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Développement d'une nouvelle génération de pansements antimicrobiens à base d'enzyme à activité lactonase

Development of a new generation of antimicrobial bandages containing lactonases

Soutenue le 19 Septembre 2018 devant le jury :

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Abréviations

3-OH-PAME : **3**-hydroxy**pa**lmitate **m**éthyl **e**ster

3-oxo-C_{12} HSL : N-(3-oxo-dodécanoyl)-L-**H**omo**s**érine **L**actone

5-FU : 5-**F**luoro**u**racil

AHL : **A**cyl **H**omosérine **L**actone(s)

AI : **A**uto**i**nducer(s)

AI-1 : **A**uto**i**nducer-**1** ou Acyl Homosérine Lactone

AI-2 : **A**uto**i**nducer-**2** ou Furanosyl borate diester

AI-3 : **A**uto**i**nducer-**3**

AIP : **A**uto**i**nducing **P**eptide(s)

C_4-HSL : N-butyryl-L-**H**omo**s**érine **L**actone

C_6-HSL ou **HHL** : N-**h**exanoyl-L-**H**omo**s**érine **L**actone

C_{10}-HTL : *N*-décanoyl-L-**H**omocystéine**t**hio**l**actone

Cas : CRISPR **as**sociated protein

CDC : **C**enter for **D**isease **C**ontrol and Prevention

CNP : **C**-type **N**atriuretic **P**eptide

CRISPR : **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats

CWNA : **C**hemical **W**arfare **N**erve **A**gent

DSF : **D**iffusible **S**ignal **F**actor

FAO : **F**ood and **A**griculture **O**rganization

FDA : **F**ood and **D**rug **A**dministration

GC : **G**as **C**hromatography

***Gc*L** : ***G**eobacillus **c**aldoxylosilyticus* **L**actonase

HAI : **H**ealthcare **A**ssociated **I**nfection

HHQ : 4-**h**ydroxy-2-**h**eptyl**q**uinoline

IAS : **I**nfection **A**ssociée aux **S**oins

IFN γ : **I**nter**f**ero**n** gamma

IQS : **I**ntegrated ***Q**uorum* sensing **S**ignal

LC : **L**iquid **C**hromatography

MBEC : **M**inimal **B**iofilm **E**radication **C**oncentration

MLL : **M**etalo-β-lactamase **L**ike **L**actonase

MS : **M**ass **S**pectrometry

OCDE : **O**rganisation de **C**oopération et de **D**éveloppement **E**conomiques

OMS : **O**rganisation **M**ondiale de la **S**anté

PCA : **P**rincipal **C**omponent **A**nalysis

PLL : **P**hosphotriesterase-**L**ike **L**actonase (PLL)

PMSF : **P**henyl**m**ethyl**s**ulfonyl **F**luoride

PQS : **P**seudomonas **Q**uinolone **S**ignal ou 2-heptyl-3-hydroxy-4-quinolone

PTE : **P**hospho**t**ri**e**sterase

QS : ***Q****uorum* **S**ensing

QQ : ***Q****uorum* **Q**uenching

QSI : ***Q****uorum* **S**ensing **I**nhibitor

SDS : **S**odium **D**odecyl **S**ulfate

SDS-PAGE : **S**odium **D**odecyl **S**ulfate-**P**oly**a**crylamide **G**el **E**lectrophoresis

Sso*Pox** : ***S*ulfolobus* ***S****olfataricus* **P**araoxonase

$\mathbf{T_m}$: Melting Temperature

WHO : **W**orld **H**ealth **O**rganization

I. Introduction

Introduction

Les bactéries sont des organismes procaryotes unicellulaires qui ont longtemps été considérées comme des entités individuelles et « asociales » jusqu'à la découverte du *quorum* sensing (QS), un système de communication intercellulaire [1–3].

La première preuve d'une forme de communication chez les bactéries a été rapportée en 1965. Alexander Tomasz a observé que l'induction de la compétence chez *Streptococcus pneumoniae* (anciennement *Pneumococcus*) ne nécessite pas de contact direct entre les bactéries et peut s'effectuer via l'utilisation d'une molécule purifiée du surnageant de culture [4]. Quelques années plus tard, en 1970, des observations similaires sont décrites chez *Vibrio fischeri* (anciennement *Photobacterium fischeri*) pour la régulation de la bioluminescence [5]. En effet, chez *V. fischeri*, la production des enzymes responsables de la fabrication de photons est induite de manière importante seulement à partir de la phase exponentielle et non dans une culture fraichement inoculée à faible densité cellulaire. Les auteurs ont alors émis l'hypothèse d'une autoinduction dans la régulation de la bioluminescence chez cette bactérie. Il en découlera le terme d'autoinducteur pour décrire la molécule qui s'accumule dans le milieu de culture et qui est capable de déclencher la production de bioluminescence [6,7]. Par la suite, le terme de *quorum*[l] a été employé pour décrire l'idée d'un nombre minimal de bactéries nécessaire au déclenchement de comportements de groupe tel que la bioluminescence [8]. Au début des années 2000, le QS a été décrit et étudié chez de nombreuses bactéries à Gram positif et négatif et semble intervenir dans la régulation de processus variés comme la sporulation, la virulence, le biofilm ou encore la synthèse d'antibiotiques [9].

Le QS est donc un système de communication basé sur la sécrétion dans l'environnement de petites molécules organiques ou de courts peptides [10,11]. Ces molécules, ou autoinducteurs, s'accumulent au cours de la croissance jusqu'à atteindre une concentration seuil permettant l'activation de récepteurs membranaires ou directement de facteurs de transcription **(Figure 1)**. Ce mécanisme permet la synchronisation de l'expression de gènes lorsque la population atteint une haute densité cellulaire. L'objectif est d'optimiser l'utilisation des ressources et le coût énergétique de certaines voies métaboliques en fonction de la taille de la population. Bien que le QS soit principalement décrit et étudié chez les bactéries, des systèmes comparables ont aussi été découverts chez les archées [12] et les champignons [13,14].

Pour les bactéries à Gram négatif, le QS repose fréquemment sur la synthèse et perception des Acyl Homosérine Lactones (AHL) qui varient dans la longueur et les modifications de la chaîne aliphatique [10,11]. Ces molécules se fixent à un récepteur ou

[l] Dans la Grèce antique, le *quorum* constituait le nombre minium de citoyens (supposé de 6000) nécessaire pour l'établissement de nombreuses décisions comme le vote des lois ou l'ostracisme. Par extension le *quorum* représente aujourd'hui le nombre de membres présents exigé dans une assemblée délibérante pour que le vote soit valable (dictionnaire Larousse).

directement à un facteur de transcription. D'autres molécules peuvent également être utilisées par les bactéries à Gram négatif comme des quinolones (e.g. PQS ou *Pseudomonas* Quinolone Signal), des acides gras (DSF ou Diffusible Signal Factor) ou encore des composés cétoniques [10,11]. Chez les bactéries à Gram positif, des peptides (AIP) de 5 à 20 acides aminés sont secrétés et se fixent par la suite à des récepteurs membranaires induisant l'activation d'un facteur de transcription intracellulaire [10]. Enfin, un système commun aux deux types de bactéries, pouvant permettre une communication inter-espèce, utilise un furanosyl borate diester (AI-2) qui se fixe aussi à un récepteur membranaire activant un facteur de transcription responsable de la réponse intracellulaire [10,11].

Outre l'utilisation de systèmes de communications complets (*i.e.* voie de synthèse, AI et récepteur), certaines bactéries sont capables de percevoir les molécules signal produites par d'autres espèces. C'est le cas de *Escherichia coli* et *Salmonella enterica* qui, bien qu'incapables de produire des AHL, peuvent détecter ces dernières, ce qui peut influencer notamment la virulence ou encore la défense face aux bactériophages [15–18]. Bien que les études sur le QS s'intéressent majoritairement à la communication au sein d'une même espèce, quelques travaux ont rapporté des modifications de microbiotes suite à la modulation de la molécule signal AI-2 par des probiotiques [19,20]. Ceci souligne le rôle du QS dans l'architecture des écosystèmes bactériens.

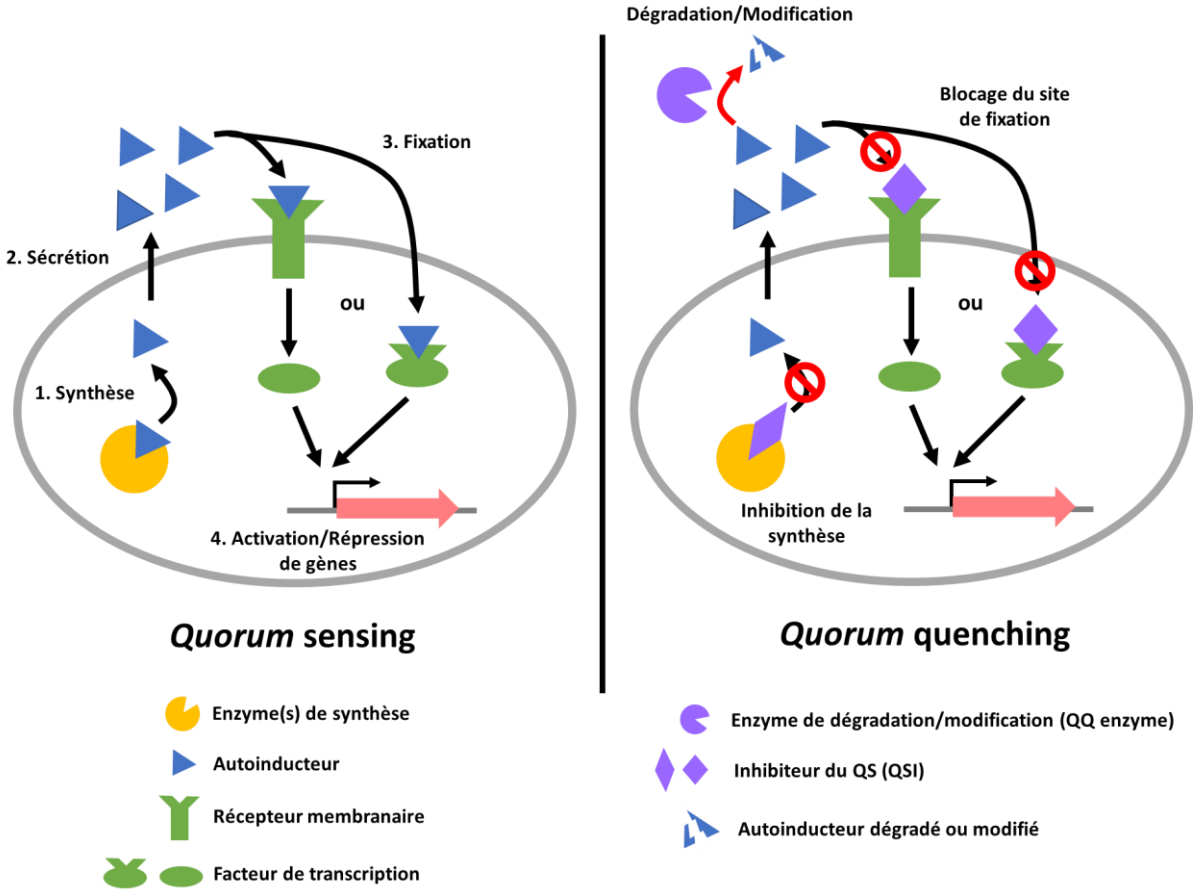


Figure 1 : Schéma simplifié du fonctionnement du QS chez une bactérie et des cibles du QQ.

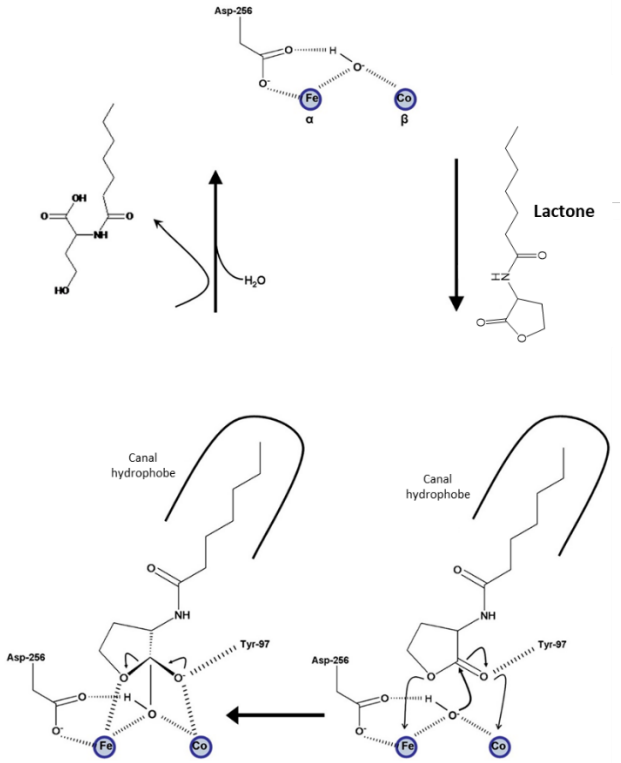
Le QS, communément associé à la mise en place de facteurs de virulence et du biofilm, constitue une cible privilégiée pour le développement de thérapies basées sur l'antivirulence [21]. Ces approches, rassemblées sous le terme *quorum* quenching (QQ), englobent tout moyen d'interférer avec la communication de type QS sans pour autant être bactéricide **(Figure 1)**. Elles peuvent agir sur la production ou la perception du signal mais aussi sur le signal lui-même [10]. Des molécules chimiques, mieux connues sous le nom de QSI (pour « *quorum* sensing inhibitor » en anglais), ont été développées pour inhiber les enzymes de synthèse, le récepteur ou le facteur de transcription en se fixant à la place de la molécule signal ou de son précurseur. Des anticorps pouvant séquestrer les autoinducteurs ont également été décrits, ainsi que des enzymes capables de dégrader ou modifier les molécules de signalisation sécrétées dans l'environnement. La diversité des molécules qui peuvent être employées pour le QQ offre de nombreuses possibilités pour contrer la virulence des bactéries et ouvre la voie à de nombreuses applications thérapeutiques. Ainsi le QQ pourrait être utilisé sous forme de médicament ou encore de dispositifs médicaux fonctionnalisés pour prévenir les infections.

Dans ce travail de thèse, une attention particulière a été portée à l'étude du QQ enzymatique en vue de développer des dispositifs médicaux d'un nouveau genre. Le développement de pansements antivirulents, contenant une enzyme capable d'interférer avec les communications bactériennes a ainsi été considéré. Plus particulièrement, les travaux se sont axés sur l'utilisation de la lactonase *Sso*Pox (*Sulfolobus solfataricus* paraoxonase) issue d'une archée hyperthermophile isolée des sources chaudes du Vésuve [22,23]. *Sso*Pox appartient à la famille des « phosphotriesterase like lactonases » (PLL) qui sont des métallo-enzymes ayant une activité naturelle lactonase et une activité de promiscuité phosphotriestérase [22]. Cette enzyme présente aussi d'autres activités plus marginales (arylestérase et phosphodiestérase) [23]. Le site actif comporte un centre bimétallique (fer/cobalt) activant une molécule d'eau en ion hydroxyde ce qui permet l'attaque nucléophile du groupement carboxyle de la lactone entrainant l'ouverture du cycle (**Figure 2**). Cette enzyme est capable de dégrader efficacement un large panel de lactones dont la N-(3-oxo-dodécanoyl)-L-homosérine lactone (3-oxo-C_{12} HSL) intervenant notamment dans le QS de *Pseudomonas aeruginosa*, une bactérie pathogène opportuniste de l'Homme fréquemment associée aux personnes atteintes de mucoviscidose, aux plaies infectées et aux infections associées aux soins (IAS) [24,25].

Le potentiel de *Sso*Pox pour inhiber *in vitro* la virulence de *P. aeruginosa* en bloquant la communication bactérienne a été préalablement mis en évidence [26]. Outre son potentiel pour interférer avec le QS, *Sso*Pox est particulièrement intéressante d'un point de vue applicatif compte tenu de son extrême résistance. En effet, cette enzyme, issue d'une archée extrêmophile, est résistante à des températures élevées (T_m[II] de 106°C) ainsi qu'à de nombreux agents dénaturants (e.g. SDS, urée) ce qui la rend compatible avec des contraintes

[II] T_m : la « melting temperature » ou température de fusion correspond à la température à laquelle la moitié de la population de protéines est dénaturée.

industrielles [27,28]. La stabilité présentant généralement un avantage pour l'évolution dirigée des enzymes, *Sso*Pox a fait l'objet de travaux de mutagénèse en vue d'accroitre son efficacité pour la dégradation du 3-oxo-C_{12} HSL [29] et a permis d'obtenir le variant *Sso*Pox W263I (*i.e.* monovariant de l'enzyme sur le résidu 263) dont l'efficacité catalytique a pu être augmentée d'un facteur 45 sur ce substrat. Ce variant s'est par la suite révélé extrêmement efficace pour réduire les facteurs de virulence et le biofilm produit *in vitro* par *P. aeruginosa* (e.g. pyocyanine et protéases) ainsi que la mortalité par un facteur quatre dans un modèle d'infection pulmonaire par cette bactérie chez le rat [30].



<u>Figure 2</u> : Mécanisme d'action de l'activité lactonase de *Sso*Pox. Figure adaptée de [22].

Le projet de thèse s'est inscrit dans la continuité des travaux précédents en poursuivant la caractérisation du variant *Sso*Pox W263I en vue de son utilisation dans des pansements. Son efficacité a ensuite été évaluée sur des souches modèles et cliniques de *P. aeruginosa*. Enfin, un dernier projet a consisté à déchiffrer les impacts phénotypiques et moléculaires de *Sso*Pox W263I sur *P. aeruginosa* en comparaison avec une autre lactonase récemment décrite, l'enzyme *Gc*L issue de *Geobacillus caldoxylosilyticus*, possédant un spectre d'action sur les AHL différent. En parallèle de ce travail expérimental, un intérêt particulier a été porté à l'analyse bibliographique du QS et des perspectives du QQ. Un premier état de l'art a permis de dresser l'inventaire des différentes applications biotechnologiques du QQ avec un focus particulier sur l'utilisation d'enzymes. Un deuxième travail bibliographique a ensuite été réalisé pour mettre en avant le potentiel du QQ dans le domaine médical en soulignant la complémentarité avec les traitements antibactériens existants (antibiotiques et bactériophages) et l'intérêt pour l'élaboration de dispositifs médicaux tels que les cathéters ou les pansements.

II. Synthèse Bibliographique

1. Les applications biotechnologiques du *quorum* quenching : focus sur les enzymes

Les agents antimicrobiens sont devenus incontournables dans la médecine moderne que ce soit les antibiotiques pour traiter les infections ou encore les solutions antiseptiques pour désinfecter des surfaces, des objets ou des plaies. Leur utilisation très répandue entraine l'augmentation de phénomènes de résistance via la sélection des bactéries les moins sensibles [31]. Récemment des résistances aux solutions hydroalcooliques pour la décontamination des mains ont également été décrites chez des entérocoques et pourraient être à l'origine de la recrudescence des infections à *Enterococcus faecum* dans certains services de soins [32]. Outre leur utilisation dans le domaine de la santé, les antimicrobiens sont aussi utilisés dans l'élevage pour limiter les infections (e.g. poissons, crevettes, porcs, poulets) [33,34], dans l'agriculture pour réduire la colonisation des plantes par des pathogènes [35] et même dans les peintures marines pour empêcher l'accumulation des micro- et macroorganismes sur les coques de bateaux [36].

L'omniprésence des agents antibactériens n'est pas sans conséquence pour les écosystèmes et leur utilisation est aujourd'hui remise en question dans de nombreux secteurs en manque de solutions alternatives. Ces molécules sont rejetées dans l'environnement via notamment les eaux usées des hôpitaux et les déchets animaux [37,38]. Elles favoriseraient ainsi l'émergence, la propagation ou le transfert de résistance entre bactéries [33,39]. Cette utilisation répandue a non seulement un impact sur l'apparition de résistances dans le domaine d'utilisation mais peut aussi favoriser leur transfert chez des pathogènes de l'Homme [38,40]. On estime aujourd'hui que les bactéries résistantes aux antibiotiques sont responsables au niveau mondial d'environ 700 000 morts par an dans le domaine médical [41]. Pour l'élevage, l'émergence de bactéries résistantes est aussi préoccupante car susceptible d'augmenter les risques d'épidémies difficiles à traiter chez ces animaux. Par ailleurs, les antibiotiques peuvent aussi poser des problèmes environnementaux car ils peuvent affecter non seulement certaines bactéries du sol et des eaux usées mais aussi les chloroplastes des plantes et le développement de certains microorganismes marins [39,42]. Des composés biocides à base de métaux sont également utilisés comme agent antifouling dans les peintures marines et ont un impact néfaste sur la faune aquatique [43]. Le tributylétain utilisé pour la protection des coques de bateaux a été banni des formulations commerciales à cause de sa grande toxicité envers les crustacées et sa bioaccumulation chez les animaux (e.g. phoques, poissons). D'autres agents biocides sont néanmoins encore utilisés comme le cuivre qui risque d'être banni à son tour.

Il y a donc un besoin croissant d'alternatives ou de compléments aux antibactériens utilisés pour contrer les effets des bactéries en diminuant l'occurrence de résistance et les impacts sur l'environnement. L'interférence avec la communication bactérienne, ou QQ, constitue une alternative prometteuse car elle présente une faible pression de sélection et permettrait de réduire la virulence chez des bactéries pathogènes de plantes, d'animaux et d'humains [10]. De plus, la grande variété de molécules utilisée pour le QQ permet d'envisager

de nombreuses applications allant du médicament aux systèmes de filtration en passant par les prébiotiques et les revêtements anti-biofilm.

