder, ARG-
145 ANNOT, Card and Plasmid Finder. The genetic environment of *mcr* genes has been analyzed
146 by BLASTX in NCBI database. Then the data generated were used to represent the plasmid and
147 chromosome genome with CG view software.

148 **Multilocus Sequence Typing:** The clonal complex of *E. coli Q4552* strain and the set
149 of close typing sequences have been determined by PubMLST then analyzed and characterized
150 among (N=11808) ST of *Escherichia* spp (https://pubmlst.org/). using the following software:
151 Cytoscape and layout.

152
153
154 **Results:**
155 **Screening of colistin resistance genes:** Performed real time PCR of extracted DNA
156 from the 80 stool samples was positive for one sample. Indeed, this sample was positive for
157 *mcr-1* with CT value = 24 and for *mcr-3* with CT value = 21. This co-occurrence was confirmed

158 then by standard PCR targeting both genes *mcr-1* and *mcr-3* (fig. 1A). Besides the other *mcr*
159 genes: *mcr-2, mcr-4, mcr-5, mcr-8* were negative and total absence of fluorescence in RT-PCR.

160 **Culture and bacterial identification:** Out of 80 collected fecal stools sample, one
161 sample was interesting, as a carrier of atypical strain. After bacterial enrichment, the solution
162 was cultured on the LBJMR selective medium allowing the selection of colistin and
163 vancomycin resistant strains. Strains growing on this medium had a yellow color, then
164 identified with MALDI-TOF. Identification allowed to obtain the following strains: *E. coli*
165 (score 2.4), *Proteus mirabilis* (score 2.5) and *Enterococcus gallinarum* (score2.6). The *E. coli*
166 is conserved with other strains collection with the number Q4552.
167 Gram-positive bacteria are naturally resistant to colistin, which means that *Enterococcus*
168 *gallinarum* is not a carrier of *mcr-1* and *mcr-3*. On the other hand, some of gram-negative
169 bacteria are naturally resistant to colistin but carry the mcr gene. In this study, *proteus mirabilis*
170 does not show any positive *mcr* gene. *E. coli Q4552* is the strain with both *mcr* genes, it was
171 detected by RT-PCR and ST-PCR. After sequencing, BLAST on the database followed by an
172 alignment showed the presence of the two genes *mcr-1* and *mcr-3.*
173
174 **Antibiotic resistance patterns**: According to EUCAST the breakpoint for
175 Enterobacteriaceae for colistin is 2µg/ml. The *E. coli* Q4552 had a colistin minimal inhibition
176 concentration at 4µg/ml (fig. 1B et 1C). The strain is also resistant to Amoxicillin, Amoxicillin
177 acid clavulanic, Oxacillin, and Penicillin G. It was sensible to all most of Carbapenem and
178 Cephalosporin antibiotics class respectively (Ertapenem, Imipenem and Meropenem)
179 (Cefalotin, Cefotaxime, Ceftriaxone and Cefepime). In the other side the resistome analysis
180 indicated the presence of genes encoding resistance for β-lactams $bla_{AMPC2}$, $bla_{AMPC1}$, $bla_{TEM}$,
181 $bla_{ampH}$, *AcrF, AcrE and AcrS* (tab. 2). The resistome analysis revelated also the presence of
182 genes encoding resistance for macrolides *erm(B) mph(A)* which confers resistance to

Clindamycin and Erythromycin. Regarding Quinolone it is only resistant to Ciprofloxacin by *mdtH*. Bothe of genes *aadA1* and *aadA2* was found in the genome encoding for aminoglycoside 6-acteyltransferase, conferring resistance against Streptomycin. Regarding Polymyxin E, *E. coli* Q4552 is resistant to colistin. Strangely it is sheltering two variant of phosphatidylethanolamine transferase *mcr1.1* and *mcr3.5*.

**Genomic and Bio-informatic analysis:** After Miseq sequencing statistic assembly analysis generated various genomic data. *E. coli* Q4552 has a genome size of (4'702'528-pb) assembled into 165 Scaffolds with the longest one of (202'992-pb). The sequenced genome has a good quality with GC 50.8%, Raw cov 34.13x, Median cov 31x, reads passing EC 98.79%. Concerning MINION sequencing Scaffolds < (800-pb) and scaffolds with depth value lower than 25% of the mean depth were removed, so fare the assembly generated 11 scaffolds. The chromosome sequences are found in node 1, its length (4'581'575-bp) and cov 22.81x. Further sequence analysis shown that *mcr-1.1* is chromosomic (fig. 2) and the *mcr-3.5* is plasmidic in IncFII found in the node 2 length (84'331pb) cov 26.57x. In vitro it has been found that the plasmid carrying *mcr-3* is transferable.