De nombreux travaux ont démontré l'efficacité du QQ contre des bactéries pathogènes retrouvées en agriculture (e.g. *Pectobacterium carotovorum*), aquaculture (e.g. *Aeromonas hydrophila*) et en santé humaine et animale (e.g. *P. aeruginosa et Staphylococcus aureus*) [10]. Le QQ, permettant de lutter contre la formation du biofilm, s'est révélé efficace pour limiter l'encrassement de réacteurs à membrane pour le traitement des eaux usées et pourrait représenter une alternative au cuivre dans les peintures antifouling [44,45].

Parmi les molécules capables d'interférer avec le QS, les enzymes sont particulièrement prometteuses car elles agissent sur les molécules de communication sécrétées dans l'environnement. Elles n'ont pas besoin d'un contact direct avec les bactéries pour être efficaces contrairement aux inhibiteurs chimiques du QS qui ont besoin de se fixer aux récepteurs de la bactérie et peuvent se montrer bactéricides à haute concentration (e.g. azithromycine) [46]. De plus, les enzymes sont des protéines qui engendrent moins de pollution car elles sont biodégradables ne causant pas de phénomène de bioaccumulation contrairement à certains antibiotiques ou autres agents biocides.

Néanmoins, de nombreuses enzymes souffrent d'un défaut de stabilité qui peut limiter leur potentiel applicatif et la viabilité économique vis à vis de molécules chimiques classiques. Les enzymes issues d'organismes extrêmophiles constituent des candidats de choix grâce à leur robustesse qui peut leur permettre de répondre aux contraintes imposées par les procédés industriels.

Une première revue bibliographique a recensé les différentes applications du QQ depuis l'aquaculture en passant par les dispositifs médicaux, l'antifouling ou l'agriculture [47]. L'utilisation des enzymes est particulièrement mise en avant et une attention particulière est portée à l'enzyme *Sso*Pox issue de l'archée *S. solfataricus*. J'ai participé à la rédaction de cette revue ainsi qu'à la réalisation des figures, la relecture et la soumission du manuscrit. Ce travail a été publié dans Chemico-Biological Interactions.

1.1 Introduction

Bacterial communication, referred to as quorum sensing (QS), is the molecular mechanism by which bacteria sense their overall population density, allowing them to synchronize their behavior [7]. Bacteria produce small molecules known as autoinducers (AI) which are secreted in the environment and can be perceived by specific receptors within neighboring cells. This mechanism regulates gene expression patterns [9]. The response of microorganisms to QS is organism-dependent, but some traits are commonly regulated through QS, such as: production of antibiotics, exopolysaccharides, or exoenzymes, expression of secretion systems, swarming motility, and biofilm formation.

This review first summarizes the main aspects of bacterial QS and its implications in virulence and biofilm formation. Consequently, disrupting QS is particularly promising to modify bacterial behavior and moderate their undesirable traits. Different strategies have been considered for this purpose, including the use of QS inhibitors (QSI) or quorum quenching (QQ) enzymes. Special attention is then dedicated to applications involving QQ enzymes in various fields such as agriculture, animal and human health, and antifouling. The phosphotriesterase-like lactonase (PLL) family is then discussed as many of these enzymes have been found in extreme environments conferring attractive biotechnological capabilities. In addition, the possibility of resistance mechanisms to QQ strategies is discussed. The strengths and the weaknesses of this approach are emphasized in light of recently published research.

1.2 Quorum sensing

Several autoinducers have been identified as QS molecules. Gram-positive bacteria mainly use autoinducing peptides (AIP), also referred to as peptide-pheromones, which are specific to species and strains. Gram-negative bacteria use different types of QS systems: (i) acyl homoserine lactones (AHL), also known as autoinducer-1 (AI-1), are mostly produced by Gram-negative bacteria: it is a molecule composed of a lactone ring and an aliphatic chain whose length and nature may vary (e.g. *Pseudomonas* spp., *Acinetobacter* spp., *Burkholderia* spp.), (ii) autoinducer-2 (AI-2), a furanosyl-borate diester which is found in a wide range of both Gram-negative and Gram-positive bacteria (e.g. *Vibrio* spp., *Pectobacterium* spp.) , (iii) AI-3 (epinephrine and norepinephrine) are commonly found in human opportunistic pathogens (e.g. *Enterobacter* spp., *Escherichia* spp., *Klebsiella* spp., *Salmonella* spp.). Other molecules such as (iv) fatty acids (*Xanthomonas* spp.), (v) esters (*Ralstonia* spp.), (vi) α-hydroxy-ketones (*Legionella* spp., *Vibrio* spp.) or (vii) quinolones (*Pseudomonas* spp.) have also been reported [48–54]. Numerous Gram-negative bacteria utilize more than one QS system and may combine these systems either in additive models [55,56], or in hierarchical models when one system induces a second one [52], or with distinct or partially overlapping systems [57]. Considering the variety of signals and complexity of signaling networks, QS is a

sophisticated communication system used by bacteria to sense their population density and their surrounding environment [58].

Bacterial pathogens represent increasing concern to human health due to the rapid dissemination of antibiotic-resistant strains. Infections with these pathogens result in increased lethality risks and greater costs for health care systems. In several bacterial pathogens, QS is involved in the switch between commensal, or saprophytic lifestyles, to pathogenic cycles. This is the case for *P. aeruginosa* which is naturally present in water and humid environments. Moreover, *P. aeruginosa* is an opportunistic human pathogen that can proliferate in wounds; in such confined environments, QS signals accumulate and QS is triggered leading to the expression of virulence factors and the development of disease [59].

Bacterial pathogens also represent great financial burdens in industries other than health care. For example, bacterial infections of plants result in significant economic losses in agriculture [60]. The most widespread plant bacterial pathogens were recently listed according to their economic or scientific impact (e.g. *Ralstonia solanacearum*, *Xanthomonas* spp., *Pseudomonas syringae* pv., *Erwinia amylovora*) [61,62]. All of the selected bacteria use complex regulation networks where QS plays a central role to induce virulence. Additionally, fish or crustacean bacterial pathogens (e.g. *Vibrio* spp. [63,64]) have economic impacts in aquaculture, causing losses in livestock and contaminations that may be spread to humans.
QS also regulates the formation of biofilms. Biofilms are a specific mode of life where bacteria adhere to a surface and stick together. They build communities embedded in extracellular polymeric substances mainly made of DNA, proteins and polysaccharides that confer protection to environmental stresses (UV, dessication, antimicrobial compounds). Biofilms are particularly challenging as they can be formed on a wide range of surfaces, biotic or abiotic, and they contribute to the virulence and resistance of bacteria affecting numerous industries, spanning health care (contamination of medical devices), fisheries, and the oil industry [65–69].

Interfering with QS is an attractive strategy to inhibit biofilm formation and limit the pathogenicity of bacteria. This strategy was first described in 2000 through the identification of an enzyme that degrades AHL QS signal molecules [70]. Two QQ strategies can be distinguished: (i) to prevent bacteria from producing or perceiving QS signals and (ii) to degrade QS signals. The first strategy is mainly based on the identification of molecules QSI by screening natural compounds that will inhibit QS by different means. Halogenated furanones are one of the most common families of QSI and they were first isolated from a red macroalga, *Delisea pulchra* [71]. Further studies showed that they both target AHL or AI-2 mediated QS with distinct modes of action: they reduce the stability or binding affinity of the LuxR regulator and they inhibit the synthase, LuxS, by covalent interaction to prevent AI-2 synthesis [72–74]. Many screens have already been performed to identify such molecules. Most results were obtained in laboratory conditions but few direct applications using QQ compounds have been described. Following the example of QSI, QQ enzymes have also been investigated for their ability to disrupt QS without the need to enter the bacterial cell. Among these, AHL-

lactonases, acylases, or oxidoreductases have proved to display QQ activities. The next section is focused on the description of QQ biotechnological applications.

1.3 Applications

Plant pathogens

Bacterial plant pathogens rely on sophisticated regulation networks to synchronize the infection process and induce specific virulence factors when in contact with the host plant. Besides the perception of plant signals or nutrient availability, QS plays an essential role in the establishment of the pathogenic cycle. Therefore, QQ strategies are now considered as possible alternatives or complementary strategies to the use of pesticides [61]. Depending on the bacterial pathogens, different QS signaling molecules are produced: AHL for *Agrobacterium tumefaciens, Dickeya* spp., *Erwinia* spp., *Pantoea* spp., *Pectobacterium* spp. and *P. syringae*; AI-2 for *Erwinia* spp., *Pantoea* spp., *Pectobacterium* spp., 3-hydroxypalmitate methyl ester (3-OH-PAME) for *R. solanacearum,* and diffusible signal factors (DSF family) for *Xanthomonas* spp. and *Xylella fastidiosa* [75]. Most of these signals can be degraded by QQ enzymes: an esterase produced by the soil bacterium *Ideonella* sp. 0-0013 degrades 3-OH-PAME from *R. solanacearum*, the enzyme CarAB (a carbamoyl phosphate synthetase) produced by several *Pseudomonas* spp. degrades DSF signals. Lactonases or acylases are produced by many organisms to degrade AHL signals [76,77].

Some soil bacteria such as *A. tumefaciens* or *Bacillus* sp. naturally produce lactonases to degrade AHL [70,78,79]. For example *Bacillus thuringiensis* was shown to produce a lactonase, called AiiA, which degrades the AHL's produced by *Pectobacterium carotovorum,* thereby reducing its pathogenicity on potato slices [80]. In order to improve the efficiency of the *B. thuringiensis* lactonase AiiA, a fusion with a secretive protein was generated to enhance the dispersion of the lactonase in the environment, resulting in an increased tolerance to *P. carotovorum* on potato [81]. Since the 1960s, *B. thuringiensis* is commonly used as a biological pesticide against insects due to its natural ability to produce endotoxins lethal to moths, butterflies or mosquitoes [82]. Currently, its use against bacterial pathogens in fields has, to our knowledge, not been reported.

Another QQ strategy was also tested against bacterial plant pathogens: some plants were genetically modified using bacterial genes from *Bacillus* spp. or *A. tumefaciens* to produce lactonases. The first transgenic lines were reported in 2001, transforming tobacco and potato lines with the *aiiA* gene from *Bacillus*. The resulting transgenic lines showed an increased tolerance to *P. carotovorum* with symptoms only appearing after inoculation with very high bacterial concentrations [83].

These results showed that QQ has been used as a successful approach to protect plants from bacterial pathogens in laboratory conditions. Nevertheless, this demonstration was only achieved using plant GMO producing lactonases. QQ enzymes that may be used to treat and protect plants from bacterial infections is an attractive alternative to genetically modified

plants but is however impaired by the poor stability of enzymes. To circumvent this issue, the development of environmentally stable and chemical-resistant enzymes is crucial.

Another possible drawback in the use of QQ strategies for pest control could be the impact on beneficial or symbiotic bacteria that are naturally found in the environment. The ecological impact of tobacco lines expressing the lactonase AttM from *A. tumefaciens* was shown to be minimal, as no major difference was recorded between the root microbiota of transgenic and WT tobacco lines [84]. Nevertheless, if the bacterial populations were not impacted, some functions of bacteria using AHL-mediated QS might have been altered. This may prove incompatible with the use of *Pseudomonas* spp. as biocontrol agents. Indeed *Pseudomonas* spp. produce antibiotics and antifungal agents under control of AHL-mediated QS and using QQ strategies may prevent their beneficial effects [85]. To date, all experiments were performed in laboratory conditions with all interacting partners being inoculated simultaneously and at relatively high concentrations. The situation in the field is obviously different and further studies are needed to assess the impact of QQ and balance its drawbacks against its beneficial impact; controlling QQ enzyme specificity might be a way to modify this balance.

Aquaculture

According to the Food and Agriculture Organization (FAO), aquaculture is the farming of aquatic organisms in both coastal and inland areas involving interventions in the rearing process to enhance production. Economically, world aquaculture production represented about 97 million tonnes in 2013 (live weight) with an estimated value of 157 billion USD and 575 aquatic species registered [86]. Bacterial infections comprise a significant constraint to the development of aquaculture in the world, involving billions of USD in annual losses [87,88]. In the United States in 2012, infectious diseases are a top limiting factor that accounts for approximately 45% of losses in aquaculture [89]. The use of disinfectants and antibiotics has only limited success in treating or preventing aquatic diseases [87,88]. In developed countries, such as in the US, Canada or Norway, antibiotics have been restricted to limit the selection for resistant human pathogens [33]. However, the situation is much more problematic in countries with no or less stringent controls [33]. For example, in Chile, more than 385 tonnes of antibiotics were used in 2007 to produce a yield of 300,000 tonnes of Atlantic salmon [89]. It is estimated that about 1,500 tonnes of tetracycline and 478 tonnes of florfenicol were used in salmon aquaculture in Chile between 2000 and 2007, and 950 tonnes of quinolones between 2000 and 2008 for this purpose [90]. More importantly, massive use of antibiotics in aquaculture systems is leading to rapid evolution and spread of multiple antibiotic resistant strains that could potentially threaten human health security in significant ways [91,92]. As a consequence, numerous resistant human pathogens were isolated from aquacultures [93]. Various approaches are used to reduce contamination risks, such as the prevention of pathogen transmission between farms, stress reduction, or increasing hygiene [87]. Biological methods such as the utilization of probiotics [94], bacteriophage therapies, or immunostimulants were also investigated to prevent fish infections [87,95].

Immunostimulants are able to increase non-specific and/or specific immune response. Among these, β-glucans, alginate, or ergosan have been studied for their capacity to stimulate innate immune resistance or to enhance physiological and immunological factors [96,97]. Vaccines are also employed to control the majority of fish pathogens [98]. The methods of administration are immersion, injection, or addition in the food [99]. Injection is preferentially used, but is laborious and not effective for small or young fish [100]. However, none of these methods seem to significantly solve the problems of bacterial infections.

QQ is an appealing strategy that might reduce bacterial infections with a limited possibility that resistances will develop [87]. Numerous Gram-negative bacteria possess a QS system, including major fish pathogens such as *Aeromonas* or *Vibrio* spp. These bacteria use an AHL-LuxR/LuxI QS-like system with LuxR-LuxI homologues and the signal molecules are specific to each bacterium. More precisely, *Aeromonas hydrophila* mainly uses a N-butyryl-L-homoserine lactone (C_4-HSL) as signal molecule [101], whereas the *Vibrio parahaemolyticus* QS system is regulated by N-hexanoyl-L-homoserine lactone (C_6-HSL or HHL) [102]. AHL-degrading enzymes were thus investigated for disrupting QS of fish pathogens. Purified lactonases were tested and in particular oral administration of AHL-lactonase from *Bacillus* sp. strain AI96 was shown to decrease *A. hydrophila* infection in zebrafish [101]. Similar results were observed using AHL-lactonase (AiiAB546), from *Bacillus* sp. B546 produced by *Pichia pastoris*, in common carp [103]. Another pathogen, *V. parahaemolyticus*, is responsible for significant infections in shrimp and involves gastroenteritis for people who consume infected shrimp. The pathogen colonization of Indian white shrimp was successfully reduced after injection into the abdominal cavity of AHL-Lactonase from *Bacillus licheniformis* DAHB1 [102].

The use of lactonase-expressing whole cells was investigated as an economically-friendly alternative. For example, *Tenacibaculum* sp. strain 20J was shown to degrade C_4-HSL to C_{14}-HSL and was successfully used to decrease *in vitro* concentration of AHL produced by *Edwardsiella tarda* strain ACC35.1*,* a bacterium responsible for the Edwardsiella septicemia [104]. In order to select natural AHL's degrading microbial communities, shrimp fed with AHL's were used. One of the generated communities sampled from shrimp intestinal track was shown to protect a euryhaline rotifer from *Vibrio harveyi*. This microbial community proved to be able to degrade the *V. harveyi* HAI-1 autoinducer *in vitro* and *in vivo* [105].

Altogether these results suggest that QQ strategies are particularly attractive for limiting bacterial infections in the aquaculture industry. Moreover, QQ enzymes may be used in combination with prebiotics, probiotics, immunostimulants and vaccines to control and protect fish against a wide spectrum of pathogens.

It should be noted that biofilms in aquaculture pools act as reservoirs for the pathogenic bacteria that are responsible for recurring diseases [106], hence the importance of biofilm and biofouling treatments.

Antifouling

a. Membrane bioreactors and filters

Membrane bioreactors (MBR) are used in many industries or processes to combine a classic bioreactor system with a membrane filtration step. This technology is widely used in wastewater treatment for the bacterial cleaning of soluble pollutants and the retention of microorganisms and solid particles [107]. Fouling is a major concern encountered in such systems due to the accumulation of biological material both onto and into the membrane. In wastewater treatment, the filtration membrane is quickly colonized by organisms, thus reducing the efficiency of the process. To circumvent this drawback, high pressure is often required which represents an important energy consumption [108], as well as increased cleaning frequency, causing additional costs.

In order to counter biofouling, several approaches have been developed, including chemical, physical and biological strategies, for either preventing formation or cleaning [109,110]. In this context, QQ has emerged as a promising technology to inhibit the early stages of biofouling development.

The presence of AHL-producing bacteria in membrane biofouling has been demonstrated, highlighting the presence of species potentially using QS [108]. AHL-degrading enzymes or QS inhibitors are particularly attractive for minimizing the consequences of biofouling. Obviously, this would not avoid using classic cleaning methods, but it could reduce their frequency and decrease overall maintenance costs.

A study using membrane filters has shown the potential of vanillin to reduce biofilm formation, presumably due to the QQ effect of the molecule [111]. Two more practical studies have described the QQ effect of *Piper Betle* extract to reduce biofouling in MBR [112,113]. Other QS inhibitors could be used to prevent biofouling, but such molecules are usually soluble and would pass through the membrane, causing a secondary contamination.

Another approach is to use QQ enzymes that could disrupt bacterial communication, thus limiting the formation of biofilms and reducing impairment of the filtering system. The efficiency of Porcine kidney acylase I was demonstrated and showed an increasing lifetime of the filter in presence of the enzyme as compared to the control [108]. Most enzymes offer the advantage to be efficient and more stable when immobilized. Former enzymes were thus considered for immobilization strategies onto nanofiltration membrane [114], as well as alginate capsules [115], magnetic ion-exchange resin [116], and magnetic mesoporous silica beads [117]. These all increased stability and efficiency of the enzyme. In order to avoid the immobilization step and to limit the process costs, whole bacterial cells producing QQ enzymes were investigated [45,118–121]. Indeed, by entrapping the cells in different kinds of systems, be it microvessels or beads, the continuous production of one or more lactonases or acylases was developed. All these studies showed a significant diminution in biofilm formation and an increase of the MBR efficiency at low filtering pressure. In both cases, the QQ did not seem to impact the depolluting efficiency of the wastewater bacteria, confirming the potential of the technology [115,121].

b. Marine biofouling

Biofouling is also a major issue for structures in contact with seawater, such as boats, fish nets, or pipelines. Two related phenomenon usually occur on submerged surfaces: microfouling caused by microorganisms such as bacteria or protozoans, and macrofouling linked to algae or barnacles [44]. Biofouling is responsible for friction on boats, inducing excessive fuel consumption, increased maintenance costs, and generates considerable monetary losses annually [44,122]. Since tributyltin, an efficient antifouling molecule, has been banned due to its high toxicity, current solutions for biofouling prevention include paints and coatings, mainly containing copper as antifouling agent. The use of copper is also considered an environmentally unfriendly method but is still in use [122,123]. Antifouling paints and coatings save an estimated 60 billion USD annually [66].

In the quest for non toxic alternatives, enzymes have been considered for either preventing biofouling formation or destroying biofilms. Several reviews discuss the potential of these different enzymes and even consider actual patents for their incorporation into paints or coatings [44,122,124].

However, mature biofilms remain difficult to degrade and special attention is dedicated to prevent biofouling formation. In this respect, QS disrupting strategies would be of prime interest. First, it could reduce QS-regulated biofilm formation involved in microfouling but may also impact the attraction and fixation of macro-biofouling species. Different studies have demonstrated the influence of bacterial biofilm on the settlement of spores from algae or others [125–127]. A chemical approach would be to use QS inhibitors in the coating to prevent biofouling. In this way, kojic acid has been reported as a non-toxic QS inhibitor and is incorporated into painting, conferring the ability to inhibit both microfouling from bacteria as well as diatom *Amphora coffeaeformis* macrofouling over one month [127]. Even though QQ enzymes have been described in the literature as a solution for MBR fouling, no comparable work has been done yet for marine biofouling. The main constraint would undoubtedly be the lack of stability of enzymes within paints as well as their possible limited activity in seawater. QQ enzymes from extremophile organisms would constitute promising candidates as they usually are highly robust and may be active in non-conventional environments. Furthermore, compared with QS inhibitors, QQ enzymes would constitute an environmentally friendly solution as they may be active while incorporated irreversibly into paints or coatings, their action would be localized to the ship hull. Moreover, in case of releasing, enzymes could be degraded in the environment and would not bio-accumulate. QQ-based approaches, while restrictive as it does not prevent direct settlement of macrofouling species, would be a good complement of available approaches to reduce marine biofouling.