The sequence containing the plasmid (node 2) were subjected to a BLAST analysis on NCBI GenBank. The set of plasmids having a significant query coverage between 99% and 90% with a good alignment score were carried out. Then six plasmids were selected from the distribution of the top 100 Blast Hit, then compared with the replicon plasmid type IncFII harboring *mcr-3*. So, fare Plasmid of *E. coli* Q4552 with *mc-3* was compared to high homology plasmids (accession number AP018353.1; LC549806.1; CP041102.1; CP042621.1; MG838205.1; CP046718.1) (fig. 3). According to the genetic environment of *mcr-1.1* and *mcr-3.5*. The genes located around *mcr-3.5* (1626-pb) are: Family transposase *Tn3* (795-pb), multidrug efflux pump *Tap_2* (1239-pb), *IS6* (705-pb), *Tn3*(534-pb), Diacylglycerol kinase

208 *dgKA_2* (381-pb), *IS6* (705-pb), *IS110* (1005-pb), 23S rRNA methyltransferase (96-pb),
209 adenine-N-6-methyltransferase *erm* (111-pb), rRNA adenine methyltransferase (111-pb). So
210 far, the replicon type plasmid IncFII confers to the strain resistance to colistin and antibiotic
211 family of macrolids. The genes neighboring *mcr-1.1* (1626-pb) are Phosphatase PAP2 (324-
212 pb). The PAP2 is frequently associated with *mcr* genes[41]. The other genes are IS91 transposase
213 (1200-pb) IS30 (942-pb), GTPase Era (873-pb), Antigen43 *flu* (2847-pb), domain DUF (819-
214 pb), klcA_2 antirestriction protein (486-pb), IS30 (924pb), IS30 (732-pb), transposase (3018-
215 pb), endonuclease NucS (1164-pb), NucS (1314-pb), IS629 (327-pb). These results suggest that
216 *mcr-1.1* has been included to the chromosome via a transposon. To know more details about
217 this transposon, the *E. coli* Q4552 genome has been compared with *E. coli* K12 genome which
218 is a strain without any plasmid and sensible to antibiotics. According to the genetic environment
219 of the two strains, approximately (45-kbp) including the *mcr* genes are different from the
220 genome of the *E. coli* k12 strain (fig. 2).
221 The chromosomal genes implicated in colistin resistance are also found in the genome
222 without any mutation (http://provean.jcvi.org/index.php): PhoP, PhoQ, MicA, PmrD, SroC,
223 MgrR, EptB, EptC, PmrC, PmrB, PmrA, IpxM.
224
225 **Multilocus sequence typing analysis:** MLST classified colistin-resistant *E.*
226 *coli* Q4552 into ST-843, 100% identity. It has a human and environment origin, here we report
227 the animal source of the ST-843. Its typable allelic profile include dinB-10, icdA-148, pabB_7,
228 polB_17, putP_7, trpA_1, trpB_4, uidA_23. This clone belongs to a clonal complex Ccplx 568,
229 other clones belong to this clonal complex are shown in fig. 4. The *E. coli* Q4552 strain has a
230 CRISPR-Cas system as immune system against foreign genetic elements such as those present
231 within plasmids and provides a form of acquired immunity. The sequence description gives two

232 CRISPR is in the chromosome. The first one has 7 spacers and a CRISPER length (454-pb).
233 The second one has 11 spacers and a CRIPER length of (699-pb).
234
235 **Discussion**
236 Rarely are found strain with two *mcr* genes over the word. In 2017 it has been reported
237 in Spain *E. coli* with *mcr-1* and *mcr-3* isolated from Cattle [28]. Another one has been found in
238 2019 in New Zealand [29]. In 2020 it was also frequent reporting *mcr-1* and *mc-3* carrier strains
239 in pigs from Thailand [30]. It has been described in Thailand also *K. pneumoniae* harboring *mcr-*
240 *1* and *mcr-3* from patients [31]. A multidrug resistant *E. coli* isolated from dog feces with a co-
241 occurrence of the *mcr-1.1* and the *mcr-3.7* genes has been characterized in China [32].
242 In the current study the bacterial chromosome of *E. coli* Q4552 contains the *mcr-1.1* gene
243 associated with other resistance genes such as β-lactamases $bla_{AMPH}$, $bla_{AMPC}$; *msbA* an efflux
244 pump belongs to the superfamily of ABC transporters for nitroimidazole [33]; *acrF, acrE, acrS,*
245 and family *emrB, emrA, emrR* efflux pump for fluoroquinolone [34]; *mph* phosphorylates
246 enzymes 14-membered and 16-membered macrolides [35]; *mdtH* and *mdtG* multidrug resistance
247 efflux pumps (MDR EPs). Those proteins increase the antibiotic resistance potential and are
248 directly involved in the crosstalk with host cells [36].
249 Concerning, the variant *mcr-3.5* is found in the plasmid replicon type IncFII according
250 to plasmid Finder. It is associated with the following genes *ermB* and *mphA*. When,
251 Erythromycin is present it binds the leader peptide causing a change in conformation allowing
252 for the expression of *ermB* [37]. Regarding to *mphA*, it phosphorylates macrolides at 2'-OH
253 hydroxyl of desosamine sugar of macrolides in a GTP-dependent manner [38]. In Vietnam 2018,
254 it has already reported that IncFII is a plasmid replicon type for *mcr-3* in *E. coli* isolated from
255 food [39]. The *E. coli* Q4552 is a multidrug strain it has other replicon type plasmids IncX1_1,
256 IncFIA, IncFIB, those plasmids harbor a variety of antimicrobial resistance genes: $bla_{TEM}$, *sul3,*

*cmlA, aadA2, dfrA12, tetR, tetA*. It is frequent that colistin resistance strain is associated with other antibiotic resistance genes such as producing *E. coli* isolates collected from the faeces of diarrheic veal calves in France, it has *mcr* genes and ESBL [40]. The *E. coli* Q4552 is a special bacterium which belongs to the clonal complex Ccpx-568 and has an ST-843. The ST-843 has a close relation sheep with ST-80 and ST-1946 these clones provide from animal and human just like ST-843. ST-80 has been reported and sequenced for 30 times; this clone is pathogen (https://msphere.asm.org/content/3/4/e00179-18.full). Beside this almost of the clones owned the Ccpx-568 provide from animals.

The homologues plasmid close to that of *E. coli* with *mcr-3* are Plasmids pVE769 From *E. coli* 769 and pVNCEc59 from *E. coli* VNCEc57 both of those plasmids contain an *mcr-3* it was reported respectively in Vietnam and Japan [39,41]. It has been already reported an *E. coli* strain (FS13Z2S and FS3Z6C) possessing chromosomally and plasmidic encoded *mcr-1* [42]

In conclusion, this study describes a first phenotype and genotype of an atypical E. *coli* in Europe. We believe that is the first in France. Furthermore, this strain exhibited a multidrug resistance phenotype against a various antibiotics and harbour resistance genes. The most unusual co-occurrence is the fact that *mcr-1* is chromosomic with more than 4 IS transposases and a plasmidic *mcr-3*. These results lead to many other questions in the end. The function of *mcr* genes, according to the literature are enzymes expressing phosphoethanolamine transferase. Despite this co-occurrence of *mcr* gene does not necessarily make the strain hyper resistant to colistin which challenges us and requires a deep explanation on the mechanism of these co- occurrence genes.

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The authors declare that they have no competing interests.

**Authors' contributions**

designed the study, drafted and revised the manuscript.

performed medical examinations.

performed microbiology analyses.

All authors have read and approved the final manuscript.

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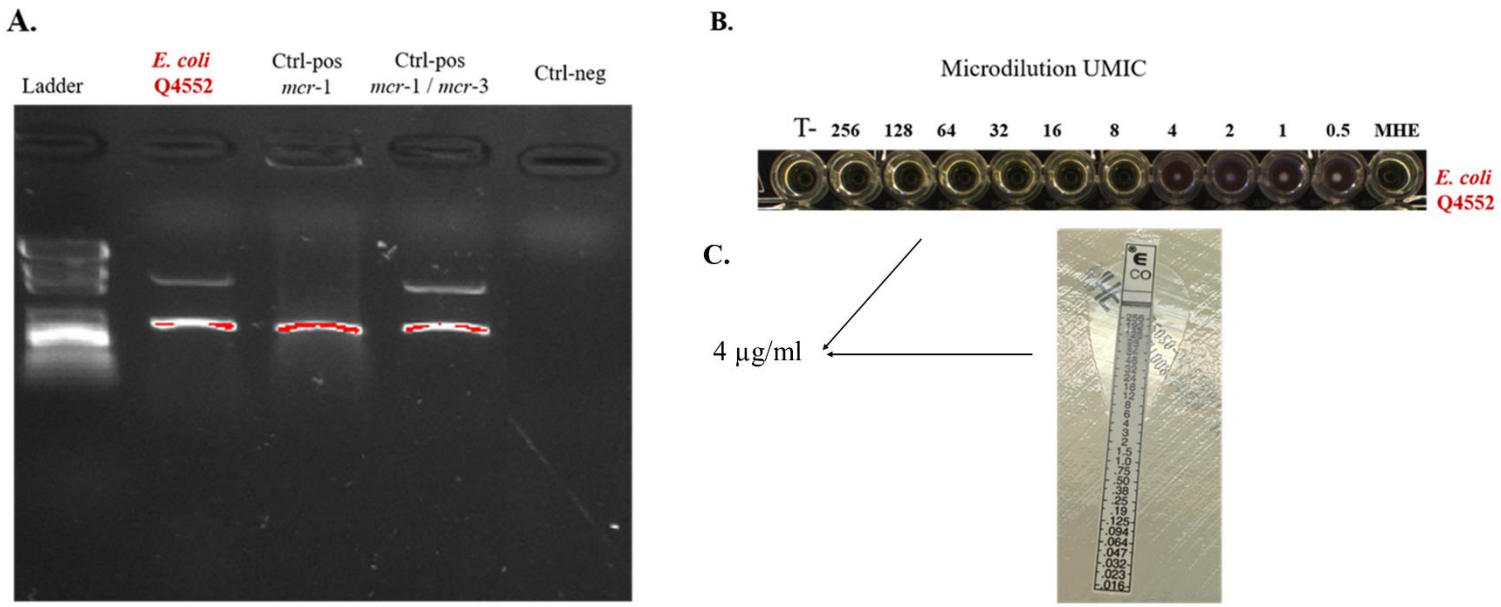


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399 **Figure 1:** Phenotypic Characterization of the *Escherichia coli* strain carrying the two genes of resistance to colistin. **1A:** Colistin resistance genes

400 detection: St-PCR multiplex for *E. coli Q4552* to detect *mcr*-1 and *mcr*-3. **1B.** AST antimicrobial susceptibility test according to EUCAST: E-

401 test. **1C.** AST antimicrobial susceptibility test according to EUCAST Microdilution UMIC and antibiogram.