Medical devices

QS-induced bacterial infections are particularly problematic in medical environments. In the US, *P. aeruginosa* accounts for 7.5 % of general healthcare associated infections. Other pathogens, such as *Acinetobacter baumannii, Proteus* spp., and *Serratia* spp., are overall responsible for 6.4 % of the infections. These pathogens are often found with catheter

associated urinary tract infections and ventilator associated pneumonia [128]. As such, healthcare facilities could directly benefit from research on AHL-targeted QQ. Furthermore, QS is responsible for a number of problematic complications such as antibiotic resistance, biofilm formation, competence, and virulence factor expression [129–131]. Hereafter are presented QQ-based devices, mainly based on enzymatic functionalization, to circumvent QS-mediated bacterial infections.

a. Quorum quenching membrane

The immobilization of the hyperthermostable Phosphotriesterase-Like Lactonase (PLL) from *S. solfataricus*, referred to as *Sso*Pox, onto nanoalumina membranes to quench bacterial communication was investigated [26]. About 95 % of the enzyme was successfully immobilized with interactions tight enough to resist high ionic strength washes. The enzymatic activity after immobilization was 25 % that of the free enzyme. The addition of membranes containing *Sso*Pox in bacterial cultures of *P. aeruginosa* PAO1 resulted in an overall decrease of pyocyanin expression and elastase activity. For the first time, the authors demonstrated that immobilization of QS-disrupting enzymes may be useful to decrease the virulence of bacterial pathogens and paved the way for the development of innovative medical devices.

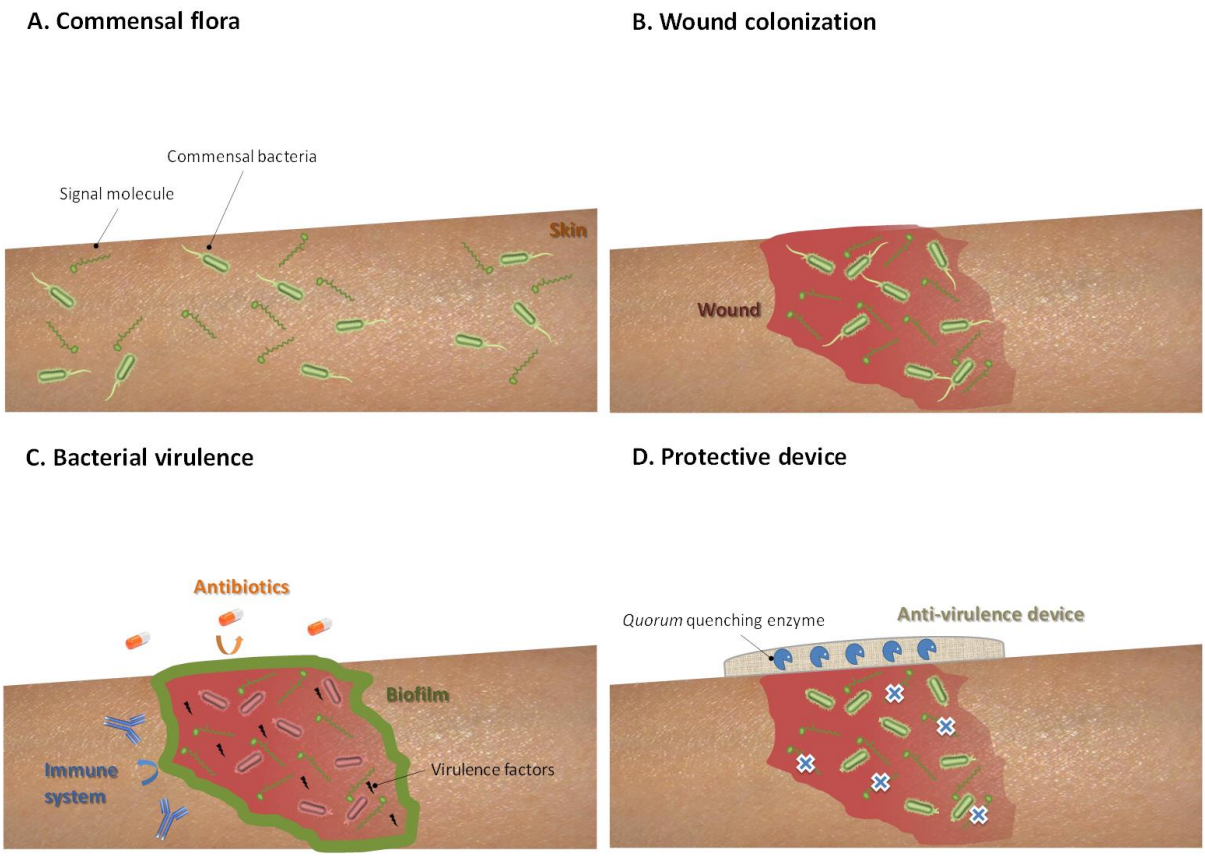
b. Functionalized catheter

The persistence of pathogens in catheters is quite problematic both in terms of costs and patient health [132]. To address this issue, a central venous catheter coated with the QSI 5-fluorouracil was developed and clinically evaluated [133]. The study was performed with a total of 960 adult patients in 25 US intensive care units and demonstrated that 5-fluorouracil coated catheter is a safe and promising alternative to catheters coated with silver sulfadiazine or chlorhexidine. Catheter functionalization with catalytic quenchers such as enzymes has also been investigated using a silicon catheter coated with multiple layers of an acylase from *Aspergillus melleus* [134]. The adherence of *P. aeruginosa* ATCC 10145 was assessed and showed to be strongly inhibited as compared to the non-treated control. Biofilm quantity was also reduced by about half in the acylase coated catheter, both in static and dynamic models. In itself, the coated catheter proved to be non-toxic against cultured skin fibroblast. Very recently, the development of functionalized urinary catheters combining both a QQ acylase and a matrix degrading α-amylase was reported. The acylase and amylase limited the biofilm formation of *P. aeruginosa* and *Staphylococcus aureus* respectively. This device was further evaluated in an *in vivo* animal model and was shown to delay biofilm development for up to 7 days [135].

c. Topical treatment and dressing perspectives

A burn infection model on mice using *P. aeruginosa* PAO1 was developed to assess the efficiency of a purified lactonase from Bacillus sp. ZA12 [136]. Animals were burned to the third degree and infected with a lethal dose of 106 bacteria subcutaneously. Topical application with a lactonase-containing gel prevented systemic spread of *P. aeruginosa*

through burned skin and reduced animal mortality. When the lactonase was combined with ciprofloxacin no mortality was observed, underlining the synergistic effect of the treatments. This report highlighted the efficiency of quenching enzyme by topical administration alone or in combination with antimicrobial treatments to prevent bacterial infection of wounds. Histological studies demonstrated that fewer damages and inflammatory markers were observable in tissues when the enzyme and antibiotics were used together. This would offer new perspectives for the investigation of enzymatically functionalized dressings and bandages **(Figure 3)**.



<u>Figure 3</u> : Enzyme-functionalized anti-virulence device and mechanism of action. A) Commensal bacteria are naturally present on healthy skin and use signal molecules to communicate without being virulent. B) In case of wound, the bacteria have a favorable medium for their growth and start their colonization step. C) When bacterial concentration is over a certain threshold the bacteria adapt their behavior and start being virulent by reducing motility [137], synthesizing a biofilm and secreting virulence factors D) Enzyme-containing devices hydrolyze QS signal molecules and prevent infection by decreasing virulence factor secretion, biofilm synthesis and motility.

d. Aerosolization

The *in vivo* use of an exogenous, engineered hyperthermostable lactonase derived from *Sso*Pox, was reported in a rat pulmonary infection model. The purified enzyme was administered concomitantly to an infection by *P. aeruginosa* PAO1 resulting in a mortality drop, from 75% to 20%, 48h post infection [30]. The enzyme was also shown to be able to

reduce, *in vitro*, the biofilm formation of *P. aeruginosa* PAO1 by up to 65% as compared to the untreated condition. The aerosolized lactonase proved to be efficient to diminish the expression of QS dependent virulence factors and would be particularly relevant for the treatment of cystic fibrosis related infections.

As previously described, many studies have reported the potential of QQ strategies to inhibit bacterial virulence and limit biofilm formation of a wide range of bacterial strains and the corresponding applications are summarized in **Table 1**. Among these, the use of AHL degrading enzymes was particularly emphasized. Such enzymes may be used in a broad spectrum of applications, from agriculture, bioprocess and anti-fouling, to human and animal healthcare. However, many issues have to be addressed to strengthen their potential. Large-scale production, stability, tolerance to industrial processes, or storage have to be investigated as they represent major constraints to the use of enzymes for biotechnological purposes. In this context, phosphotriesterase-like lactonases appear to be particularly relevant as many of these may be isolated from extreme environments and display both high activity and large substrate specificity. The possible emergence of resistance phenomenon to QQ strategies has also to be considered.

Table 1 : Summary of QQ applications

Field	Application	Quencher	Reference
Agriculture	Transgenic lines of tobacco and potato	AiiA lactonase from Bacillus sp.	[83]
	Transgenic lines of tobacco	AttM lactonase from A. tumefaciens	[84]
Aquaculture	Oral administration in zebrafish	AI96 lactonase from Bacillus sp.	[101]
	Oral administration in common carp	AiiAB546 lactonase from Bacillus sp.	[103]
	Injection in indian white shrimps	Lactonase from B. licheniformis DAHB1	[102]
Antifouling			
MBR	Reduce biofouling in MBR	P. betle extract	[112,113]
	Increase filter lifetime	Porcine kidney acylase	[108]
	Nanofiltration membrane	Porcine kidney acylase	[114]
	Alginate capsules	Porcine kidney acylase	[115]
	Magnetic ion-exchange resin	Porcine kidney acylase	[116]
	Magnetic mesoporous silica beads	Porcine kidney acylase	[117]
Marine biofouling	Coating	Kojic acid	[127]
Medical devices	Nanoalumina membrane	PLL SsoPox from S. solfataricus	[26]
	Coated catheters	5-fluorouracil	[132,133]
		Acylase from A.melleus	[134]
		Acylase from A. melleus and α-amylase from B. amyloliquefaciens	[135]
	Topical treatment	Lactonase from Bacillus sp. ZA12	[136]
	Aerosol	PLL SsoPox from S. solfataricus	[30]

1.4 Phosphotriesterase-Like Lactonases (PLL)

Highly promiscuous enzymes

PLL are natural lactonase (EC 3.1.1.25) enzymes with promiscuous catalytic activity against organophosphates (e.g. paraoxon). These enzymes are strongly related to phosphotriesterases (PTE), constituting their most probable ancestor [138–140]. They belong to the amidohydrolase superfamily and their 3D-structure is formed by a $(\beta/\alpha)_8$-barrel fold, shows an active site containing two metal cations involved in catalysis and coordinated by four histidine residues, as well as a carboxylated lysine and an aspartic acid [22]. The bi-metallic center participates to the catalysis as a Lewis acid involved in the activation of a water molecule into a hydroxide anion that subsequently acts as a nucleophile to attack the substrate. Two subfamilies, PLL-A and PLL-B, were identified according to their sequence similarities as well as the length of the two characteristic loops 7 and 8. PLL-A have been shown to degrade AHL as well as oxo-lactones, while PLL-B's are specific to oxo-lactones [141] . As such, both PLL-A and -B may be considered for QQ strategies and PPL-A are particularly promising considering their broad substrate promiscuity. Furthermore, PLL are attractive because this family encompasses numerous representatives from extreme environments. These enzymes exhibit high thermal stability and robustness that is desirable in biotechnological applications. Among these, *Gk*L (isolated from the thermophile *Geobacillus kaustophilus*) [142], *Sac*Pox (isolated from the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius*) [143], *Sis*Lac (isolated from the hyperthermophilic archaeon *Sulfolobus islandicus*) [28], *Sso*Pox (isolated from the hyperthermophilic archaeon *S. solfataricus*) [22,28,29,144], or *Vmo*Lac (isolated from the extremophilic crenarchaeon *Vulcanisaeta moutnovskia*) [141,145], have drawn special interest. Regarding their intrinsic stability (e.g. T_m values of 106°C and 128°C for *Sso*Pox and *Vmo*Lac, respectively) some of these PLL have been further considered for directed evolution experiments to improve their potential for QQ purposes [29,146,147].

PLL have distinct sequences and structures from another family of QQ lactonases belonging to the metallo-β-lactamase superfamily **(Figure 4)**. This superfamily includes the above-mentioned enzyme AiiA from *B. thuringiensis* [70,148]. Although these proteins share neither sequence nor structural similarity, their active sites show striking similarities [140].

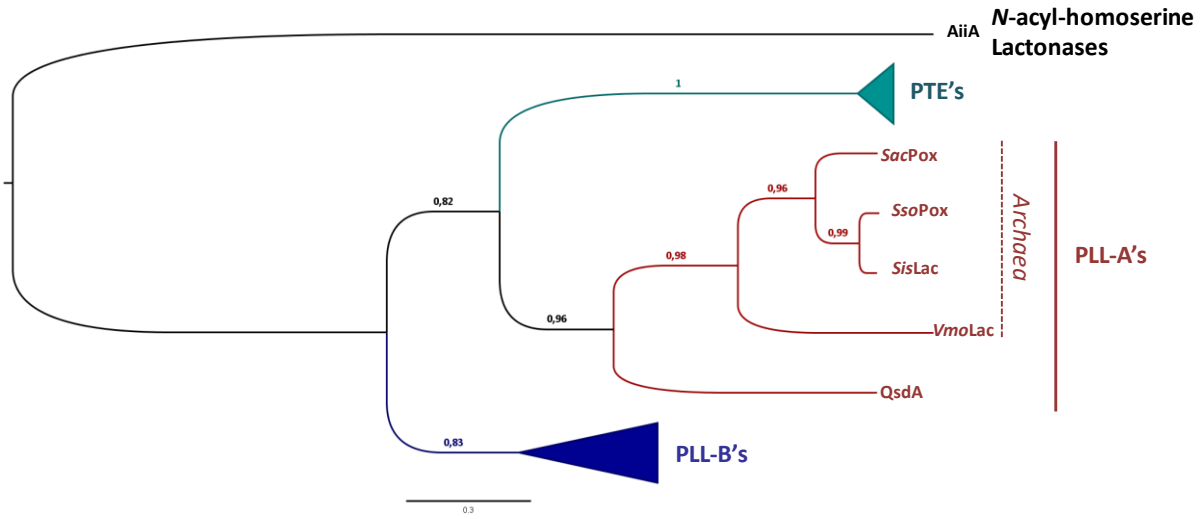


Figure 4 : Phylogenetic tree of PLL's and enzyme-related families. Webtool www.phylogeny.fr/simple_phylogeny.cgi was used for sequence alignment and phylogeny and the tree was obtained with FigTree v1.4.0. Sequence used for the analysis were: AiiA (P0CJ63), PTE (Q93LD7, P0A434), SacPox (V9S7Z1), SsoPox (Q97VT7), SisLac (C4KKZ9), VmoLac (F0QXN6), QsdA (B1N7B5), PLL-B's (A4IN23, Q5KZU5, Q9RVU2)

SsoPox a promising candidate for QQ applications

One of the best characterized PLL's so far is *Sso*Pox. This enzyme, isolated from the hyperthermophilic archeon *S. solfataricus,* was initially investigated for its ability to hydrolyze phosphotriesters (widely used as pesticides and chemical warfare agents) [23]. *Sso*Pox is an extremely stable enzyme [144], active over a wide range of temperatures (10-100°C) and pH values (5.0-9.0), paving the way for a wide panel of biotechnological applications [23]. Moreover, it is a very proficient lactonase, including at room temperature. The 3D-structure of *Sso*Pox was reported and was found as being a distorted $(\beta/\alpha)_8$ barrel-fold [149]. Conversely to other known phosphotriesterases displaying a similar structural organization, *Sso*Pox differs by the length of two loops. Loop-7 and loop-8 are shorter and longer than other reported PTE, respectively. These modifications are responsible for the creation of a hydrophobic channel that perfectly accommodates the lactone substrate **(Figure 5)**.

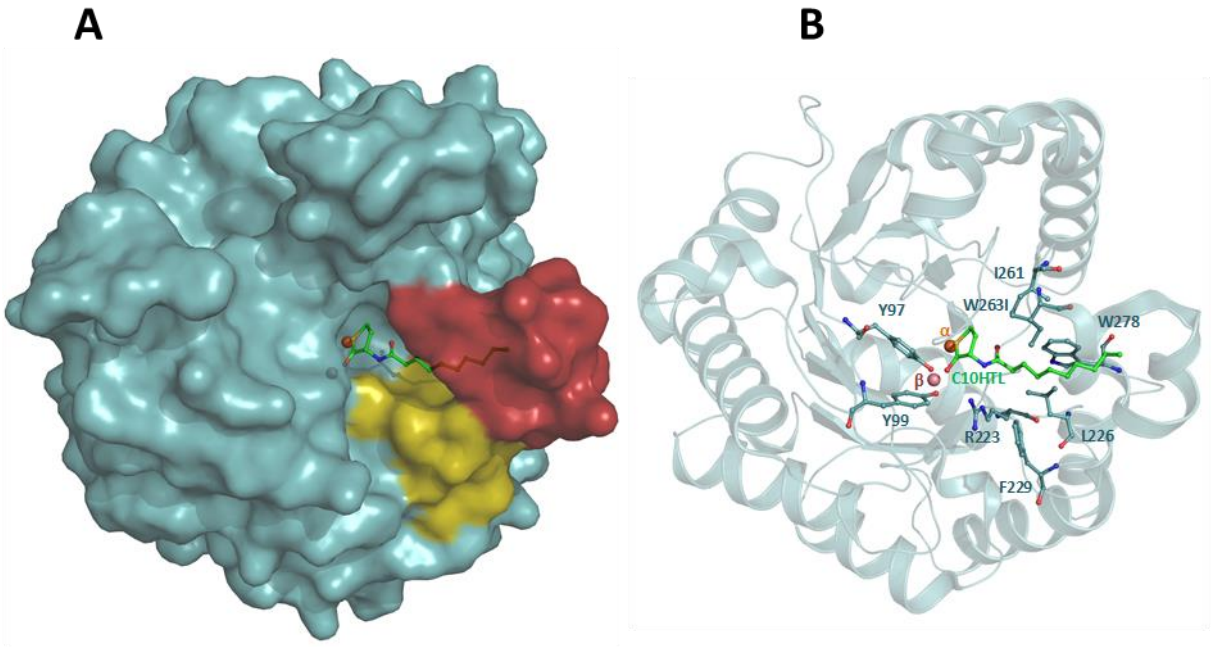


Figure 5 : Representation of SsoPox variant W263I structure (PDB ID: 4KF1) bound with substrate analogue C_{10}-HTL. (A) Structure is shown as surface and loop-7 and loop-8 are emphasized in yellow and red respectively. (B) C_{10}-HTL is shown in green stick, surrounding residues are emphasized by blue sticks and divalent cations are drawn as spheres.

Various lactones were assayed with the wild-type enzyme including AHL, γ-lactones, or δ-lactones **(Figure 6)** [22,29,138]. Catalytic parameters were determined and k_{cat}/K_M values up to $8.0x10^4$ $M^{-1}.s^{-1}$ were reported, suggesting that *Sso*Pox is a natural proficient lactonase. Protein engineering strategies were also considered to further increase its catalytic efficiency towards lactone substrates. Residue W263 was particularly considered as it is located in the active site and is involved in enzyme loop flexibility, mediating its promiscuity [29]. Saturation mutagenesis experiments were performed and led to the identification of extremely efficient variants (e.g. *Sso*Pox-W263I) with k_{cat}/K_M values up to $5.8x10^6$ $M^{-1}.s^{-1}$ at room temperature. About twenty lactones with various chemical structures are known to be hydrolyzed by *Sso*Pox and/or its variants underlining the wide promiscuity of these enzymes **(Table 2)**. Furthermore, *Sso*Pox and its variants exhibit a strong tolerance to proteases, surfactants, and also organic solvents [28,29].

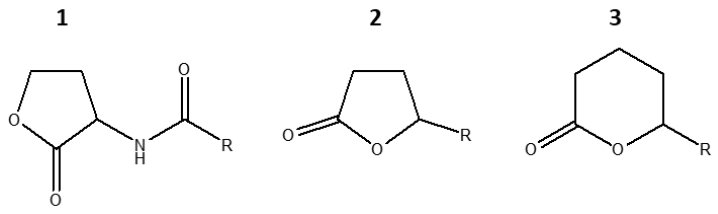


Figure 6 : Chemical structures of SsoPox lactone substrates. (1) Acyl-Homoserine Lactones, (2) γ-lactones, (3) δ-lactones.

About ten structures, either in *apo* or *holo* form, are now available in the protein database (www.pdb.org). Among these, variant *Sso*Pox-W263I was found to be particularly efficient for lactone hydrolysis and has been co-crystallized with the substrate analogue *N*-decanoyl-L-homocysteinethiolactone (C_{10}-HTL) **(Figure 5)**. This variant is of utmost interest for QQ investigations as it is both highly efficient for lactone hydrolysis and is widely promiscuous towards a large range of substrates. Moreover, *Sso*Pox-W263I retains an impressive thermostability (T_m = 88 °C), albeit lower than the wild-type, and, owing to its properties, might be suitable for a large panel of applications in the various domains afore-mentioned. Altogether, thermostable lactonases are particularly appealing for biotechnological investigations as these enzymes could be more readily compatible with material sciences, such as incorporation into coatings, materials, paints and polymers, and to develop innovative, non-toxic and environmental-friendly alternatives against bacterial infections and biofilms.

Table 2 : Catalytic parameters of SsoPox wild-type and variants towards lactones with respective reaction conditions. Data were taken from [22,29,138].