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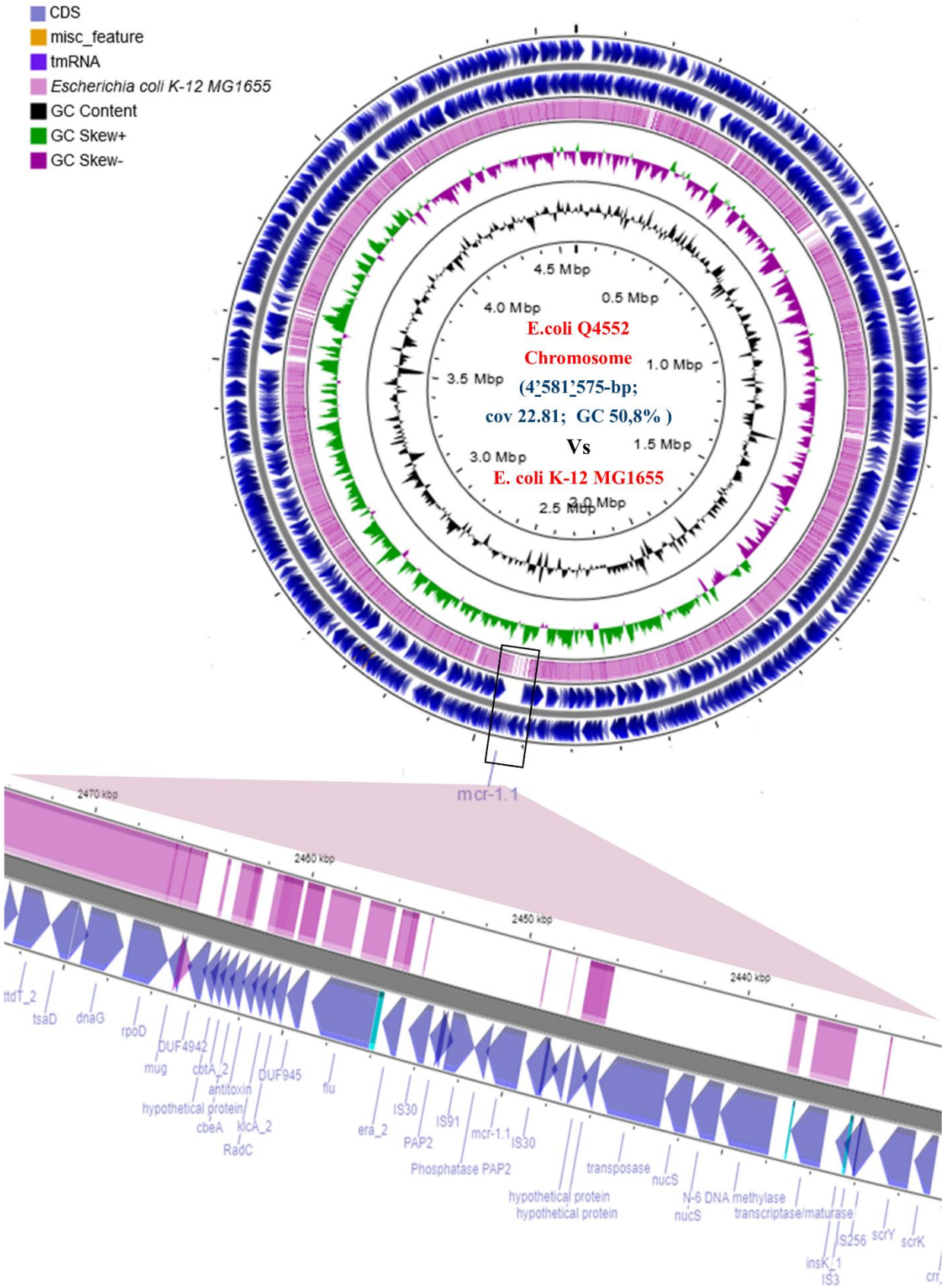