Substrate	Enzyme	Condition	k_{cat} (s^{-1})	K_M (µM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
3-oxo-C12 AHL (l)	WT	25°C	1.01	456	2.22×10^3
	W263F	25°C	0.41	146	2.81×10^3
	W263M	25°C	ND	ND	ND
	W263L	25°C	ND	ND	ND
	W263I	25°C	1.8	17.8	1.01×10^5
	W263V	25°C	3.0	24.7	1.21×10^5
	W263T	25°C	6.44	137	4.70×10^4
3-oxo-C10 AHL (l)	WT	25°C	4.52	143	3.16×10^4
	WT	25°C + 0.1% SDS	ND	ND	1.96×10^2
	WT	25°C + 0.01% SDS	0.75	243	3.09×10^3
	W263F	25°C	3.96	288	1.38×10^4
	W263M	25°C	ND	ND	ND
	W263L	25°C	ND	ND	ND
	W263I	25°C	0.6	1605	3.74×10^2
	W263V	25°C	0.19	1346	1.41×10^2
	W263T	25°C	0.11	1000	1.06×10^2
3-oxo-C6 AHL (l)	WT	25°C	0.08	558	1.49×10^2
3-oxo-C6 AHL (r)	WT	25°C	0.04	592	6.87×10^1
3-oxo-C8 AHL (l)	WT	25°C	0.54	123	4.39×10^3
3-oxo-C8 AHL (r)	WT	25°C	0.42	256	1.63×10^3
Undecanoic-δ-lactone (r)	WT	25°C	7.38	94	7.86×10^4
	W263F	25°C	66.5	135.2	4.92×10^5
	W263M	25°C	71.2	161	4.42×10^5
	W263L	25°C	56.8	219	2.59×10^5
	W263I	25°C	58.0	<10	$>5.80 \times 10^6$
	W263V	25°C	44.8	57	7.92×10^5
	W263T	25°C	93.3	130	7.17×10^5
Undecanoic-γ-lactone (r)	WT	25°C	4.95	2099	2.36×10^3
	WT	25°C + 0.1% SDS	2.23	1250	1.78×10^3
	WT	25°C + 0.01% SDS	0.46	94	4.89×10^3
	W263F	25°C	4.63	373	1.24×10^4
	W263M	25°C	4.25	334	1.27×10^4
	W263L	25°C	3.92	371.8	1.05×10^4
	W263I	25°C	1.94	361	5.37×10^3
	W263V	25°C	5.64	1760	3.20×10^3
	W263T	25°C	4.55	13	3.49×10^5
γ-butyrolactone	WT	25°C	ND	ND	1.20×10^3
γ-heptanolide (r)	WT	25°C	2.92	166	1.76×10^4
Nonanoic-γ-lactone (r)	WT	25°C	5.54	2943	1.88×10^3
Dodecanoic-γ-lactone (r)	WT	25°C	2.72	1220	2.23×10^3
δ-valerolactone	WT	25°C	ND	ND	ND
Nonanoic-δ-lactone (r)	WT	25°C	15.32	359	4.27×10^4
Dodecanoic-δ-lactone (r)	WT	25°C	12.65	1678	7.54×10^3
ε-caprolactone	WT	25°C	4.45	234	1.90×10^4
Dihydrocoumarin	WT	25°C	7.32	1376	5.32×10^3
5-thiobutyl-γ-butyrolactone	WT	70°C	29.0	80	3.60×10^5
	R223H	70°C	0.42	273	1.54×10^3
	Y97W	70°C	75.7	1540	9.58×10^5
5-thioethyl-γ-butyrolactone	WT	70°C	9.0	15	7.00×10^5
5-thiohexyl-γ-butyrolactone	WT	70°C	6.0	70	8.00×10^4

ND corresponds to an undetermined value.

1.5 QQ strategies and resistance

Antibiotics have been widely used over the past decades for treating chronic and acute bacterial infections. Antibiotics induce a strong selection pressure on bacteria by either killing them or inhibiting their growth. However, the intensive use of antimicrobial agents has led to the emergence of adaptive resistance that considerably limits their efficiency and is associated with treatment dose increase [150–152]. QQ has emerged as a promising therapeutic alternative as it can be used to inhibit both the secretion of virulence factors and the formation of biofilm [153], but does not kill bacteria [154–156]. Therefore, QQ strategies are believed to induce a milder selection pressure. However, recent evidence suggests that the effect of QS disruption on bacterial growth was dependent on the culture medium used (*i.e.* nutrient-rich or not) [157], and might thereby introduce a selection pressure, albeit milder than a biocide strategy, and select for resistant bacteria [158,159]. Using QS-disrupted variants, studies have shown that bacterial resistance to QS may arise. Mutations increasing efflux of C-30, an efficient QQ furanone, as well as compensatory mutations were observed as mechanisms to overcome QS disruption [160,161]. Moreover, "Social cheaters", (*i.e*. bacteria that ceased production of quorum regulated factors), were reported [162]. Such QS-insensitive mutants might interfere with QQ efforts [163], but recent experimental studies suggest that QQ resistance would spread slowly, as these mutants were found to be less fit than their counterparts [164].

The emergence of resistance to QQ strategies will certainly depend on the actual used strategy. The use of QQ enzymes is possibly the least resistant-prone of all QQ strategies because enzymes can act remotely and do not need to enter the bacterial cells. Moreover, contrary to QSI that need to bind to a target protein (the signaling receptor), QQ enzymes act independently. Putative resistance mechanisms to QQ enzymes have been proposed [158,159] and suggest that bacteria may evolve for an increased production of the autoinducer molecule (AHL) to counteract the hydrolysis by QQ enzymes. This could be bypassed by increasing the total enzymatic activity in the environment. Another resistance scenario would consist of modifications to the chemical structure of the autoinducer. This possibility is reduced by the fact that QQ enzymes are naturally broad-spectrum enzymes and can be engineered for altered specificity. Another mechanism would consist of the selection of modified LuxR receptors with tremendous affinity for the autoinducer, or with improved response to AHL [165,166], therefore the QQ enzymes would not be active enough at these low concentrations of autoinducers. In this case, enzyme engineering may offer solutions to produce enzymes with higher affinity for AHL.

1.6 Conclusion and perspectives

Although it may affect bacterial fitness, QS disruption is a promising strategy to substitute or at least supplement antibiotics. Many studies have shown that biofilm is associated with antibiotic and antimicrobial agent resistance for a wide range of bacteria

[65,167–172]. Biofilm formation governs many mechanisms involved in antibiotic resistance, such as limited penetration of the antibiotic, horizontal gene transfer within the bacterial community and changes in gene expression that may influence resistance [173,174]. QS disruption often results in decreased biofilm formation, therefore QQ could be an efficient tool for the restoration of bacterial susceptibility to antibiotics or antimicrobial agents in biofilms [174].

QQ strategies, particularly catalytic quenchers, such as enzymes are appealing to develop new alternatives for QS-disruption and antifouling. Since stability is a major constraint that usually impairs enzyme utilization, intensive efforts have been dedicated to the isolation of robust enzymes from extreme environments. Among these, PLL's (particularly *Sso*Pox) are highly promising as they have already been successfully incorporated into devices while retaining their lactonase activity. Moreover, enzymes are highly attractive as these molecules are usually not toxic and may be integrated into various matrices without being released. The proofs of concept have been widely described and further investigations would obviously permit to develop concrete applications (e.g. medical devices, paintings, coatings…) in order to address the issues of bacterial virulence and biofouling. Furthermore, the applications of QQ enzymes are focused on the disruption of the AI-1-based QS mechanism. The quest for enzymes targeting AI-2, AI-3, or even AIP is of utmost interest for extending the potential of QQ strategies to a wider panel of Gram-negative and Gram-positive bacteria.

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2. Les perspectives pharmaceutiques de l'interférence avec le *quorum* sensing

Le XXème siècle a connu l'âge d'or des antibiotiques avec la découverte et l'avènement de ces molécules dans la médecine moderne. Ils sont devenus les piliers de la lutte contre les infections mais leur utilisation croissante a entrainé la sélection et la dissémination de bactéries résistantes. Ce phénomène serait responsable de 700 000 décès par an dans le monde [41]. En 2013, le « Center for Disease Control and Prevention » (CDC) a recensé 2 millions d'infections par des bactéries résistantes dont au moins 23 000 décès aux États-Unis[III]. La même tendance est observée en Europe avec environ 25 000 morts par an selon la Commission Européenne[IV]. Les bactéries résistantes sont donc impliquées dans de nombreuses infections pouvant compliquer la prise en charge voire entrainer la mort du patient. Dans le cas de la tuberculose, le traitement préconisé repose sur une combinaison de 4 antibiotiques pour une durée minimum de 6 mois mais qui passe à 7 antibiotiques pendant 9 mois dans les cas d'infections par des bactéries multi-résistantes[V]. Le traitement de remplacement est donc moins efficace, plus long et nécessite une observance accrue de la part du patient pour obtenir une guérison complète. Les antibiotiques sont également souvent utilisés en prophylaxie pour les patients temporairement immunodéprimés comme lors d'actes chirurgicaux, de transplantation d'organe ou de traitement anticancéreux [175,176]. L'apparition de résistance peut donc entrainer des complications à la suite de ces interventions.

La dissémination des bactéries résistantes représente une menace pour la santé et a aussi un impact économique non négligeable. En effet, la diminution de l'efficacité des antibiotiques entraine un surcoût dans les dépenses de soin dû à des traitements antibiotiques plus complexes et plus longs et à la nécessité d'un suivi plus poussé par une autorité médicale. Ce coût est estimé à 1,5 milliard d'euros pour l'Europe et à 20 milliards de dollars pour les États-Unis par an. En général, les pays de l'Organisation de Coopération et de Développement Economiques (OCDE) s'accordent sur un surplus de 10 000 à 40 000 dollars par patient[IV].

La découverte de nouvelles classes d'antibiotiques s'est faite de plus en plus rare depuis la fin des années 1960 au profit de la synthèse ou découverte d'analogues des classes déjà répertoriées [177,178]. La recherche de nouveaux analogues a souvent pour but de réduire la toxicité envers l'Homme, d'élargir le spectre d'actions ou d'augmenter l'activité envers les bactéries. De plus, la recherche de nouveaux analogues de classes existantes représentent moins de risque pour les industries pharmaceutiques que la découverte de nouvelles classes qui nécessitent plus de recul sur leur toxicité et mode d'action [179]. Cependant, les mécanismes de résistance aux analogues peuvent être les mêmes que pour la

[III] https://www.cdc.gov/drugresistance/threat-report-2013/index.html

[IV] https://ec.europa.eu/health/amr/antimicrobial-resistance_en

[V] http://apps.who.int/iris/bitstream/10665/250125/1/9789241549639-eng.pdfhttp://www.who.int/tb/areas-of-work/drug-resistant-tb/treatment/FAQshorter_MDR_regimen.pdf?ua=1

molécule d'origine et leur utilisation peut se retrouver très vite limitée [177,179]. Néanmoins, la mise sur le marché d'analogues reste plus fréquente que celle de nouvelles classes. Par exemple, en 2017, sur 45 autorisations, la « Food and Drug Administration » (FDA) a approuvé trois nouveaux médicaments à activité antibactériennes dont deux qui utilisent de nouveaux analogues (Solosec®[VI] et Baxdela®[VII]).

Des compléments ou des alternatives ont été développés ou sont en cours de développement afin de proposer des thérapies plus durables pour la lutte contre les bactéries. Des inhibiteurs de pompes à efflux ou des inhibiteurs d'enzymes impliquées dans la dégradation des antibiotiques ont été étudiés pour traiter certaines bactéries résistantes [180,181]. Par exemple, en 2017, le troisième médicament à activité antibactérienne approuvé par la FDA est le Vabomere® qui est l'association d'un antibiotique déjà utilisé (méropénème) avec un inhibiteur de bêta-lactamases[VIII] (vaborbactam). Ces compléments aux antibiotiques sont très utiles mais ne ciblent qu'une fraction des mécanismes de résistance.

En parallèle, la découverte de nouvelles molécules antibiotiques se poursuit notamment du côté des peptides antimicrobiens [182,183]. Ces derniers font l'objet de beaucoup d'attention car leur grande variété permet d'obtenir des spectres d'action antimicrobiens plus ou moins larges permettant de moduler la spécificité du traitement [182,184]. Ils peuvent avoir des activités antibiotiques directes ou un effet immunomodulateur ce qui ouvre la voie vers de nouvelles thérapies [183,185]. A ce jour, leur efficacité a été démontrée en application topique et d'autres essais cliniques sont encore en cours [183,184].

D'autre part, des thérapies alternatives aux molécules antibiotiques classiques sont également considérées. La phagothérapie par exemple suscite un regain d'intérêt général [183,186]. D'abord envisagée comme remède aux infections au début du XXème siècle, cette stratégie thérapeutique a été délaissée dans les pays occidentaux au profit de la pharmacopée chimique, notamment les antibiotiques. Néanmoins certains pays de l'est de l'Europe tels que la Géorgie, la Pologne et la Russie utilisent communément la phagothérapie et bénéficient d'un recul de près de 70 ans sur cette stratégie. La phagothérapie est à nouveau sérieusement considérée en occident et des études cliniques sont en cours de réalisation par des entreprises anglaises, américaines et françaises pour traiter notamment les infections à *P. aeruginosa* [183]. L'utilisation de bactériophages a été autorisée en France en traitement compassionnel pour traiter avec succès deux patients atteints d'une infection ostéoarticulaire par *S. aureus*[IX]. Les phages constituent une alternative sérieuse aux antibiotiques présentant une spécificité plus importante que les traitements antibiotiques pouvant limiter ainsi les effets secondaires.

A l'inverse des antibiotiques ou des phages, d'autres stratégies non bactéricides et non bactériostatiques ont été considérées pour lutter contre les infections bactériennes. Ces stratégies ne visent directement ni le développement ni la survie de la bactérie mais ciblent

[VI] Secnidazole (5-nitroimidazole)

[VII] Delafloxacin (fluoroquinolone)

[VIII] Enzyme de dégradation des antibiotiques contenant un noyau lactame.

[IX] http://www.chu-lyon.fr/en/viruses-heal-antibiotic-resistant-infections-0

les mécanismes liés à la virulence [21,187]. L'avantage principal de ces stratégies est de diminuer la pression de sélection appliquée aux bactéries pour limiter l'émergence de résistances. Cette stratégie a été testée dès 1893 par Emil von Behring qui utilisait du sérum de patient immunisé contre la toxine diphtérique pour traiter des enfants malades. Cette stratégie, délaissée dans un premier temps au profit des antibiotiques, s'est par la suite répandue et le développement d'anticorps neutralisants est aujourd'hui en pleine expansion [21,187]. Certains ciblent des toxines extracellulaires comme dans le cas d'infection à *Bacillus anthracis* (anthrax) et d'autres ciblent des composés membranaires comme les appareils de sécrétion et la matrice extracellulaire chez *P. aeruginosa* [21]. Des enzymes et des inhibiteurs de la communication bactérienne de type QS sont aussi très étudiés car ils permettent de bloquer de nombreux facteurs de virulence à la fois sans affecter directement la survie des bactéries [21].

La virulence se traduit notamment par la production de facteurs visant à favoriser la colonisation du site d'infection [187]. Des toxines, appareils de sécrétion et exoenzymes sont souvent utilisés pour endommager les cellules de l'hôte. Par ailleurs, la virulence d'une bactérie est aussi liée à des mécanismes d'adhésion et de formation de biofilm qui est une matrice extracellulaire secrétée par les bactéries. Ceci leur permet d'une part de coloniser l'hôte mais aussi de se protéger contre l'action du système immunitaire. Ainsi le biofilm est souvent associé à des infections chroniques comme celles à *P. aeruginosa* chez les patients atteints de la mucoviscidose ou d'ulcères du pied diabétique [24,188]. Le biofilm permet également aux bactéries de coloniser de nombreuses surfaces et est fréquemment impliqué dans les IAS lors de la mise en place d'implants, de sondes urinaires ou encore de cathéters et autres dispositifs médicaux [24]. Enfin, il contribue à la tolérance accrue des bactéries envers les agents antimicrobiens [168].

Le QS, impliqué dans la régulation de mécanismes de virulence ainsi que dans la formation de biofilm, constitue une cible de choix pour lutter contre certaines infections bactériennes [10]. De plus, le QS régulerait différents mécanismes liés à la résistance aux antimicrobiens et aux bactériophages, dont le système CRISPR-Cas[X], ce qui pourrait contribuer à diminuer l'efficacité des traitements [189–191].

De nombreuses études ont mis en évidence le lien entre QS et virulence dans une large gamme de modèles *in vivo* allant de l'amibe unicellulaire jusqu'aux mammifères (e.g. souris, rats). La mutation du récepteur ou de la voie de synthèse du signal diminue la virulence de nombreuses bactéries se traduisant par une diminution de la mortalité ou de la charge bactérienne au point d'infection [192,193]. L'utilisation d'inhibiteurs (QSI), de peptides ou d'enzymes s'est montrée efficace pour lutter contre le développement d'infections *in vivo* de bactéries à Gram positif (ex : *S. aureus, S. epidermidis)* et négatif *(ex : P. aeruginosa, B. cepacia)* [30,194,195]. De plus, un effet synergique avec des antibiotiques a pu être observé *in vivo* confortant l'idée de complémentarité des traitements [196,197].

[X] Clustered regularly interspaced short palindromic repeats and CRISPR associated protein

Le QQ étant efficace pour la prévention des infections, il pourrait être utilisé dans des dispositifs médicaux pour réduire le nombre des IAS. De nombreuses molécules ou enzymes ont été immobilisées avec succès sur différents matériaux tout en conservant leur activité de QQ [134,197,198]. Les enzymes sont particulièrement intéressantes pour ces applications car elles agissent sur les molécules de communication extracellulaires et ne nécessitent pas un contact direct avec la bactérie.

Interférer avec le QS bactérien est particulièrement prometteur et a fait l'objet d'une deuxième revue bibliographique [199]. Dans une première partie, les différentes molécules intervenant dans les différents systèmes de communication, ainsi que celles utilisées pour les bloquer, ont été répertoriées. Les synergies avec les traitements antimicrobiens ont ensuite été décrites, puis l'effet *in vivo* du QQ dans plusieurs modèles et pour plusieurs bactéries a été résumé. Enfin une dernière partie fait l'inventaire des différents dispositifs médicaux basés sur cette technologie notamment en vue de lutter contre les IAS. J'ai participé à la rédaction de cette revue depuis la structure de son plan, la rédaction de la partie sur les modèles animaux, la création de tableaux et figures et jusqu'à son dépôt et sa soumission au journal *Frontiers in Pharmacology*.

2.1 Introduction

Quorum sensing (QS) is a molecular mechanism by which bacteria communicate to collectively adapt their behavior according to cell density and the surrounding environment **(Figure 7)**. This communication system enables bacteria to undertake processes that are costly and non effective at low cell density but that become useful for the whole community at high cell density such as virulence factor synthesis, biofilm formation, and protease and siderophore production [200]. QS consists in the production and sensing of small extracellular molecules, known as autoinducers (AI), that are released in proportion to cell density [11]. In Gram-positive bacteria, autoinducing peptides (AIP) were widely studied and reported to induce QS. AIP are specific to species and strains and have been described in *Staphylococcus* spp., *Clostridium* spp., or *Enterococcus* spp., among others, AIP **(Figure 8)** [201]. Many Gram-negative bacteria, including *Pseudomonas* spp., *Acinetobacter* spp. or *Burkholderia* spp., were reported to use a different class of autoinducers: the acyl-homoserine lactones (AHL) [202]. AHLs are composed of a lactone ring and an aliphatic acyl chain varying in length and modifications [202]. A wide variety of other signaling molecules was also identified[203], including fatty acids used by *Xanthomonas* spp., *Burkholderia* spp*., Xylella* spp. [204], ketones (*Vibrio* spp. and *Legionella* spp. [205]), epinephrine, norepinephrine and AI-3 (enterohemorrhagic bacteria [51]) or quinolones (*Pseudomonas aeruginosa* [206]). Finally, AI-2, a furanosyl borate diester, is used by both Gram-negative and Gram-positive bacteria [48] **(Figure 8)**. Most Gram-negative bacteria combine several QS systems to integrate different signals either hierarchically, as *P. aeruginosa* in which four QS systems (*las*, *rhl*, *iqs* and *pqs*) act in a network [52], or in parallel, as in *Vibrio harveyi* in which three systems are integrated into one regulatory cascade [55].

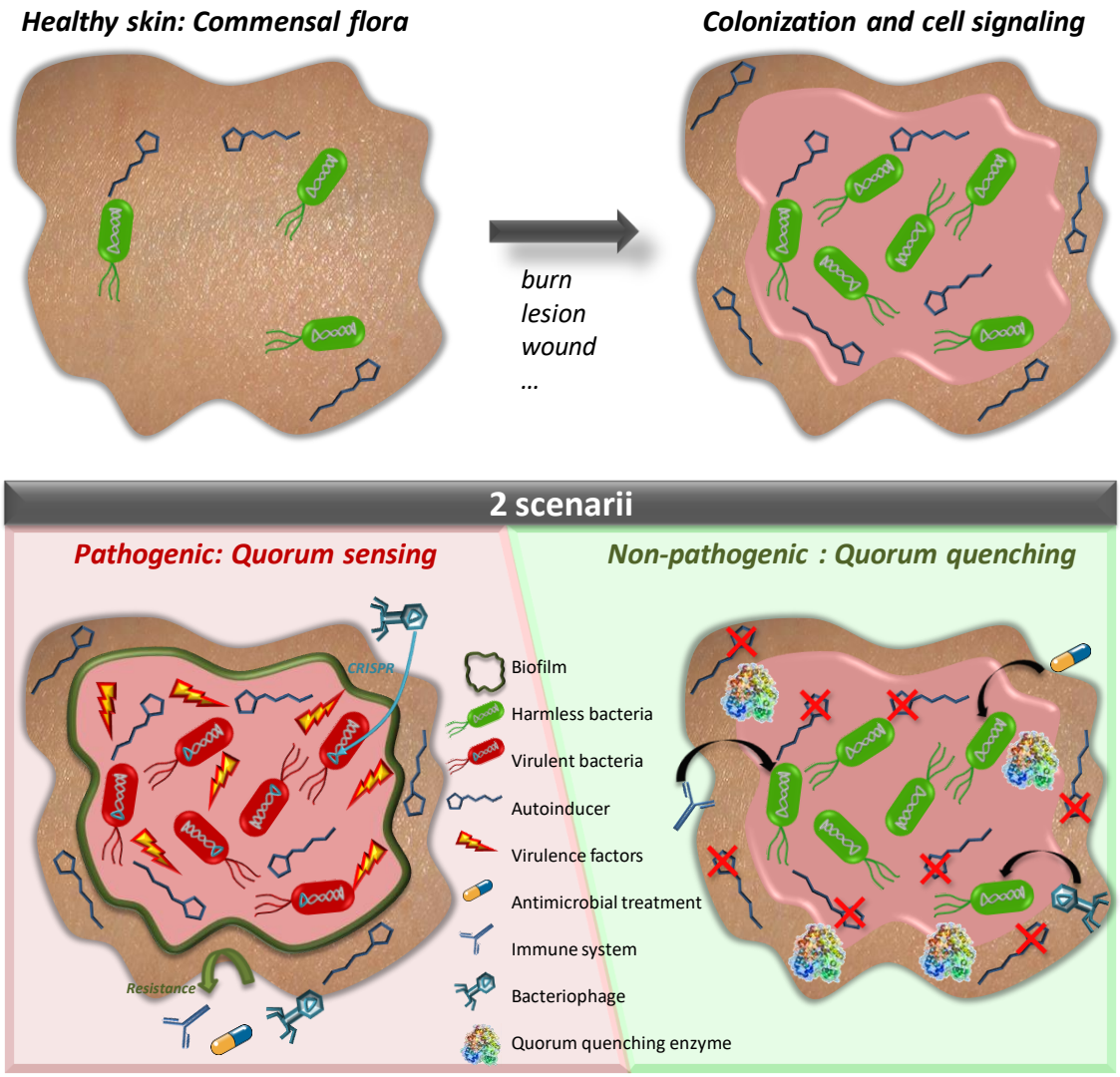

Figure 7 : Quorum sensing and quorum quenching in a wounded tissue. The skin usually harbors a natural and commensal flora which is not pathogenic (upper left). When a wound or a lesion occurs, bacteria colonize the wounded tissue and further develop being in a favorable environment (upper right). While growing, bacteria produce communication molecules (autoinducers). If the molecules are not degraded (bottom left), bacteria can synchronize their behavior to secrete virulence factors and produce biofilms which may prevent efficiency of antibiotic or phage therapy. The wound is infected. If the autoinducers are degraded (bottom right) bacteria do not synchronize their behavior and remain harmless and defenseless. The wound remains colonized but no infection occurs.

Interferences with QS are termed quorum quenching (QQ) **(Figure 7)**. QQ was discovered as a naturally occurring phenomenon first described in 2000 with the identification of a QQ enzyme able to degrade AHL signals from *Erwinia carotovora* [70]. The enzymatic hydrolysis of AHL led to the disruption of the QS signal. The disruption of bacterial communication can be achieved by several processes: (i) interfering with the production or perception of AI via small molecules referred to as quorum sensing inhibitors (QSI) [207], (ii) scavenging of AI by quorum quenching antibodies [208] and macromolecules such as cyclodextrins [209–211] or (iii) by extracellular hydrolysis of the AIs using QQ enzymes [212] **(Figure 9)**. Several antagonist peptides have been identified among natural compounds or

designed to quench Gram-positive bacteria and many QSI, mainly targeting Gram-negative QS and AI-2 mediated QS, have also been reported [207,213]. Such compounds can be natural products, like polyphenols isolated from tea or honey, ajoene from garlic, eugenol from clove or many others produced by marine organisms and fungi [207,214], or they can be synthetic, such as 5-fluorouracil (5-FU) or azithromycin [215,216]. Many QQ enzymes and macromolecules [212,217] as well as natural or synthetic QSI [214,218–221] have been reported to date and exhaustively reviewed. Patents associated with these compounds [155,222,223] as well as routes to access novel molecules [224] have also been discussed. The mechanisms used by the different QSI are not always known and most probably differ from one QSI to another [225]. Some molecules inhibiting QS such as azithromycin are also considered as antibiotics as they can inhibit bacterial growth above a certain concentration [226].

Currently identified QQ enzymes mainly target AHL and AI-2 mediated QS: phosphotriesterase-like lactonases (PLL), lactonases, acylases and oxidoreductases degrade AHL signals (Fetzner, 2015) and oxidoreductases target AI-2 [70,227]. As QS induces noxious traits such as biofilm formation or virulence, the disruption of bacterial communication appears as a promising strategy to prevent bacteria from synchronizing their virulent behavior. Therefore, QQ approaches may have applications in numerous fields such as agronomy, water engineering, and the marine industry and is particularly relevant in health care [47].

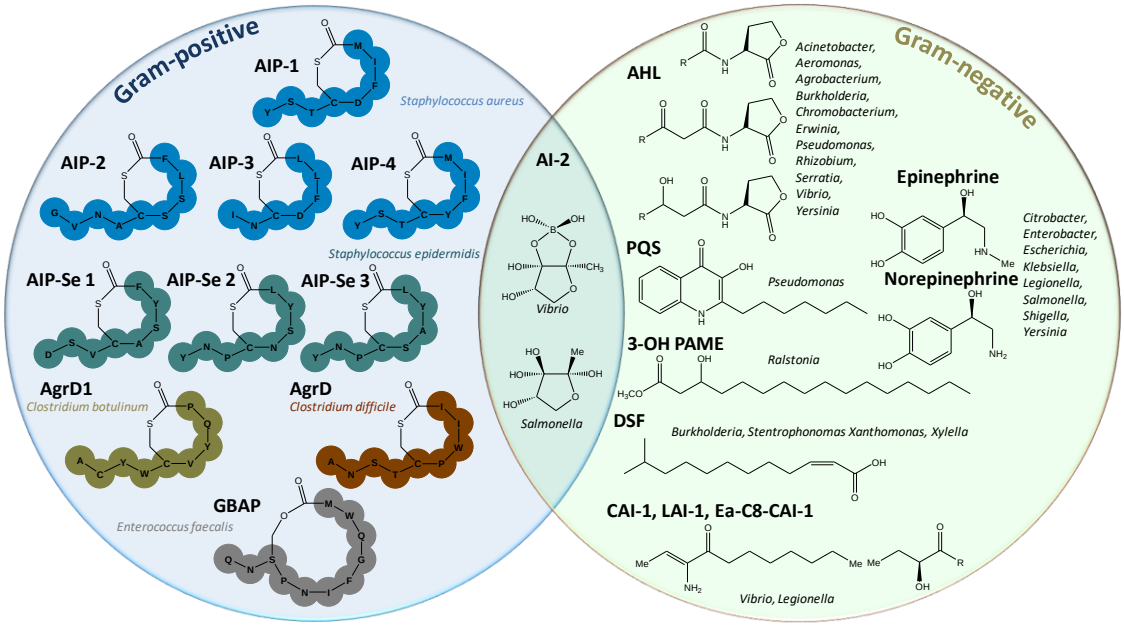

Figure 8 : Representation of autoinducer molecules. The left circle represents autoinducing peptides used by Gram-positive bacteria such as Staphylococcus spp., Clostridium spp., Enterococcus faecalis [201]. The right circle gives an overview of the different molecules used in Gram-negative quorum sensing: acyl homoserine lactones (AHL) [202], quinolones (PQS), 4-hydroxypalmitate methyl ester (3-OH-PAME) [50], fatty acids (DSF) [204], epinephrine and norepinephrine [51]. In the middle, the different forms of AI-2, a furanosyl diester, used by both Gram-positive and Gram-negative bacteria are depicted [48].

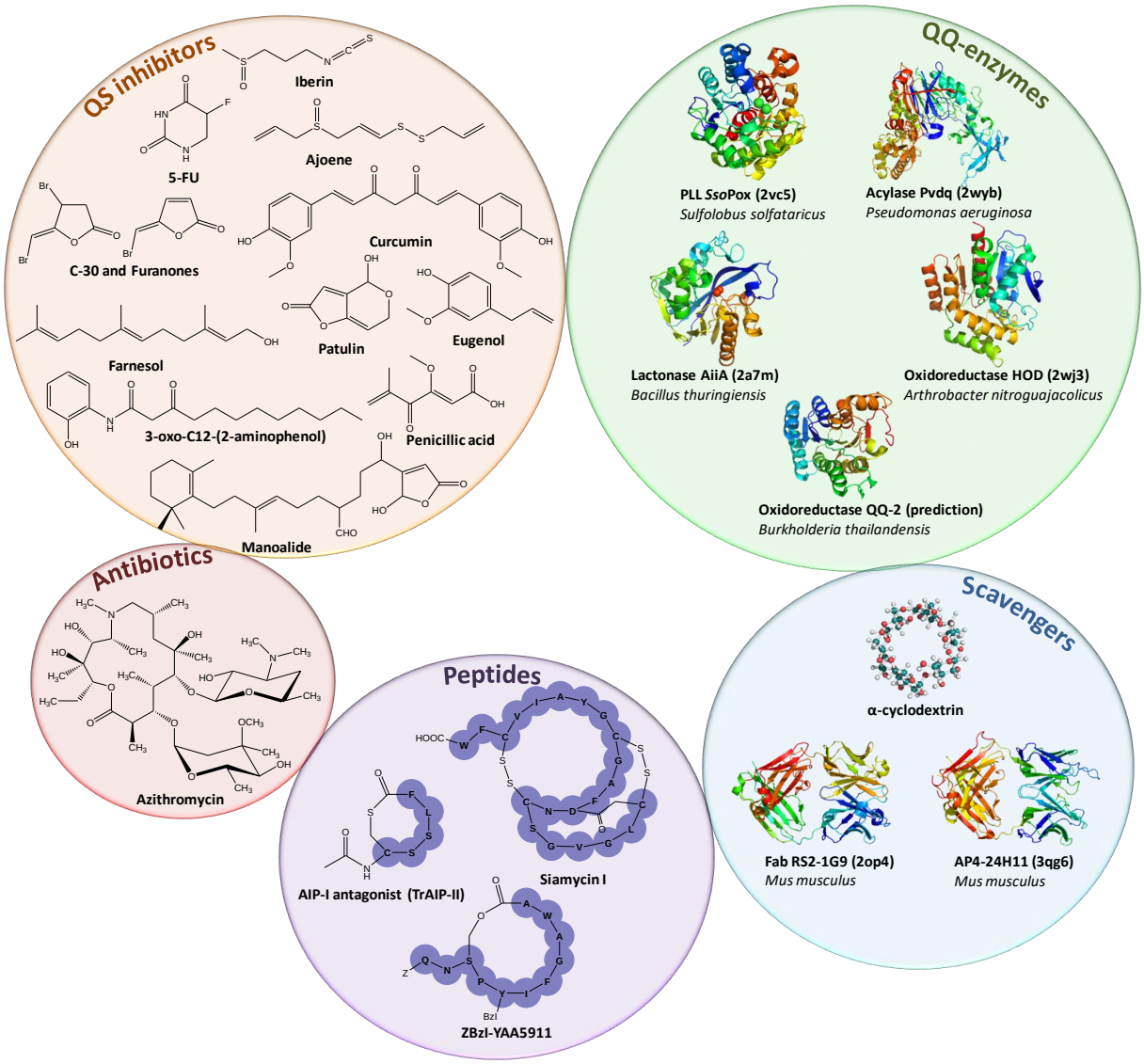

Figure 9 : Representation of quorum quenching agents. Quorum sensing inhibitors, mainly acting against AHL or AI-2-based QS, are depicted in the orange circle [207]. Antibiotics such as azithromycin can be used as QSI at sub-inhibitory concentrations [215]. Purple circle represents the QQ peptides used to inhibit Gram-positive QS [213]. Blue circle represents molecules used to scavenge AIs such as cyclodextrins or derivatives [211] and antibodies scavenging AHL (Fab RS2-1G9) or AIP (AP4-24H11) [208]. Green circle depicts QQ enzymes that disrupt AHL (*Sso*Pox, Pvdq and AiiA), the quinolone PQS (HOD) and AI-2 signals (QQ-2) [212].

In the current context of the rise of antibiotic tolerance and resistance, novel therapeutic approaches are needed [59]. The ability of QQ approaches to inhibit bacterial virulence and biofilm is appealing as this latter is associated with increased antibiotic tolerance [172]. Biofilm formation is triggered via QS and consists in a heterogeneous multi-cellular structure attached to a solid surface, embedded in an extracellular matrix [228]. The extracellular matrix, made of polysaccharides, proteins and extracellular DNA, may prevent some antibiotics from successfully penetrating the cells, inducing antibiotic tolerance [229]. The bacterial cells embedded in the matrix also have a slower growth rate and an altered

metabolism which further reduces antibiotic efficiency [230]. In addition, biofilm environments combine high cell density and high selection pressure increasing the rate with which resistant cells appear through mutations or gene transfer [231]. Biofilms also shelter persistent cells, a non inheritable trait denoting a subpart of cells present in any bacterial population that will survive antibiotic treatment as a result of being in a different physiological state at the time of the treatment [232]. Bacteria in biofilms are considered to be 100 to 1,000 times more tolerant to antimicrobial compounds as compared to their planktonic lifestyle [230]. Biofilms can also be at the source of infections: it is considered that between 65 and 80% of infections are biofilm associated infections, either by directly infecting the tissue such as lung infection in the case of cystic fibrosis or via a contaminated device such as a catheter [233]. Hospital-acquired infections (HAI) affect between 6 and 10% of health care patients in developed countries, the most frequent type of infections being urinary tract infections [234,235]. Eliminating biofilms in health care devices and environment is a challenge to limit and treat HAI. It is, therefore, essential to develop alternative or complementary treatments to conventional antimicrobial and antibiotic products. To this end, QQ and phage therapy are increasingly studied [236,237].

This review highlights the latest findings and biopharmaceutical perspectives of QQ as well as its potential complementarity with antimicrobial agents, antibiotics and bacteriophages. The eukaryotic models used to prove the efficiency of QQ as a successful anti-virulence and anti-biofilm strategy and the medical applications with QQ devices are also summarized.

2.2 Quorum sensing and the sensitivity to antimicrobial agents

As QS involves a global change in bacterial gene expression and cell physiology, the relationship between QS and antibiotic tolerance is multi-faceted. For example, the addition of AHL to a logarithmic culture of *P. aeruginosa* was shown to increase the number of persister cells in the population after treatment with carbenicillin and ciprofloxacin [190]. Furthermore, transcriptomic analysis with the QS transcription regulator MvfR (PqsR) in *P. aeruginosa* PA14 revealed that QS induces the expression of peroxidases which provide protection against reactive oxygen species (H_2O_2) and β-lactam antibiotics [238]. In another study using *P. aeruginosa* PAO1, VqsM, a global regulator that induces QS, was shown to mediate antibiotic tolerance by inducing the expression of *nfxB*, an antibiotic resistance regulator, providing increased tolerance to quinolones, tetracycline and kanamycin via regulation of *mexC-mexD-oprJ* operon expression [239,240].

Although some physiological aspects may be involved in QS-mediated tolerance to antibiotics, many reports focus on the importance of biofilm in antibiotic tolerance of bacteria [168], causing many difficulties for treatments of clinical infections [241]. Those effects have been frequently observed with *P. aeruginosa* [242] in both model and clinical strains[243] as well as in other species such as *Klebsiella pneumoniae* [244,245] and *S. aureus* [246]. The particular conditions that the biofilm mode of growth provides to bacteria favors the

development of different defense mechanisms and phenotypes: physical barrier, modification of gene expression, and cellular physiological states (e.g. persister cell) [228]. In *P. aeruginosa*, the *rhl* and *las* QS systems are essential for biofilm formation and their disruption is correlated with a higher sensitivity to the host immune system and antimicrobial compounds [247–249]. Moreover in *P. aeruginosa*, another QS system, the *pqs* system, has been demonstrated to mediate a programmed cell death inducing extracellular DNA release which promotes biofilm formation and antibiotic tolerance, benefitting the rest of the cell population [250]. In clinical isolates of *A. baumannii*, the presence of levofloxacin or meropenem antibiotics was reported to induce the overexpression of an efflux pump which stimulates the release of AHL and thus enhances the formation of QS-mediated biofilm, increasing antibiotic tolerance [251].

Regarding the important role of QS in biofilm formation and antibiotic tolerance, combination therapy with QQ was investigated. In *P. aeruginosa*, the use of a pharmacological compound, benzamide-benzimidazole, inhibiting the QS regulator MvfR (PqsR) decreased biofilm formation and restored antibiotic susceptibility [252,253]. Baicalin hydrate and hamamelitannin, an AHL-targeting QSI and a peptide-based QSI, enhanced biofilm disruption in both Gram-negative (*P. aeruginosa* and *Burkholderia cepacia* complex) and Gram-positive (*S. aureus*) bacteria and showed synergistic effects in cotreatment with tobramycin and clindamycin or vancomycin respectively both *in vitro* and *in vivo* [194]. From aminoglycosides [254,255] to quinolones [256], polypeptides [257,258], cephalosporins [252] and glycopeptides [259], the efficiency of a large range of antibiotics is enhanced by the addition of QSI.

Taken together these results suggest that using QSIs is a potential way of increasing antibiotic sensitivity and thereby lower antibiotic active doses. Additionally, similar trend and efficiency have also been observed with a lactonase QQ enzyme and the antibiotic ciprofloxacin in a mice model [136]. Combining antimicrobial agents and QQ was showed to have encouraging synergistic effects, highlighting that QQ is a good strategy to decrease antibiotics use and fight against the increasing problem of antibiotic resistance. Nevertheless, if QQ can help to prevent and treat infections, it cannot be used on its own to treat acute infections by antibiotic resistant strains.

2.3 Relationship between quorum sensing and the sensitivity to bacteriophages

Recently, interest has considerably increased in phage therapy as a way of treating infections caused by multi-drug resistant bacteria [260]. Bacteriophages are the most abundant bacterial predators on the planet and they are still used to treat bacterial infections in Eastern Europe [261]. Although bacteriophages represent an interesting solution to circumvent antibiotic resistance, bacteria have also developed resistance mechanisms to counteract phage actions [262]. Firstly, phage entry can be decreased by extracellular matrix production, or by modifying the phage receptor structure or expression [262,263]. Once inside the cell, phage DNA can be recognized and degraded by restriction enzymes or the adaptive inducible CRISPR-Cas (clustered regularly interspaced short palindromic repeat and CRISPR

associated proteins) system [262,264]. Other potential metabolism adaptations could also provide bacteriophage resistance [265]. As the risk of phage infection rises with cell density, QS-mediated resistance would provide protection during high risk conditions while limiting the overall fitness cost of those mechanisms [266].

The relationship between QS and bacteriophage sensitivity was originally observed in *P. aeruginosa* [267], but the ability of QS to regulate phage defense mechanisms in *Escherichia coli* was only demonstrated years later [16]. The authors showed that AHLs induced a reduction in the phages lambda and chi adsorption rate by reducing the number of receptors at the cell surface. In *Vibrio cholerae*, a deficiency in AI synthase genes, and thereby in QS induction, reduced phage resistance which could be restored by the addition of exogenous AIs, AI-2 and CAI-1 [189]. This increased phage resistance upon QS activation was explained by the downregulation of O-antigen synthesis which decreased phage adsorption and by an increase in hemagglutinin protease production which was shown to inactivate phages [189]. Similarly, the addition of synthetic AHLs to a *Vibrio anguillarum* QS deficient strain improved phage resistance [268]. Indeed, AHL production was negatively correlated with phage receptor *ompK* expression. Recently, it was shown in *Serratia marcescens* that the CRISPR-Cas immune system was also under QS regulation [191]. Both the acquisition of immunity and DNA degradation mechanism coordinated by this system were negatively impacted by the absence of QS signal in a synthase mutant. Besides, by analyzing former datasets [269,270], the authors suggested that this type of regulation might occur in *Pectobacterium atrosepticum* as well as in *Burkholderia glumae*. Similar QS control of CRISPR-Cas system was demonstrated in *P. aeruginosa* PA14 in which the expression of the CRISPR-Cas genes is down-regulated in a strain deleted for both AI synthase genes *lasI* and *rhlI* [271]. To evaluate whether QQ would increase phage sensitivity, QSI effects were investigated on resistance mechanisms and on phage susceptibility phenotypes. The use of penicillic acid increased *P. aeruginosa* sensitivity to phages by increasing the proportion of sensitive viable cells in the population [265]. Finally, the QSI baicalin was shown to inhibit the QS stimulation of CRISPR-Cas system in *P. aeruginosa* which could prevent the use of this adaptive system by bacteria in case of phage infection [271].

In light of these findings, the use of QQ compounds is a highly promising way to develop new therapeutic applications. Indeed, their use in combination with phage therapy treatments could increase bacterial sensitivity to phages by synergistic effects. In addition, disturbing the QS of one species was demonstrated to induce a reduction in total biomass in multimicrobial cultures under phage infection, leading to the consideration that QQ combined with phage therapy could as well be efficient against polymicrobial infections [272]. To prove the efficiency of QQ as antivirulent agent proper *in vivo* assays and proof of concepts on animal models should be performed.

2.4 Antivirulence activity of quorum quenchers *in vivo*

In order to evaluate the role of QS in pathogenicity several models have been developed over the past few years. Three models, from a simple unicellular model to complex models, commonly used to assess the benefits of QQ and studies conducted on humans are summarized below.