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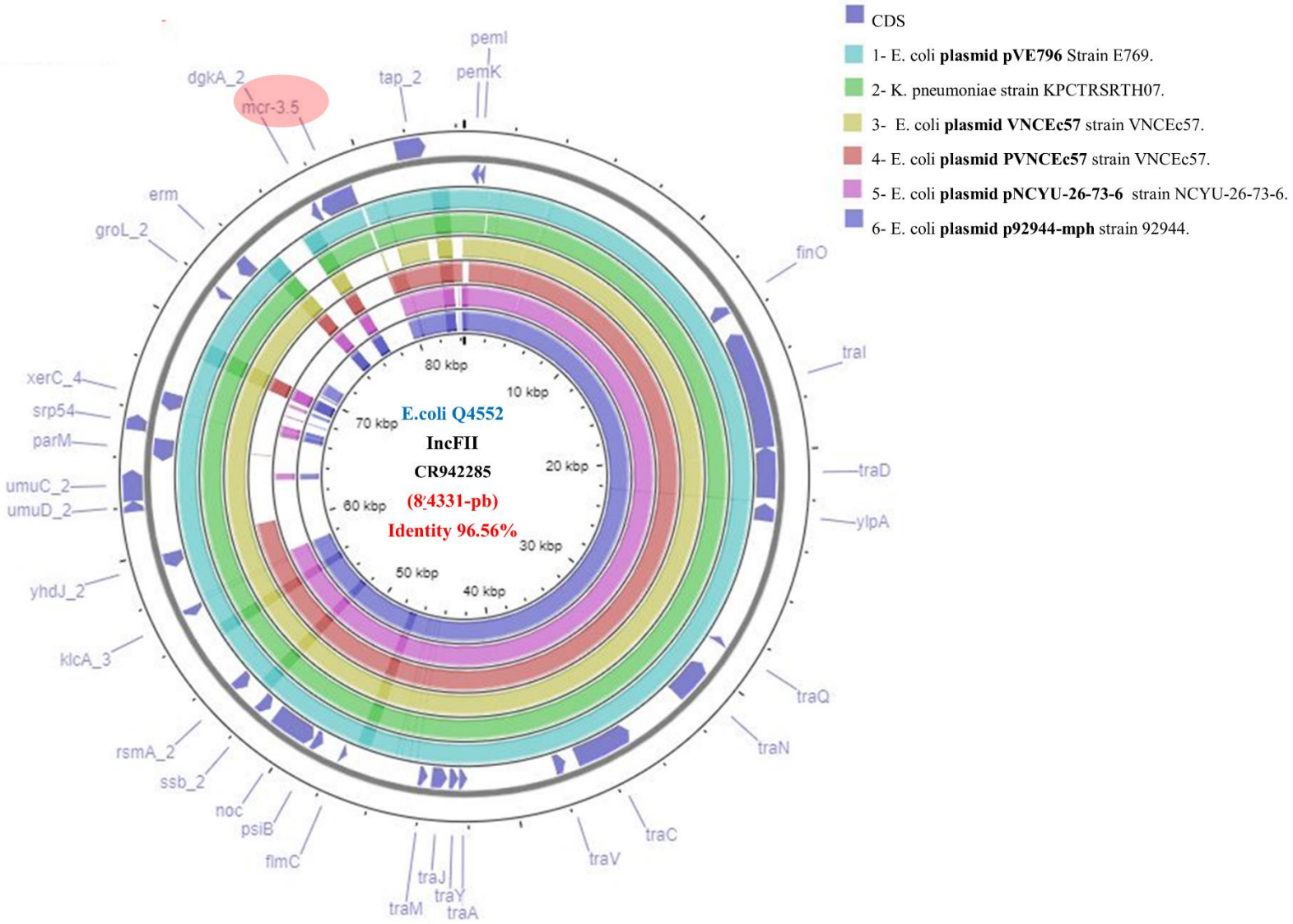
406 **Figure 2:** Circular maps of the chromosomal genome with *mcr-1.1* and zoom on genetic
407 environment of the transposon carrying the *mcr-1.1* gene.

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409 **Figure3**: Circular map of the plasmid carrying *mcr-3.5* compared to its 6 homologous plasmids with considerable query coverage**.**