Amoebal infection models

Free-living amoebae are eukaryotic organisms found either in a resting (cyst) or a vegetative (trophozoite) form feeding on bacteria among other organisms (algae or fungi). In this way, they use phagocytosis coupled with lysosomal digestion which is close to macrophage bacterial elimination pathway [273–275]. Considering these close interactions, amoeba were considered to test bacterial production of virulence factors [276,277], biofilm [278,279] and secretion systems [280–282]. Classically, the evaluation of bacterial virulence in amoebae relies on the capacity of amoebae to grow or not to grow in the presence of pathogenic bacteria [277,281]. The link between virulence factors and QS in different bacterial species was evaluated using this approach and was extensively described for *P. aeruginosa*. QS-deficient mutants of *P. aeruginosa* had a decreased virulence towards the amoeba *Dictyostelium discoideum* [277,281]. Although this model is fast and convenient for large scale experiments such as screening assays, the use of this model is limited, as both culture conditions and amoeba species may strongly affect the results [283]. Despite these limitations the amoeba model was recently used to test a QQ enzyme based on the well-characterized assay with *P. aeruginosa* and *D. discoideum* [276]. The over-production of *P. aeruginosa* PA14 aliphatic amidase AmiE resulted in a disruption of QS and reduction of virulence in a *D. discoideum* plate killing assay [276].

Caenorhabditis elegans infection models

The roundworm *C. elegans* is a widely used multicellular organism model to study microbial virulence [284–287]. Like amoebae, *C. elegans* is a convenient model for high throughput evaluation of QS impact on bacterial virulence [286–289]. However, in contrast to amoebae, *C. elegans* has an innate immune system which results in a closer comparison with the human immune response [285] and is particularly relevant for studying pathogenicity. Classically, *C. elegans* is fed using the bacteria of interest and the survival rate is followed. Two types of assays can be performed: (i) a fast killing assay which leads to worm death in a few hours to assess the presence of toxins, and (ii) a slow killing assay with death occurring after several days to evaluate bacterial colonization [290–292].

In order to decipher the importance of QS in virulence, many experiments were dedicated to study the pathogenicity of bacterial mutants impaired in AI synthesis or perception. QS inactivation in different *P. aeruginosa* strains resulted in a decrease in worm mortality [287,288,293,294]. The *C. elegans* model was also used to show the link between QS and virulence of other various Gram-negative bacteria including *Chromobacterium*

violaceum [295], *E. coli* [296], *Yersinia pseudotuberculosis* [297], *B. cepacia* [290], *Burkholderia cenocepacia* [298] or *Burkholderia pseudomallei* [299]. Moreover, the link between QS and pathogenicity was also shown for Gram-positive bacteria such as *Enterococcus faecalis* [192,286] and *S. aureus* [300]. Considered as a whole, these studies highlight that QS triggers virulence in many bacteria.

In addition to genetic mutations, the roundworm model was used, alongside traditional *in vitro* tests, to prove the efficiency of QSI as well as QQ enzymes or bacteria **(Table 3)**. Though the impact on survival may vary according to the assay used and the culture conditions, all the QQ agents tested were shown to efficiently decrease virulence in both Gram-positive and negative bacteria and thus enhancing *C. elegans* survival up to 100% notably with the QQ enzyme BpiB09 targeting AHL [301]. The QSI having the more drastic effect on *C. elegans* survival after infection with *P. aeruginosa* PAO1 is 4-nitro-pyridine-N-oxide, a non toxic chemical compound, which almost fully restored worm survival [289]. The most efficient natural QSI are extracts from *Conocarpus*, *Callistemon vinimalis* or *Bucida buceras* with a restoration of survival up to 87% [302] **(Table 3)**. Moreover, a synergistic effect with antibiotics was reported for the QSI baicalin and hamamelitannin [194].

C. elegans is a highly valuable invertebrate model enabling high throughput screening (for bacterial mutants or QQ compounds) and gives a very deep insight into virulence regulation, modulation by QQ agents and, in general, by anti-infective molecules [303]. In most cases, QQ with either QSI or enzymes seems to be able to reduce mortality due to a wide range of bacteria in *C. elegans* and thus gives a relevant proof of concept of QQ as antivirulent agent in a multicellular organism. However, it also has some limitations, such as the living parameters of the worm which differ from bacterial ones (e.g. growth temperature around 20°C), and the physiopathology of the roundworm which is very different from the human one. Furthermore, as for amoebae, the influence of assay conditions on the outcome of the assay have been highlighted by several studies [292,304,305].

Table 3 : *C. elegans* survival rate upon quorum quenching of several virulent bacteria.

BACTERIA	STRAIN	QQ AGENT (Concentration)	SURVIVAL RATE QQ/control (Time)*	REFERENCE
Quorum sensing inhibitors (QSI)				
B. cepacia	LMG16656	Baicalin hydrate (100µM)	≈50%/≈25% (48h)	[194]
	LMG18828		≈35%/≈15% (48h)	
C. violaceum	ATCC31532	Chloro lactone (20µM)	100%/0% (48h)	[295]
E. coli	O157:H7	Broccoli extract (0,5% v/v)	50%/21,5% (8 days)	[296]
P. aeruginosa	PAO1	4-nitro-pyridine-N-oxide (100µM)	95%/0% (5h)	[289]
		Garlic extract (2% v/v)	60%/0% (5h)	[289]
		Extract from *Conocarpus*, *Callistemon viminalis* or *Bucida buceras* (1mg/mL)	84-87%/0% (4h)	[302]
		Curcumin (3 µg/mL)	28%/0% (100h)	[306]
		2,5-piperazinedione (100µg/mL)	66%/0% (84h)	[307]
		Phenylacetic acid (200µg/mL)	53%/0% (84h)	[308]
		Clove oil (1,6% v/v)	62%/0% (96h)	[309]
		Fractionated methanol extract of *Terminalia chebula Retz.* (0,5mg/mL)	50%/0% (72h)	[310]
		Menthol (800µg/mL)	58%/0% (96h)	[311]
		Methanol extract of *Trigonella foenum-graceum* (1mg/mL)	48%/0% (96h)	[312]
		Oleanolic aldehyde coumarate (200µM)	48%/20% (4h)	[313]
		Mangifera indica methanol leaf extract (800µg/mL)	72%/0% (48h)	[314]
	PAO1	Baicalin hydrate (100µM)	≈30%/≈10% (48h)	[194]
	ATCC9027		≈50%/≈25% (48h)	
	PA14	Extract from *Conocarpus*, *Callistemon viminalis* or *Bucida buceras* (1mg/mL)	53-90%/0% (4h)	[302]
			57-60%/0% (58h)	
		Meta-bromo-thiolactone (50 µM)	77%/≈20% (24h)	[288]
	Pa1 (clinical isolate)	Tea polyphenols (3,125mg/mL)	63%/20% (48h)	[315]
S. aureus	Mu50	Hamamelitannin (250µM)	≈55%/≈15% (48h)	[194]
V. anguillarum	LMG441	3,4-dichloro-cinnamaldehyde (10µM)	≈90%/71% (48h)	[316]
V. harveyi	BB120	3,4-dichloro-cinnamaldehyde (10µM)	≈80%/49% (48h)	[316]
Vibrio vulnificus	LMG16867	3,4-dichloro-cinnamaldehyde (20µM)	≈80%/15% (48h)	[316]
Quorum quenching enzymes				
B. cepacia complex	46 strains	AiiA, lactonase from *Bacillus sp.* 240B1	100%/0-100% (5 days)**	[317]
P. aeruginosa	PAO1	AiiD, acylase from *Ralstonia* strain XJ12B	≈85%/5% (4h)	[318]
		PvdQ, acylase from *P. aeruginosa* PAO1	≈75%/0% (4h)	[319]
			≈60%/≈35% (72h)	
		BpiB09, short chain dehydrogenase reductase	100%/0% (4h)	[301]
		MomL, lactonase from *Muricauda olearia* Th120 (0,5 $U.mL^{-1}$)	≈95%/≈50% (24h)	[320]
			≈90%/≈40% (48h)	
Y. pseudotuberculosis	YpIII	AiiA, lactonase from *Bacillus subtilis*	Reduce biofilm infection severity***	[297]
Quorum quenching bacteria				
B. cenocepacia	LGM16656	Rhizosphere, water, mucus or intestines of flounders isolated bacteria	Increased survival (48h)***	[321]
P. aeruginosa	PAO1	*Pseudomonas, Pseudoalteromonas, Delftia, Arthrobacter, …*	Increased survival (48h) ***	[321]

* survival or not paralyzed at given time
** estimated from score and strains dependent
*** no survival rate (only increased in survival rate or other)

Murine infection models

Mammalian models, such as rats or mice, are commonly used to decipher the impact of QS in bacterial infections. Indeed, mutations or deletions of QS related genes were shown to reduce the mortality or severity of the infections in the lungs [193,322–324], wound burns [287,325], peritonitis [192], the prostate [326] and the intraperitoneal foreign body model [327], with the exception of *Staphylococcus epidermidis* [328]. In the vast majority of cases, QQ approaches result in a decrease in mortality, accelerate recovery and reduce bacterial colonization.

In lung infection models, *P. aeruginosa* colonization or related mortality was reduced by furanones [153,329], sub-minimal inhibitory concentrations of azithromycin [330], garlic extract [331] and also by the inhalation of the lactonase *Sso*Pox [30]. In skin wound models, a wide range of QSIs reduced *S. aureus* pathogenicity [195,197,332,333]. Similar inhibition of pathogenicity was observed with an AIP-targeting antibody [208]. Treatment with QS inhibiting peptide strongly reduced *S. epidermidis* colonization in a graft associated infection [334]. The efficiency of QQ was also demonstrated in a burn wound infection model with *P. aeruginosa* and an AHL degrading enzyme [136] or PqsR (MvfR) inhibitors [335] and, in an excision injury model, with the use of tea polyphenols as QSI [315]. Moreover, the combination effect of QQ molecules and antibiotics *in vivo* was demonstrated against both Gram-positive and negative bacteria. Indeed, for *B. cenocepacia,* the combination of baicalin and tobramycin allowed to reduce lung colonization by 2 and 1 log of CFU compare to control and antibiotic alone respectively [316]. The use of ciprofloxacin and a lactonase to treat *P. aeruginosa* wound burn infection enabled to reduce mortality and global bacterial dissemination to internal mice organs [136]. Furthermore, the cotreatment with a QSI and an antibiotic also drastically reduced colonization of artificial foreign body (e.g. catheter or implants) by *S. aureus* [195,197], *S. epidermidis* [334] and *P. aeruginosa* [196,259]. Those examples increased the interest of reducing antibiotic tolerance by QQ either in infected organs or medical device associated infections.

Murine models are useful and common tools to investigate the QQ impact on bacterial infections thanks to their adaptive and innate immune systems together with a physiology closely related to human beings. Furthermore, they are usually necessary and required as preclinical tests before starting human trials. At this stage of drug development, QQ seems to demonstrate great efficiency to reduce either morbidity or deleterious impacts for a wide variety of infections. However, murine models are less prone to screening steps because of practical and ethical problems unlike *C. elegans* or amoeba [336]. Furthermore, some physiological aspects of a pathology are not fully mimicked in murine models like wound healing or inflammation [336–338].

Clinical Trials in Humans with Quorum Sensing Inhibitors

So far, only previously approved or commercialized QSI were used in clinical trials, even if their primary use and approved biological activity did not relate to bacterial QS at all, but rather to their bactericidal, antimicrobial activities (antibiotics) or their cytotoxicity (anti-cancer molecules) [133,339].

In the early 2000s, azithromycin **(Figure 9)** was used in clinical trials to treat cystic fibrosis [340,341] and pulmonary transplanted patients [342]. This macrolide antibiotic improved patients' quality of life but did not lead to a decrease of bacterial load [340]. At the same period, the ability of azithromycin at non bactericidal concentrations to disrupt bacterial signalling in *P. aeruginosa* was demonstrated *in vitro* [343]. Later, the impact of azithromycin on *P. aeruginosa* QS in ventilator-associated pneumonia patients was evaluated [339]. The authors described beneficial anti-virulence effects of azithromycin in a high-risk group of patients, yet results were not significant enough.

Garlic is also known for its QQ properties [289] and was used in a trial to treat cystic fibrosis patients, although, no clear evidence has emerged of the curative effect of garlic extract on patient health [344].

Finally, the anti-cancer drug [345], 5-FU, a pyrimidine analogue **(Figure 9)**, was demonstrated to inhibit QS-regulated virulence in *P. aeruginosa in vitro* [216] and was further used for the coating of functionalized catheters, which were shown to be efficient during clinical trials [132,133].

In the end, very few QQ molecules reached human clinical trials but they tend to demonstrate some beneficial effects of QSI. Although many proofs of concept were performed in animal models, further efforts have to be dedicated to the validation of this approach in clinical phases to confirm its therapeutic relevance.

2.5 Use of quorum quenching molecules in medical devices

Medical devices are involved in numerous HAI [346]. Multi-drug resistant and/or biofilm forming bacteria are mainly responsible for HAI causing severe medical complications, high morbidity and risk of mortality. Considering the ability of QQ to prevent bacterial virulence [10], the development of novel medical devices using QQ agents is of outmost interest. New generations of catheters [347], dressings [47,237], aerosols [30], contact lenses [348], implantable devices [349] or orthopedic and trauma devices [350] are currently being developed **(Table 4)**.

QSIs were first considered for the functionalization of catheters. Covalently-attached furanones were shown to decrease biofilm formation by *S. epidermidis* ATCC 35984 and to control infection for 65 days in an *in vivo* sheep model [351]. 5-FU was used to coat a central venous catheter and was demonstrated to be efficient and comparable to classically used chlorhexidine/silver sulfadiazine coated catheters in a clinical study involving 960 adult patients in 25 US intensive care units [132,133]. Although the link to QS was not made by the

authors, the 5-FU coated catheters showed reduced contamination levels, by Gram-negative bacteria, as compared to the traditional coating which could be a clue as to interference with AHL dependent QS in this study. Poly(ethylene glycol)-based coating containing the QSI DHP(5-methylene-1-(prop-2-enoyl)-4-(2-fluorophenyl)-dihydropyrrol-2-one) was recently shown to reduce *S. aureus* strain 38 and *P. aeruginosa* MH602 colonization [352]. Combinations of DHP and furanone derivatives were also covalently attached onto glass surfaces and significantly reduced the adhesion of *S. aureus* SA38 and *P. aeruginosa* PAO1 [353]. A delivery system based on varnishes releasing the QSI thiazolidinedione-8 (TZD-8) was used on catheters and were active against *Candida albicans* biofilms [354]. Notably, honey polyphenols were introduced into a scaffold of selenium nanovectors for quenching *P. aeruginosa* PAO1 *in vitro* and *in vivo* [355].

For *agr*-based QS in *S. aureus*, inhibiting peptides were also successfully incorporated into biomaterials. Macrocyclic peptides were loaded into non-woven polymer nanofibers by electrospinning and showed to retain biological activity against *S. aureus* after releasing over a three-week period [356]. Click chemistry was also considered for covalently coating surfaces with pro- and anti-QS peptides, AIP-I and TrAIP-II respectively and showed efficacy against *S. aureus* strains [198]. The synergy of QS inhibiting peptide FS3 with antibiotics was also demonstrated, with daptomycin being highly effective against staphylococcal infections when combined with a FS3-coated prosthesis [197]. Similarly, for several strains of *S. epidermidis*, the RNAIII-Inhibiting peptide (RIP) was efficient in reducing infection when incorporated into a Dacron graft [334].

Although QS inhibiting materials were obtained after covalent immobilization of QSIs or peptides, QQ enzymes were also thoroughly investigated as these compounds, acting on secreted autoinducers, do not need direct contact with the cells to disrupt communication. Acylase from *Aspergillus melleus* was successfully incorporated into polyurethane coatings and silicon catheters reducing biofilm formation of *P. aeruginosa* ATCC 10145 and PAO1 respectively [134,357]. Combination of the acylase with α-amylase from *Bacillus amyloliquefaciens* delayed biofilm development of both *P. aeruginosa* ATCC 10145 and *E. coli* ATCC 25922 for up to seven days in an *in vivo* rabbit model [135]. Acylase from porcine kidney was also immobilized on carboxylated polyaniline nanofibers for the development of nanobiocatalysts limiting biofilm formation of *P. aeruginosa* PAO1. Topical treatment involving lactonase from *Bacillus* sp. ZA12 was also investigated in a burn infection model on mice using *P. aeruginosa* PAO1 [136]. Application of a lactonase-containing gel after 10^6 bacteria burn infection prevented systemic spread, decreased mortality and showed synergistic effect with ciprofloxacin.

Because enzyme stability is a major bottleneck in the development of bio-based materials, catalysts from extremophile environments were considered. Particularly, PLL *Sso*Pox from *S. solfataricus* was found to be a highly attractive way of quenching bacterial virulence [47,237]. This highly robust enzyme [28,358], was first immobilized onto nanoalumina membranes while retaining strong efficacy for reducing virulence factor secretions, pyocyanin and elastase of *P. aeruginosa* PAO1 [26]. The variant enzyme *Sso*Pox-

W263I was further shown to significantly reduce the virulence of 51 clinical isolates of *P. aeruginosa* from diabetic foot ulcerations and kept its efficiency toward PAO1 after immobilization into polyurethane coating via glutaraldehyde crosslinking [359]. The *in vivo* use of this variant was also reported through intratracheal administration and drastically enhanced the survival rate in a rat pneumonia model infected by *P. aeruginosa* PAO1 [30].

In addition to the studies using AHL-based QS quenchers, a recent report described the use of the AI-2 processing kinase LsrK. This enzyme was attached to a capsule of biological polymers chitosan and alginate supplemented with ATP substrate and reduced AI-2 mediated QS [360].

QQ-based devices have raised special attention considering that they could prevent HAI by limiting bacterial virulence and biofilm formation. However, further efforts have to be dedicated to validate the proof of concepts *in vivo* and in clinical phases. The efficacy of these devices has to be demonstrated not only in model bacterial strains but also on genetically and phenotypically diverse clinical isolates. Although the development of medical devices is less constrained than for drugs, further regulatory concerns have to be considered to confirm the potential of the techniques for therapeutic applications. Nevertheless, the wide spectrum of both QSI and QQ enzyme as well as the numerous examples of their medical relevance would pave the way to the emergence of innovative devices.

Table 4 : Quorum quenching based medical devices.

QQ STRATEGY	QQ AGENT	APPLICATION	REFERENCE
QSI	5-FU	Catheters	[132,133]
	Furanones	Catheters	[351]
	DHP	Coatings	[352]
	TZD-8	Urinary catheters	[354]
	Furanone and DHP derivatives	Implanted medical devices	[353]
Peptides	TrAIP-II	Colonization-resistant materials	[198]
	Macrocyclic peptides	Nanofiber coatings	[356]
	FS3	Prosthesis	[197]
	RIP	Dacron graft	[334]
QQ Enzymes	PLL *Sso*Pox from *S. solfataricus*	Coatings, membranes, aerosols	[26,30,359]
	Acylase from *A. melleus*	Catheters and other coated devices	[134,357]
	Acylase from *A. melleus* and α-amylase from *B. amyloliquefaciens*	Catheters	[135]
	Lactonase from *Bacillus* sp. ZA12	Topical treatments	[136]
	Acylase from porcine kidney	Nanofibers	(Lee et al., 2017)
	AI-2 processing kinase LsrK	Capsules	[360]
Natural compounds	Polyphenols of honey	Nanovectors	[355]

5-FU: 5-fluorouracil; Furanone: 3-(10-bromohexyl)-5-dibromomethylene-2(5H)-furanone; DHP: 5-methylene-1-(prop-2-enoyl)-4-(2-fluorophenyl)-dihydropyrrol-2-one; TZD-8: Thiazolidinedione-8; TrAIP-II: a truncated autoinducer peptide (AIP-II) with the exocyclic tail replaced by and acetyl group; FS3: RNA-III inhibiting peptide (RIP) analogue (YAPWTNF-NH_2)

2.6 Conclusions and perspectives

Over the past 15 years, many studies have demonstrated that QQ molecules and QQ approaches have great potential as anti-infective agents against a broad range of bacteria. This is evidenced by the numerous studies demonstrating the benefit of these approaches in functionalizing medical devices. To date, little is known about potential resistance mechanisms that bacteria could develop to overcome QQ [158,159]. The apparition of resistance phenomenon results from the natural process of evolution in a context of selection pressure which favors the growth of resistant strains. This is the case for antibiotics that apply high selection pressure, through growth inhibition, for sensitive strains [361]. If some QSI such as azithromycin lead to severe growth inhibition, others have only moderate or no effect on

growth rate [362]. Apparition of QQ resistant bacteria is possible but its rate might be slower as compared to antibiotic resistance and will depend on the type of QQ (QSI or QQ enzymes) and its impact on bacterial growth [363]. QQ resistant strains have already been reported either from laboratory experiments or from clinical samples, notably strains with lower uptake or higher efflux of QSI [161]. Nevertheless, little is known on how QQ resistant strains would over grow QQ sensitive strains nor on how the population would evolve. Most studies performed to address this question were performed *in vitro* and using QS mutants or QSI, the results obtained so far were not consistent [164,363,364]. To limit QQ resistance, QQ agents should be carefully chosen to keep growth deleterious effects minimal. Many QSIs have toxic activities and need to enter the cells in order to be active. QQ enzymes may represent ideal candidates combining a minimal, if any, selection pressure and potent inhibitory effects on biofilm formation and virulence [359]. Further studies involving QQ enzymes should be performed to evaluate potential QQ resistance mechanisms using QQ enzymes. Nonetheless, QQ is a promising strategy to extend the therapeutic arsenal available to treat bacterial infections in complement to classical antimicrobial agents and antibiotics or reemerging bacteriophages.

The broad effect of QS on the physiology of bacteria shows that QQ would be an appropriate strategy not only to reducing bacterial virulence but also in terms of restoring antibiotic tolerance by decreasing biofilm formation and in terms of decreasing bacterial phage resistance, paving the way for future combination therapies.