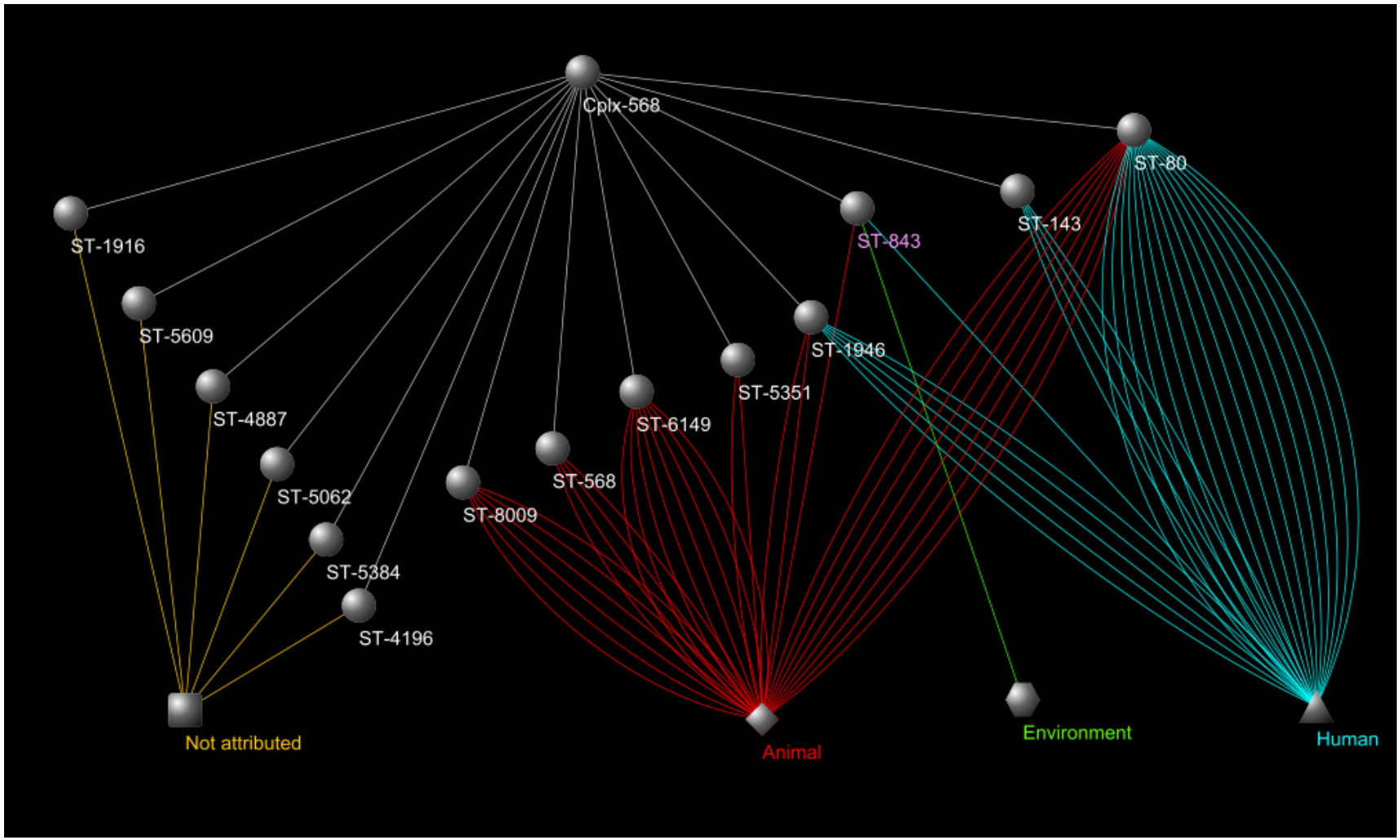


**Figure 4:** MLST and cytoscape analysis of *Escherichia coli* Q4552 (ST843) clone compared with the other clones associated to Ccpx568.

413 Table 1: Phenotypic and genotypic comparison of *Escherichia coli* Q4552 according to antibiotic family

| Antibiotic Family | Antibiotics | CMI (mm) | CMI (mg/ml) | ARG's | encoded gene for | %COVERAGE ARG ANNOT | % COVERAGE Resfinder |
|---|---|---|---|---|---|---|---|
| **β-lactams** | Penicillin A: Amoxicillin | R:0 | | (Bla)AmpC2 | β-lactamase Class C | 99% | 100% |
| | Amoxicillin + Clavulanic acid | R:16 | >256 | (Bla)AmpC1 | β-lactamase Class C | 99,88% | 100% |
| | Oxacillin | R | >256 | (Bla)blaTEM | BLSE Class A | 100% | 100% |
| | Piperacillin | S | 2 | | | 100% | 100% |
| | Aztreonam | S | 0,46 | | | | |
| | Penicillin G | R | >32 | | Carboxypeptidase endopeptidase | | |
| | Tazobactam piperacillin | S: 24.2 | 0,38 | | | | |
| **Cephalosporins** | Cephalosporin G1: cefalotin | I:16,1 | 0,32 | acrF | Inner membrane transporter | 100% | |
| | Cephalosporin G2: cefotaxime | S | <0,25 | AcrE | Membrane fusion protein | 99.66 % | |
| | Cephalosporin G3: ceftriaxone | S:38,8 | | acrS | Efflux pump | 99% | |
| | Cephalosporin G4: cefepime | S:30 | | | | | |
| **Carbapenems** | Meropenem | S | <0,125 | | | | |
| | Ertapenem | S: 39.3 | 0,03 | | | | |
| | Imipenem | S:35.6 | <0,32 | | | | |
| **Glycopeptides** | Vancomycin | R | >256 | | | | |
| **Macrolides** | Clindamycin | R | >256 | erm(B)_1 | | 100% | 100% |
| | Erythromycin | R | >256 | mph(A) | | 100% | 100% |
| | Levofloxacin | S | 0,125 | emrA, emrB, emrR | Multidrug efflux pump | 99% | |
| **Quinolone** | Ciprofloxacin | R | 1024 | mdtH | Multidrug resistance protein | 97,87% | |
| **Tetracycline** | Doxycycline | R:10.4 | 32 | (Tet)tetA, | Tetracycline efflux pump | 94,12% | 99,92 |
| | Minocyclin | | 12 | (Tet)tetR | Tetracycline efflux pump | 100% | |
| **Sulfamids** | Trimethoprim sulfamethoxazole | R:0 | | dfrA12_8 | | | 100% |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Sulfamid | R | 64 | (Sul)sul3 | | 100% | 100% |
| **Aminoglycosides** | Streptomycin | R | | (AGly)aadA1-pm<br>(AGly)aadA2 | Aminoglycoside 6-Acetytransferase | 93.86%<br>100% | 98.48%<br>95.24% |
| | Gentamicin | S:37.6 | <0,64 | | | | |
| | Netilmicin | | 0,25 | | | | |
| | Kanamycin | | 1,5 | | | | |
| | TGC Tigecycline | | 0,5 | | | | |
| | Amikacin | S:25.6 | 1,5 | | | 100% | |
| **Phenicols** | chloramphenicol | | | (Phe)cmlA1 | Mutation | 100% | |
| **Polymyxin E** | Colistin | R: 13,6 | 4 | (Col) mcr-1.1 | Phosphatidylethanolamine Transferase | 100% | 100% |
| | | | | (Col) mcr-3.5 | | 100% | 100% |
| **OTHERS** | Fosfomycin | | 0,125 | mdtG | Multidrug efflux pump | 99.27 % | |
| | Metronidazole | | >256 | | | | |
| | Rifampicin | | >32 | | | | |

## Supplementary data

**Table 1:** Sequences of primers and probes used for real-time PCRs and conventional PCRs in this study[23].

| Gene | PCR Type | Primers / Probes | PCR product |
|---|---|---|---|
| *mcr-1* | RT PCR | F: GCAGCATACTTCTGTGTGGTAC | 180 |
| | | R: ACAAAGCCGAGATTGTCCGCG | |
| | | Probe : FAM-GACCGCGACCGCCAATCTTACC-TAMRA | |
| | Standard and sequencing | F: GCAGCATACTTCTGTGTGGTAC | 893 |
| | | R: TATGCACGCGAAAGAAACTGGC | |
| *mcr-2* | RT PCR | F: CTGTGCCGTGTATGTTCAGC | 151 |
| | | R: TTATCCATCACGCCTTTTGAG | |
| | | Probe: VIC-TGACCGCTTGGGTGTGGGTA-TAMRA | |
| *mcr-3* | RT PCR | F: TGAATCACTGGGAGCATTAGGGC | 144 |
| | | R: TGCTGCAAACACGCCATATCAAC | |
| | | Probe: FAM-TGCACCGGATGATCAGACCCGT-TAMRA | |
| | Standard and sequencing | F: AAATAAAAATTGTTCCGCTTATG | |
| | | R: ATGGAGATCCCCGTTTTT | |
| *mcr-4* | RT PCR | F: GCCAACCAATGCTCATACCCAAAA | 112 |
| | | R: CCGCCCCATTCGTGAAAACATAC | |
| | | Probe : FAM-GCCACGGCGGTGTCTCTACCC-TAMRA | |
| *mcr-5* | RT PCR | F: TATCCCGCAAGCTACCGACGC | 126 |
| | | R: ACGGGCAAGCACATGATCGGT | |
| | | Probe: FAM-TGCGACACCACCGATCTGGCCA-TAMRA | |
| *mcr-8* | RT PCR | F: TCCGGGATGCGTGACGTTGC | 158 |
| | | R: TGCTGCGCGAAT-GAAGACG | |
| | | Probe: FAM-TCATGGAGAATCGCTGGGG-GAAAGC-TAMRA | |

# CONCLUSION ET PERSPECTIVES

**Conclusion et perspectives**

Malgré un intérêt croissant pour la colistine ces dix dernières années, les mécanismes d'action et de résistance à cet antibiotique restent encore incomplets. Cette difficulté tient dans le fait que la colistine agit sur un organe essentiel pour les bactéries, la membrane externe, dont la composition et les voies métaboliques impliquées dans la synthèse sont variées et complexes.

En conclusion, mes travaux de thèse ont contribué, à travers ma revue de la littérature, à la restitution des connaissances actuelles des mécanismes de résistance à la colistine à travers les méthodes utilisées pour leur découverte qu'elles soient anciennes ou récentes. Nous avons développé de nouveaux outils dans le but d'améliorer nos connaissances sur la résistance à la colistine. Les premiers mécanismes identifiés concernant la résistance à la colistine ont essentiellement été caractérisés par des techniques de clonage et de mutagénèse, suivies par la caractérisation des gènes et des protéines identifiés. Ces travaux longs et laborieux ont mis au jour plusieurs gènes tels que *pmrA/B*, *phoP/Q*, mais un nombre important d'autres protéines supposées impliquées n'ont jamais été caractérisées. L'avènement et la démocratisation des techniques de séquençage à haut débit, pour l'étude du transcriptome et la caractérisation des mutants *in vitro*, sont des méthodes intéressantes pour de futurs travaux. De même, les techniques de CRISPR-Cas9 permettraient d'améliorer le rendement de la mutagénèse dirigée.