Remarkably, the disruption of bacterial signaling, a communication system central to microbial communities [365], has implications that go beyond the single bacteria physiology. Indeed, the gut microbiota of fishes fed with probiotic bacteria, producing QQ enzymes, was modified and the population of pathogenic *Aeromonas hydrophila* was reduced [366]. In another approach, a recent study showed the ability of *E. coli* overproducing AI-2 to counter the impact of streptomycin-induced gut dysbiosis potentially underlining the role of quorum sensing in the context of complex microbiota [367]. More studies are needed to delineate the effects induced by QQ strategies at both the single bacterial species level and in the context of communities. Future investigations will determine the breadth of the action of QQ molecules and their potential in being used as therapy, combination therapy and as coating agents in medical devices.

Acknowledgments

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III. Projets de recherche

Objectifs de la thèse

Le travail de thèse s'est inscrit en parallèle d'un projet qui vise à développer un pansement incorporant une lactonase (ici *Sso*Pox W263I) afin de bloquer la communication bactérienne de type QS. La start-up Gene&GreenTK (qui développe l'enzyme *Sso*Pox) travaille sur la mise au point d'un prototype de textile médical fonctionnalisé avec *Sso*Pox afin d'obtenir un nouveau type de pansement antivirulent.

Le premier travail engagé lors de cette thèse a eu pour but de lever des verrous technologiques quant à la compatibilité de l'enzyme avec des procédés industriels comme les effets de la température et de la stérilisation sur la stabilité de l'enzyme. D'autres caractéristiques comme la stabilité, l'activité à basse température et l'efficacité de fixation dans des billes d'alginate ont aussi été étudiées. Ces données sont importantes pour la suite du projet afin de déterminer les forces et faiblesses de l'enzyme pour des applications industrielles.

Ensuite, l'efficacité de l'enzyme a été démontrée sur des isolats cliniques de *P. aeruginosa* et deux souches modèles (PAO1 et PA14) pour prouver l'impact sur le biofilm et les facteurs de virulence. En parallèle, l'efficacité de l'enzyme après immobilisation a aussi été évaluée afin de prouver le potentiel de la technologie pour une incorporation dans un pansement.

Enfin, un axe plus fondamental a été engagé afin d'approfondir les effets du QQ enzymatique chez *P. aeruginosa*. L'impact de *Sso*Pox, à la fois aux niveaux phénotypiques et moléculaires, a été comparé à ceux d'une autre lactonase récemment découvert (*Gc*L) possédant un spectre d'action plus large pour l'hydrolyse des lactones. Ceci constitue la première étude sur le rôle de la spécificité des enzymes dans l'inhibition de la communication bactérienne.

1. Caractérisation des propriétés biotechnologiques d'une lactonase hyperthermostable

Les procédés industriels impliquent souvent des contraintes physico-chimiques très éloignées des conditions physiologiques auxquelles les enzymes sont classiquement confrontées. Températures extrêmes, pH fortement acides ou basiques, ou encore utilisation de solvants organiques sont autant d'étapes fréquemment utilisées dans les procédés industriels. Des contraintes plus spécifiques, telles que la stérilisation dans l'industrie médicale, sont parfois strictement nécessaires dans le processus de fabrication. Ces étapes charnières sont généralement destructrices pour les molécules du vivant comme les protéines. D'autre part, le produit fini devra être stable à long terme pour permettre sa conservation, si possible à température ambiante sans altération de ses propriétés ni de son efficacité.

Les enzymes sont souvent des molécules fragiles inadaptées aux procédés industriels dont les contraintes s'avèrent délétères conduisant à leur dénaturation et à une perte de leur fonction catalytique. Néanmoins, la découverte et l'étude des organismes vivant dans des conditions extrêmes (e.g sources hydrothermales, solfatares près des volcans, lacs salins, fosses marines) ont permis d'isoler et de caractériser des enzymes plus robustes que leurs équivalents mésophiles[XI] [368,369]. Dans l'industrie agroalimentaire, les glycosyl hydrolases (e.g. amylases, xylanases) thermostables sont utilisées pour la dégradation de sucres complexes comme l'amidon car le processus fait intervenir une température élevée afin de maintenir ce dernier liquéfié [369,370]. Les enzymes entrent parfois dans la composition de produits du quotidien tels que les produits ménagers comme la lessive qui contient entre autres des protéases et lipases ayant un large spectre de tolérance à la température et aux détergents [369]. Enfin, certaines enzymes hyperthermostables ont aussi révolutionné des domaines spécialisés comme celui de la biologie moléculaire. C'est le cas de l'ADN polymérase isolée de *Thermophilus aquaticus* qui résiste à des variations de température pouvant atteindre 90°C pendant de nombreux cycles de réactions et qui est maintenant un outil incontournable des laboratoires de recherche et de diagnostic [369]. Ainsi, les enzymes issues d'organismes extrêmophiles représentent un avantage industriel et commercial certain. Bien que considérées moins actives à faible température [371], elles représentent des châssis très robustes pour la mutagénèse et l'évolution dirigées car elles ont une meilleure tolérance aux effets déstabilisateurs de certaines mutations [372].

Parmi les organismes extrêmophiles, les archées, fréquemment retrouvées dans les lacs salins ou les sources hydrothermales, constituent un vivier de protéines résistantes et ont été particulièrement étudiées pour isoler des enzymes compatibles avec des applications industrielles [373,374]. L'enzyme *Sso*Pox, isolée de l'archée thermophile *S. solfataricus,* a notamment été particulièrement étudiée. Cette lactonase hyperthermostable (T_m de 106°C) est résistante à de nombreux agents dénaturants et conserve environ la moitié de son activité

[XI] Mésophile : enzyme stable et active dans des conditions modérées de température, de pression et de salinité.

à température ambiante par rapport à sa température optimale de 70°C [27,28]. *Sso*Pox est active, entre autres, sur la 3-oxo-C_{12} HSL qui est utilisée dans le QS de *P. aeruginosa,* la bactérie ciblée dans le cadre du développement d'un pansement antivirulent. L'activité de *Sso*Pox vis-à-vis de la 3-oxo-C_{12} HSL a par ailleurs été multipliée par 45 grâce à la mutation du résidu tryptophane 263 en une isoleucine (W263I). Cette mutation confère à l'enzyme une flexibilité accrue qui lui permet d'accommoder plus efficacement son substrat augmentant ainsi son efficacité. Cette flexibilité est associée à une légère diminution de la thermostabilité de l'enzyme (T_m de 88°C pour le mutant contre 106°C pour l'enzyme sauvage) mais reste néanmoins bien supérieure aux enzymes mésophiles généralement utilisées pour le QQ (e.g. AiiA, AiiB). *Sso*Pox W263I est donc une enzyme robuste, efficace et prometteuse en vue d'applications industrielles [10,29].

Compte tenu de son fort potentiel, *Sso*Pox W263I a été produite à grande échelle en fermenteur de 500 L en vue de la caractérisation de ses propriétés biotechnologiques. Un extrait bactérien sous forme de poudre partiellement purifiée a été obtenu après atomisation. Par la suite, nous avons soumis l'enzyme à des conditions proches de celles trouvées dans la chaine de fabrication du pansement. La résistance aux hautes températures, aux solvants organiques, à la stérilisation a été démontrée ainsi que sa stabilité sur 300 jours. Nous avons par ailleurs mis en évidence que les sécrétions de bactéries à Gram positif (*S. aureus, Bacillus cereus*) et négatif (*P. aeruginosa, A. baumannii*), pouvant être rencontrées dans des plaies infectées, n'impactent pas, voire améliorent, les propriétés de l'enzyme. Des expériences préliminaires d'immobilisation de l'enzyme dans des billes d'alginate ont également été réalisées et la stabilité des billes sur plusieurs cycles d'utilisation a été évaluée. Enfin, les enzymes issues d'organismes hyperthermophiles étant généralement considérées comme faiblement actives voire inactives à basse température, l'efficacité catalytique[XII] du variant *Sso*Pox W263I a été mesurée à -18, 23 et 70°C. Cela a permis de prouver l'étonnante robustesse de l'enzyme qui s'avère particulièrement pertinente pour une utilisation industrielle engageant des conditions extrêmes pour des enzymes.

L'ensemble de ces résultats a été rassemblé dans un premier article publié dans Scientific Reports [358]. J'ai largement contribué à ce travail en effectuant la majorité des expériences à l'exception de la production à l'échelle semi-industrielle de l'enzyme et des processus de stérilisation. J'ai participé à l'écriture, la mise en forme des résultats et à la relecture du manuscrit.

[XII] L'efficacité catalytique est traduite ici par la division de la constante catalytique (k_{cat}), qui représente le nombre de réaction par seconde, sur la constante d'affinité (K_m).

1.1 Introduction

Since the emergence of directed evolution and the beginning of the third wave of biocatalysis, enzymes have gained considerable interest for biotechnological purposes [375]. As a consequence, the global market for enzymes is expected to reach US$ 7.1 billion by 2018 [376]. However, enzyme use can be limited by cost, activity levels, or incompatibility with existing industrial production plants [377]. Overcoming these limitations has been extensively investigated during the last decade by either stabilizing highly active biocatalysts or isolating enzymes from extreme environments (also known as extremozymes) [368,372,378–381]. Psychrophile and thermophile organisms are among those that have been studied in this way [369,382–384]. Psychrophilic enzymes have been proven to be efficient at low to moderate temperatures (4-25°C) and their tolerance to solvents has been highlighted [377]. Conversely, thermophilic enzymes are particularly active at high temperatures (>55°C) and are known to resist denaturing agents including proteases, surfactants or detergents [377,379]. These features increase the compatibility of these biocatalysts with industrial processes and are continuously under consideration for developing efficient enzyme-based technologies.

*Sso*Pox is a phosphotriesterase-like lactonase (PLL) isolated from the archaeon *S. solfataricus* [23]. This enzyme is a natural lactonase that is able to hydrolyze acyl-homoserine lactones (AHL) that are involved in the quorum sensing (QS) of Gram-negative bacteria (e.g. *P. aeruginosa*, *A. baumannii*) [22,30]. QS is a communication mechanism by which bacteria sense their population density and synchronize their behavior [8,9,154,157]. Virulence factor secretion and biofilm formation are for example regulated by QS [153,385,386]. Strategies aiming at counteracting QS, dubbed quorum quenching (QQ), are of prime interest for developing therapeutic alternatives to classical antimicrobial agents (e.g. antibiotics) [217,387,388]. Industrial applications using QQ strategy mainly include medical devices such as catheters, aerosols or dressings and have been exhaustively reviewed elsewhere [10,155]. QQ also appears to be a promising strategy for anti-fouling applications [108].

In addition to its lactonase activity, *Sso*Pox also displays promiscuous phosphotriesterase activity that can degrade organophosphorus chemicals (OP) [28]. OP are highly toxic compounds that inhibit acetylcholinesterase, a key enzyme for regulation of the central nervous system [389,390]. The exhaustive use of OP for agricultural purposes has led to serious contamination worldwide and is a major environmental and public health issue [391–393]. OP were also considered for military ends leading to the synthesis of noxious chemical warfare nerve agents (CWNA) [394,395]. These compounds constitute a serious threat for civil and military populations but no satisfactory external decontamination method is currently available [396]. OP-degrading enzymes have thus emerged as potential bioremediation alternatives [396,397].

Considering both its lactonase and phosphotriesterase capabilities as well as its exceptional thermal stability (T_m=106°C), many applications involving *Sso*Pox are considered [26,47,237,396,398–401]. Although its natural robustness confers outstanding biotechnological potential on *Sso*Pox [144], its catalytic activities were first increased to turn the biocatalyst into a cost-effective technology [29]. The resolution of the 3D-structure

allowed the identification of the crucial role of residue W263 in both activity and substrate promiscuity [22]. Site-saturation mutagenesis was applied and led to the characterization of catalytically improved variants maintaining strong robustness [29]. Among these, the single variant *Sso*Pox-W263I was of special interest insofar as both its AHL- and OP-degrading activities were increased as compared to wild-type enzyme while harboring a high melting temperature value (T_m=88°C).

Variant *Sso*Pox-W263I, which exhibits higher lactonase and phosphotriesterase activity, is a promising candidate for the external bioremediation of OP, including liquid decontamination solutions, filtration systems, and auto-decontaminating textiles or materials. Additionally, its ability to interfere with bacterial signaling offers a wide variety of possible uses, such as medical devices containing enzymes and biomaterials. However, the industrial feasibility of such bio-based products is dependent on the ability of the variant to meet process requirements. We evaluated the compatibility of the improved variant *Sso*Pox-W263I in harsh conditions. The variant was produced at pilot scale (500 L fermentation) and used for the biotechnological characterization under rough process-like conditions. The variant's resistance to temperature, solvents, sterilization, bacterial degradation and the enzyme's lifetime were determined. Our results show that *Sso*Pox-W263I maintains most of its activity after extremely harsh treatments, highlighting the tremendous potential for using this enzyme in existing industrial production lines.

1.2 Results

Resistance to temperature-induced stress

Industrial processes often require short but high-temperature steps for various purposes including drying, polymer reticulation, solubilization, chemical reactions [377,379]. We investigated the resistance of *Sso*Pox-W263I submitted to a heat shock from 40°C up to 120°C in liquid form (*i.e.* atomized powder resuspended in water) and 150°C in solid form (*i.e.* atomized powder) for 5 minutes using a dry bath to simulate reticulation step used for example in textile processes **(Figure 10)**. In liquid form, activity only fell slightly up to 90°C. From 100 to 120°C, the activity decreased quickly and was almost null at 120°C. The post-shock activity of the powder form was measured after resuspension in water. Activity only fell marginally with heat shock up to 120°C. From 130 to 150°C, activity decreased quickly, albeit more than 10% of the activity remained at 150°C. The temperatures required to lose one half of enzymatic activity in 5 minutes were found to be 92°C and 130°C in liquid and solid states respectively.

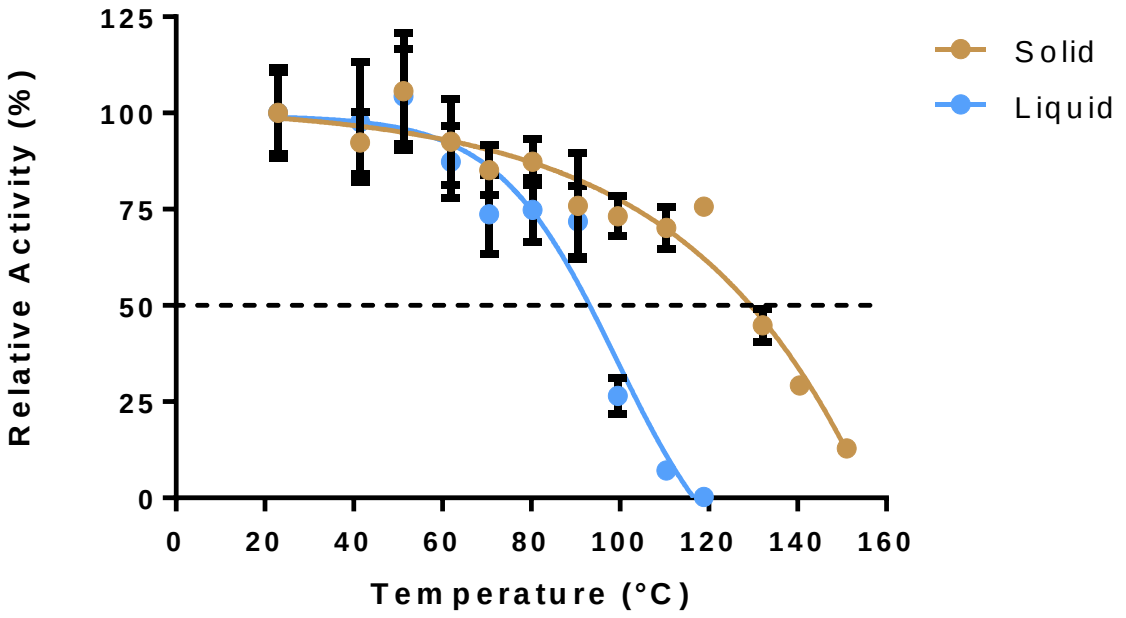

Figure 10 : Relative activity of *Sso*Pox-W263I submitted to a 5-minute heat shock in either liquid (blue) or solid form (brown). The values represent the mean ± SEM (Standard Error to the Mean) of six replicates.

Resistance to sterilization methods

The enzyme was subjected to three sterilization methods: autoclaving, ethylene oxide and β-radiation. Autoclaving is commonly used in medical applications for sterilizing materials. It uses a combination of elevated temperature and pressure and is usually destructive for proteins. Here we autoclaved the enzyme in liquid or in solid state (powder) at 121 °C for 15 minutes **(Figure 11a)**. Surprisingly, more than 30% activity remained after autoclaving the enzyme powder, while all the activity was lost in the case of the liquid form, which is consistent with the temperature resistance of the liquid enzyme.

Chemical gaseous sterilization is used for disinfecting and sterilizing instruments whose properties would be affected in liquid form. We investigated the alkylating agent ethylene oxide for sterilizing the solid enzyme **(Figure 11b)**. Only 30% of enzyme activity was lost after the first sterilization cycle, which underscores the high robustness of the protein. Interestingly, no additional loss was observed after three repeated sterilization cycles.

The last method we investigated was β-radiation **(Figure 11c)**. This process is commonly used for high-throughput sterilization with a typical dose of 25 kGy. Here, we considered three distinct doses from 25 kGy up to 100 kGy for sterilizing both liquid and solid forms. With the liquid enzyme, the remaining activity decreased down to 40% at the highest dose. Conversely, the solid form was barely, if at all, affected by radiation. This result is particularly interesting because β-radiation is probably the most convenient sterilization method for medical devices. Radiation may also be used for liquid enzyme sterilization but the dose level may impact the remaining activity.

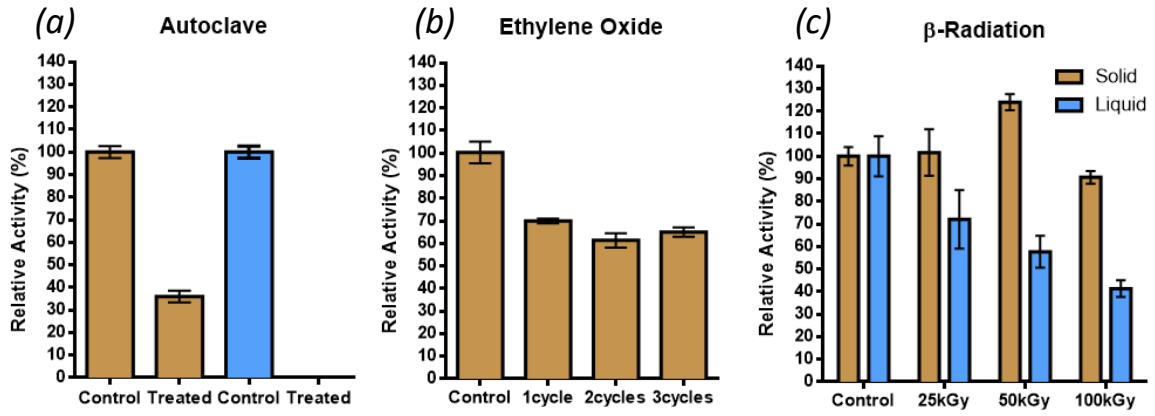

Figure 11 : *Sso*Pox-W263I relative activity after submission to three sterilization methods: autoclave *(a)*, ethylene oxide *(b)*, β-radiation *(c)*. Experiments on solid enzyme are in brown bars while those on liquid enzyme are in blue. Measures were performed in triplicate as compared to a non-sterilized control. Values represent means ± SEM.

Activation by bacterial secretion factors

We investigated the ability of bacterial secretion materials to promote enzyme activity or reactivate heat-shocked enzyme. Four strains of both Gram-negative and Gram-positive bacteria were considered. Culture supernatants of *Pseudomonas aeruginosa* PAO1, *A. baumannii* AYE, *S. aureus* ATCC 29213 as well as a clinical isolate of *B. cereus* were investigated for how they affect *Sso*Pox-W263I activity **(Figure 12)**. Many pathogenic bacteria secrete agents such as proteases or surfactants to inactivate host proteins. Surprisingly, enzyme activity did not decrease in the presence of bacterial supernatant and, in some cases, increased as compared to the LB medium control. Next we considered the ability of supernatants to reactivate heat-shocked enzyme at 150°C. All the supernatants favored enzyme reactivation, from 40% for *P. aeruginosa*, up to 80% for *B. cereus*. To evaluate the thermo-susceptibility of the reactivation phenomenon, supernatants were further heated to precipitate unstable compounds. Interestingly, the reactivation was less pronounced after heating the supernatant for 10 minutes at 100°C, underlining the fact that thermolabile metabolites and/or proteins are partially responsible for enzyme reactivation.