Nous avons montré dans la deuxième partie de notre thèse, l'utilité de la mise en place de ces techniques pour la compréhension des mécanismes de résistance dans des souches cliniques humaines. En effet, dans un pays à forte endémie de résistance à la colistine comme la Grèce, l'inactivation du gène *mgrB* chez *K. pneumoniae* était présente chez 60% des souches et représentait le mécanisme

principal de cette résistance. Cependant, plus de 30% des souches présentaient un mécanisme de résistance non identifié, et nous avons également eu la surprise de détecter des souches de *K. pneumoniae* présentant un gène *mgrB* inactivé mais qui restaient sensibles à la colistine. Ces résultats laissent penser que le mécanisme lié à l'inactivation de *mgrB* n'était pas le seul impliqué, et que d'autres voies métaboliques restent à découvrir, ce qui permettrait aussi d'expliquer la forte disparité des CMIs observées entre les souches résistantes ayant un gène *mgrB* inactivé. Il en est de même pour les facteurs favorisant l'émergence de la résistance à la colistine. Si l'utilisation massive de la colistine est un facteur bien identifié dans des pays où la consommation est forte, d'autres facteurs semblent être impliqués dans des milieux où la consommation est limitée. L'émergence de la résistance peut être liée à l'expansion d'un clone résistant ou ayant une facilité à acquérir cette résistance, cette expansion étant liée au clone lui-même et/ou à des facteurs extérieurs. De plus amples investigations concernant les particularités de ces clones sont à réaliser.

Enfin dans une troisième partie, l'étude de souches particulières présentant des mécanismes de résistance ou ayant un phénotype atypique ont été sélectionnées pour les étudier de manière approfondie par des techniques innovantes. À l'aide de la mutagénèse aléatoire, nous avons montré que les voies métaboliques impliquées dans la résistance à la colistine chez une souche clinique d'*E. coli* faisaient intervenir une multitude de gènes, ces gènes apparaissant comme co-dépendants les uns des autres. Le rôle de la membrane externe apparaît comme primordial dans le développement de cette résistance pour cette souche.

Alors que l'on pensait que la résistance à la colistine était moins complexe chez *K. pneumoniae*, l'isolement de souches sensibles ayant un gène *mgrB* tronqué nous montre que ces mécanismes sont en fait plus complexes. Nos résultats laissent penser

que d'autres régulateurs que *mgrB* jouent un rôle dans la cascade d'activation des systèmes à deux composants et dans la synthèse de sucres ou alors qu'une autre voie indépendante pourrait intervenir. De plus amples analyses restent nécessaires pour l'étude de ces souches, notamment par l'utilisation de la transcriptomique et de la protéomique. Nos résultats montrent qu'il semble exister un lien entre la résistance à la colistine et les clones bactériens, parce que certains présentent des membranes particulières, parfois des capsules, qui jouent un rôle dans la résistance à la colistine. L'étude de la clonalité des bactéries peut générer une compréhension plus complète de l'importance des clones à haut risque dans la diffusion internationale de la résistance à la colistine. Mais les mécanismes de résistance à la colistine restent très complexes. En effet, des rapports récents de protéomique, métabolomique et transcriptomique démontrent le rôle du métabolisme bactérien dans la régulation de la résistance à la colistine (Cheng *et al.*, 2018; Su *et al.*, 2018; Sun *et al.*, 2018, 2020; Li *et al.*, 2019). Le développement de ces nouvelles technologies devrait permettre de mieux comprendre l'émergence de la résistance à la colistine chez *K. pneumoniae*, *E. coli* mais également chez *E. cloacae* dont la résistance à la colistine émerge ces dernières années et chez d'autres bactéries en général. Enfin, notre travail montre qu'il est primordial de mettre en place une surveillance de cette résistance pour pouvoir détecter de nouveaux clones résistants susceptibles de diffuser dans la population humaine.

Les facteurs de risque sont également peu étudiés. En effet, peu de rapports sur les facteurs de risque associés à la résistance à la colistine ont été publiés et les études réalisées concernent essentiellement des pays à forte endémicité de la résistance aux carbapénèmes et à la colistine. Les facteurs de risque connus pour la résistance à la colistine comprennent la pression de sélection, une antibiothérapie

antérieure, une hospitalisation antérieure et une colonisation antérieure par des bactéries multi-résistantes, le sexe masculin et l'immunosuppression ont également étaient rapportés (Büchler *et al.*, 2018). D'autres pistes d'études des facteurs de risques sont en cours d'exploration comme le lien entre phages et résistance. En effet, il semblerait que les gènes plasmidiques *mcr* procurent un rôle de protection immunitaire envers les bactériophages et les défensines humaines. L'ajout de la phosphoéthanolamine par les gènes *mcr* modifient le LPS, support de fixation des bactériophages, entrainant ainsi un camouflage du récepteur de l'antigène contre les bactériophages. Tel est le cas pour les défensines qui partagent des similitudes au niveau du mécanisme d'action et de la structure chimiques avec la colistine. La phosphoéthanolamine transférase constitue un système de défense immunitaire ubiquitaire pour résister à la colistine, mais aussi au système immunitaire inné eucaryote et à la destruction par les bactériophages. Ceci montre le rôle de la membrane bactérienne spécialement chez les bactéries hyper virulentes, pour lesquelles la capsule confère une résistance à l'activité bactéricide des peptides antimicrobiens ce qui entrave également la pénétration des phages.

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