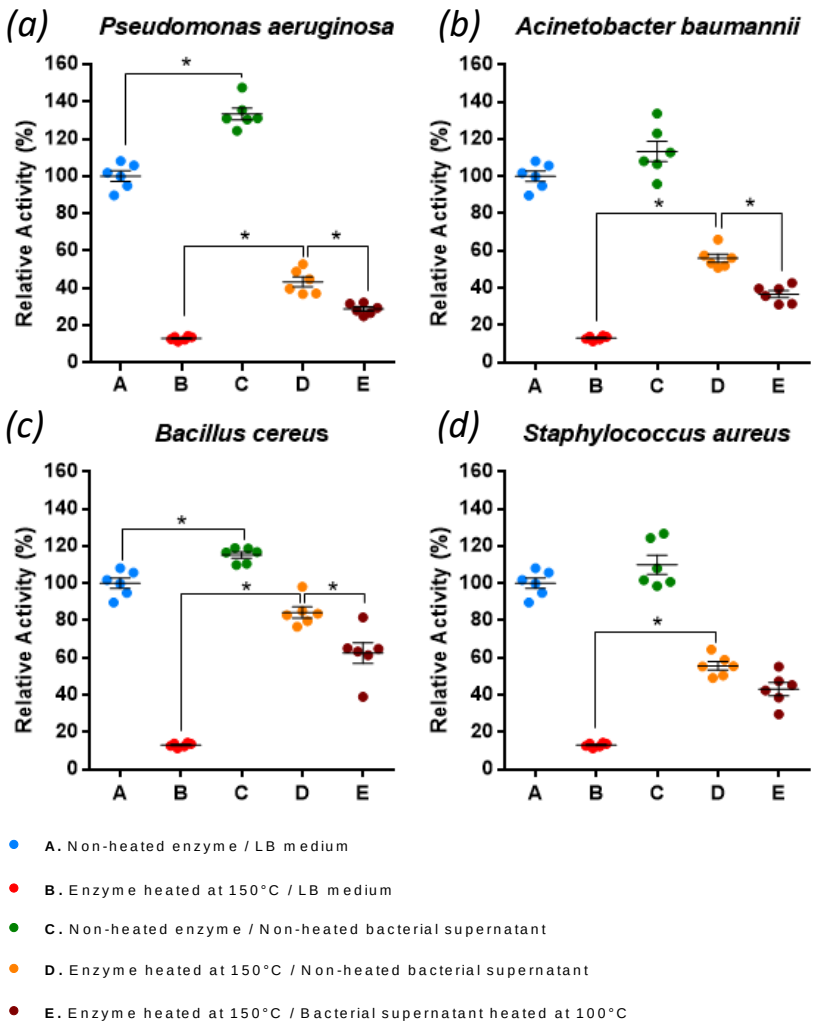

Figure 12 : Dot plots of the relative activity of *Sso*Pox-W263I in contact with bacterial culture supernatants of *P. aeruginosa* PAO1 *(a)*, *A. baumannii* AYE *(b)*, *B. cereus (c)* and *S. aureus (d)*. For each graph, blue dots correspond to the positive control (*i.e.* unheated enzyme resuspended in sterile LB medium). Red dots correspond to the heated control (*i.e.* enzyme heated at 150°C and resuspended in sterile LB medium). Green dots represent the enhanced activity of unheated enzyme resuspended in bacterial supernatant. Orange dots illustrate the reactivation of heat-shocked enzyme resuspended in bacterial supernatant. Finally, purple dots represent the activity of heated enzyme resuspended in previously 100°C heated bacterial supernatant to investigate the role of thermolabile compounds in the reactivation phenomenon. The n=6 replicates are represented with mean ± SEM (Standard Error to the Mean) in black bars. Black stars (*) indicate a significant difference ($p<0.01$) according to the corresponding contrast.

Catalytic activity at sub-zero temperatures

Hyperthermostable enzymes are commonly described as nearly inactive at room temperature, but their catalytic power is in fact greater at lower temperature [370,371,377]. Optimum *Sso*Pox-W263I activity was previously determined in the range of 80-95°C and significant activity was also observed at 23°C [23,28,29]. Here, we investigated the ability of the enzyme to work at sub-zero in glycerol-complemented buffer **(Figure 13)**. Surprisingly, the

variant was still significantly active at -18°C. The k_{cat}/K_M values were estimated using a low ethyl paraoxon concentration (250 µM) and values of $2.0x10^4$ $M^{-1}.s^{-1}$, $9.7x10^3$ $M^{-1}.s^{-1}$ and $1.4x10^3$ $M^{-1}.s^{-1}$ were determined at 70°C, 23°C and -18°C respectively **(Table 5)**. Rates only decreased by 14-fold and 7-fold at -18°C, as compared to 70°C and room temperature, and converted more than 99% of substrate in 6 hours with only 0.3 µM of enzyme. In a previous work, the temperature coefficient (Q_{10}) for > 100 enzymatic reactions was found to be 2, including those involving thermostable enzymes [371]. Thus, the average enzymatic rate decrease by ~446-fold and ~17-fold over the same temperature ranges, 70°C/-18°C and 23°C/-18°C, respectively. The temperature dependence of *Sso*Pox-W263I is therefore much lower than the average enzyme. Consequently, the Q_{10}^{kcat/K_M} temperature coefficient values calculated between 70°C/23°C, 70°C/-18°C and 23°C/-18°C varying from 1.17 to 1.60 were found to be significantly lower than for most enzymes (Q_{10}^{kcat/K_M} value is ≈2)[371].

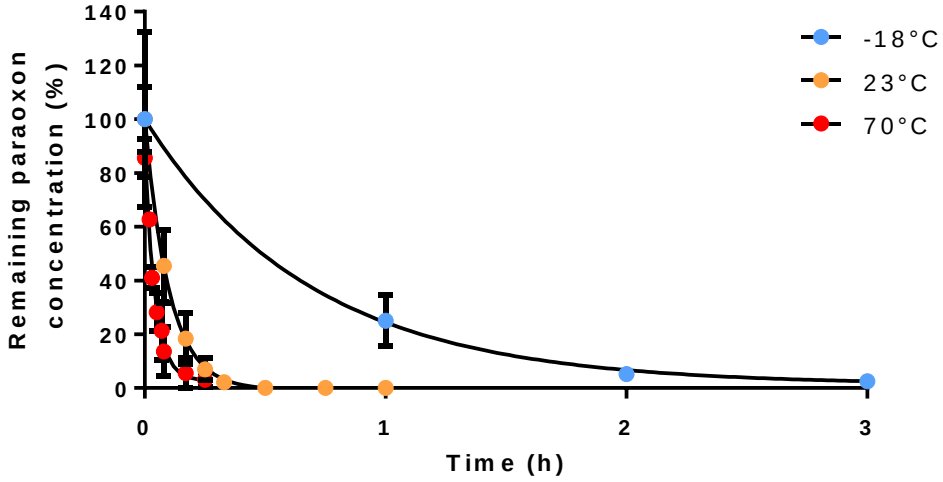

Figure 13 : Hydrolysis of paraoxon by variant *Sso*Pox-W263I over time at 70°C (red dots), 23°C (orange dots) and -18°C (blue dots) with 50% glycerol. Relative concentrations of paraoxon are presented. Measures were performed in triplicate and are represented with mean ± SEM in black bars.

Table 5 : Catalytic efficiencies of *Sso*Pox-W263I with paraoxon at different temperatures

Temperature	Reaction conditions	k_{cat}/K_M ($M^{-1}.s^{-1}$)*
-18°C	Activity buffer + 50% glycerol	$1.4x10^3$
23°C	Activity buffer	$1.2x10^3$ **
23°C	Activity buffer + 50% glycerol	$9.7x10^3$
70°C	Activity buffer	$2.5x10^4$
70°C	Activity buffer + 50% glycerol	$2.0x10^4$

* k_{cat}/K_M were estimated for a concentration of 250 µM of paraoxon. It was assumed that the substrate concentration was negligible as compared to K_M. k_{cat}/K_M was thus estimated by using the one phase decay function in GraphPad Prism v6. The resulting rate constant k was divided by the enzyme molar concentration to estimate the k_{cat}/K_M in $M^{-1}.s^{-1}$.

** Data taken from [29]

Tolerance to solvents

As for temperature, compatibility with solvents is a major prerequisite for processing viability [402,403]. Here, we evaluated the potential of *Sso*Pox-W263I to resist to a solvent-requiring industrial step. A broad panel of 16 solvents was considered: acetone, acetonitrile, butanone, butyl acetate, chloroform, dichloromethane, diethyl ether, ethanol, ethyl acetate, isopropanol, methanol, methoxypropanol, methylcyclohexane, petroleum ether, toluene and xylene **(Figure 14)**. Solid enzyme powder was resuspended in pure (100%) solvent that was further evaporated. The enzyme was subsequently solubilized in water and activity was measured and compared to the water-treated control. Only four out of the 16 solvents investigated significantly affected the activity of *Sso*Pox-W263I (*i.e.* methylcyclohexane, petroleum ether, toluene and xylene). Among these, petroleum ether and toluene only slightly reduced the activity, down to 78% and 74% respectively as compared to the water control. Xylene was the most aggressive solvent as it decreased activity by more than 75%. Conversely, methylcyclohexane surprisingly improved activity by ≈2.5-fold increase (255%). For the 12 others, no significant effect on the enzyme was detected after 2-hour long contact. *Sso*Pox-W263I showed impressive resilience to pure organic solvents.

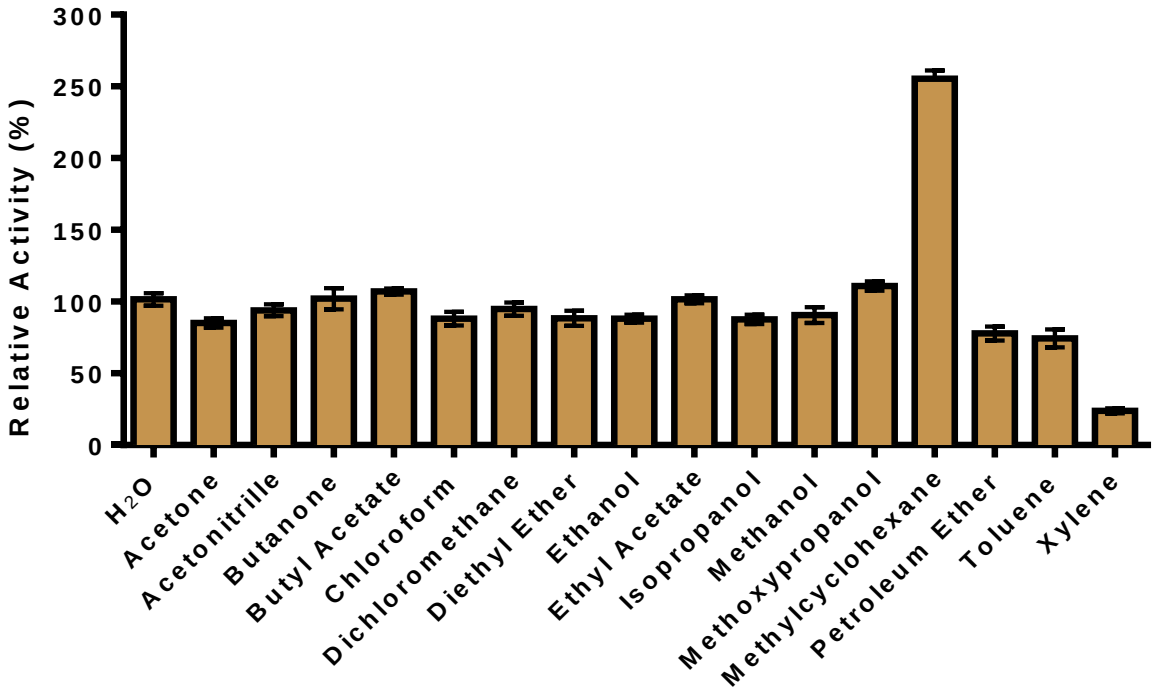

Figure 14 : Relative activities of *Sso*Pox-W263I after treatments in different solvents. For each solvent, the n=6 replicates are represented with mean ± SEM in black bars.

Storage capacity of SsoPox-W263I

Hyperthermostable enzymes, with their intrinsic robustness, usually show enhanced lifetime as compared to their mesophilic counterparts. This constitutes a major advantage for long-term storage with minimal constraints. Moreover, lyophilization and atomization usually preserve unstable compounds by minimizing water activity and/or limiting contamination by microorganisms, in addition to being convenient for transport purposes. We followed the activity of solid enzyme stored at room temperature in anhydrous conditions. Interestingly, the enzyme remained active in these experimental conditions for > 9 months. Only a very slight activity decrease can be observed **(Figure 15)**. This result strongly underlines the industrial relevance of *Sso*Pox-W263I, which may be kept for weeks without alteration.

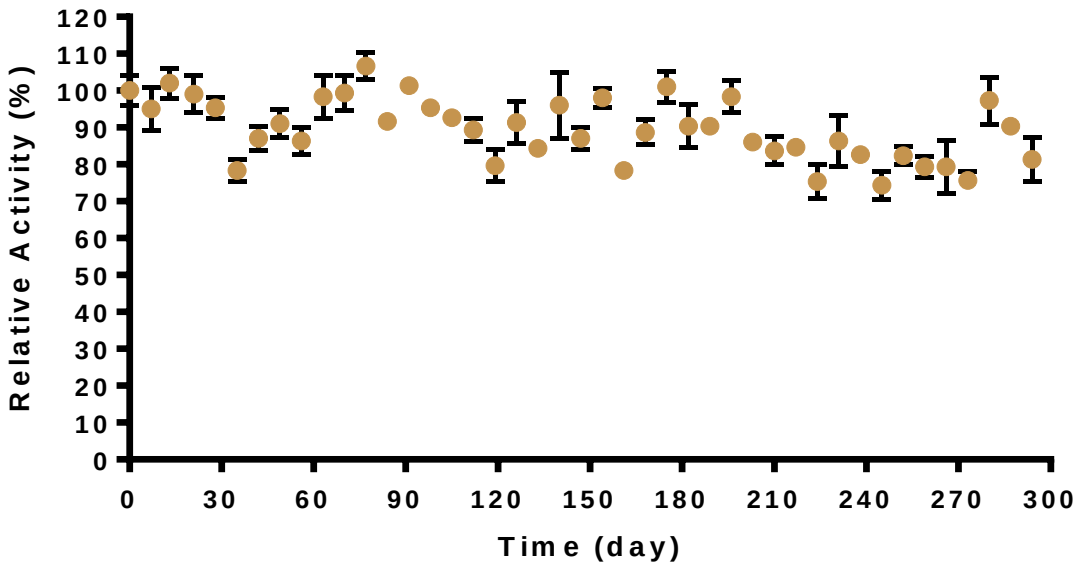

Figure 15 : Dot plots of the relative activity of solid enzyme stored at room temperature over time. For each time, the n=3 replicates are represented with mean ± SEM in black bars.

Immobilization of SsoPox-W263I

We investigated the immobilization of *Sso*Pox-W263I through crosslinking aggregation into alginate beads. Glutaraldehyde (GAD) was previously reported as an aggregating agent [404], and was used here for crosslinking the enzyme inside alginate beads. Only a small amount of enzyme leaked out of the beads during the rinsing steps and the enzyme remained active and accessible after entrapment. Up to 15% of activity was immobilized with this technique. Bead recovery was subsequently investigated and the immobilized enzyme was used ten times with only 30% decrease **(Figure 16)**. This method offers a simple procedure for the cheap and efficient immobilization of *Sso*Pox-W263I.

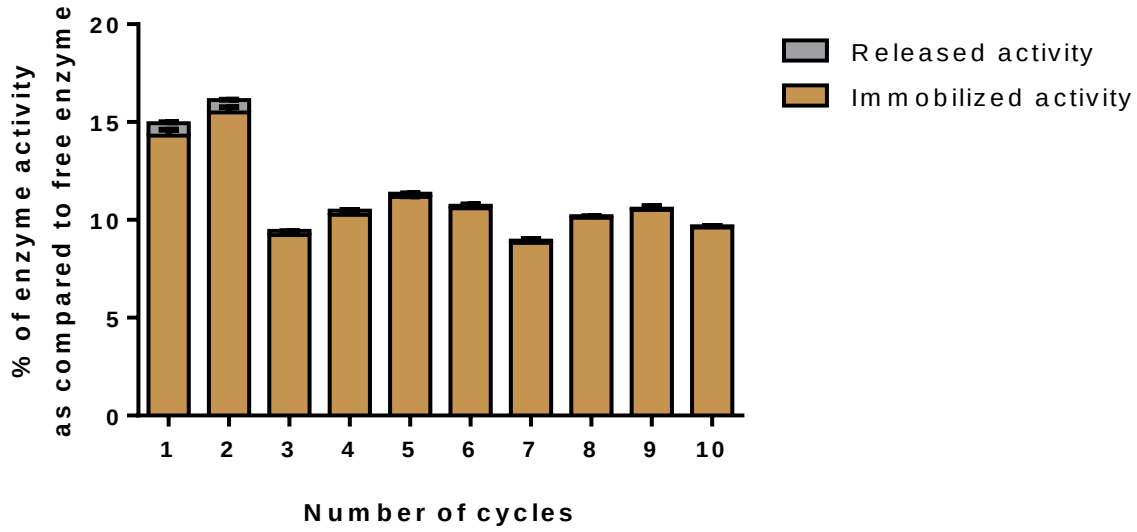

Figure 16 : Immobilization rate of enzyme by crosslinking in alginate beads as compared to the free enzyme and recovery. The results for each cycle are represented with mean ± SEM in black bars. Immobilized and released activities are shown in brown and grey respectively.

1.3 Discussion and conclusion

Enzymes isolated from extreme environments have gained considerable interest for biotechnological applications. Extremozymes were largely found to be both robust and evolvable, to be optimizable through protein engineering strategies while maintaining tremendous stability. In this work, we first investigated the robustness of a previously engineered *Sso*Pox variant improved against OP and AHL. We show that *Sso*Pox-W263I exhibits remarkable heat resistance, with no drastic effects observed up to 90°C on liquid or solid samples, which is consistent with the extremophile origin of the enzyme and the Tm value of *Sso*Pox-W263I (88°C). The enzyme in solid form was found more resistant to temperature as compared to the liquid form, which is probably due to a lower molecular agitation and water activity. Although this extreme robustness is rare for enzymes, other reports have described the tremendous resistance of solvent free protein or super-oxide dismutase which tolerates autoclaving [405,406]. Temperature resistance is a major prerequisite for the biotechnological use of enzymes, nevertheless many other aspects have to be considered as well. For example, developing enzyme-based medical devices requires a sterilization step with no or limited loss of activity. We applied three methods, autoclaving, ethylene oxide and β-radiation to sterilize *Sso*Pox-W263I with little or no impact on activity. This result is particularly promising: indeed, contrary to the atomized enzyme, the enzyme incorporated into a medical device would be partially protected by the material from external exposures and would be potentially less affected by the sterilization processes.

Medical devices functionalized with enzymes, such as dressings or catheters, would have to remain active when exposed to bacteria-rich environment such as infected wounds. In this way, we have demonstrated the ability of *Sso*Pox-W263I to resist the molecular arsenal,

such as proteases or surfactants, secreted by different bacteria for the degradation of exogenous proteins. The high resistance of the variant to bacterial secretions was observed with both Gram-negative and Gram-positive strains. Enzymatic activity was in fact increased by bacterial culture supernatants. Surprisingly, the supernatants were also able to reactivate the heat-shocked enzyme. Whereas these results match the common observation that hyperstable enzymes are resistant to surfactants or proteases that may be secreted by bacteria, the mechanisms for these two observations are unclear and will require further investigations.

In this study, we demonstrate that *Sso*Pox-W263I is active over an extremely wide range of temperature. Indeed, we have confirmed that the enzyme exhibits very similar catalytic activities at 70 and 23°C. More surprisingly, we also showed that this enzyme is active at sub-zero temperature and that its temperature dependence is lower than that of most enzymes. Our results underline the fact that extremozymes do not necessarily require high temperatures to be functional and effective. This wide range of activity may be useful for a large panel of applications including bioremediation in permanently cold regions or during winter, the treatment of low-temperature effluents, and anti-fouling in cold seas.

Along with temperature resistance, tolerance to solvent is valuable for biotechnological considerations. However, unlike most extremozymes from psychrophilic origins, hyperthermostable enzymes may be inactivated by solvents. Resuspension of the enzyme in pure organic solvents followed by an evaporation step had only mild effects on the enzyme's catalytic efficiency. Although the enzyme's solubilities in organic solvents were not evaluated, these results indicate that solvents may be used as carrier for *Sso*Pox-W263I without altering its efficiency (except for xylene) which is particularly attractive for chemical processes with solvent-required steps or functionalization steps.

Industrial applications also require the enzyme to be compatible with storage. We thus evaluated the activity of *Sso*Pox-W263I over time. We found that the enzyme remained active over months at room temperature with variation in activity in the range of <25%. Finally, we have immobilized the enzyme and tested its activity. Industrial applications using enzymes often require an immobilization step that offers many advantages as compared to the utilization of soluble enzyme. Immobilization usually increases recyclability and stability, while the downstream process is simplified and the contamination risk is reduced. Many strategies may be considered for immobilizing enzymes, including adsorption, covalent binding, entrapment and crosslinking [407–409]. Reaching a compromise between support cost and binding capacity is required to optimize the competitiveness of the product. We have immobilized *Sso*Pox-W263I on alginate beads using glutaraldehyde crosslinking. The immobilized enzyme remained active and very little (if any) enzyme release was observed in the reaction medium. This suggests that such solid biocatalysts may be used for filtration purposes for various applications including the bioremediation of OP for decontaminating soiled effluents.

To conclude, this study demonstrates the exceptional properties of *Sso*Pox-W263I in regards to its resistance to temperature, solvents, proteases, sterilization processes, storage

as well as immobilization procedures. These properties strongly support that *Sso*Pox-W263I is manipulable for biotechnological considerations as it matches several industrial requirements. Many applications involving this variant may be envisaged for either OP or AHL degradation, such as filtration systems, anti-fouling paints, medical devices, self-decontaminating materials, and textiles.

1.4 Methods

Batch characteristics

The production was performed in a 500-L tank and was adapted from a previously reported lab-scale procedure. Briefly, strain *Escherichia coli* BL21 $(DE)_3$-carrying plasmids pGro7/GroEL and pET22b *Sso*Pox-W263I for chaperone and *Sso*Pox-W263I expressions were used [28]. A first preculture of 100 mL was made from glycerol stock in LB medium (supplemented with 100 $mg.L^{-1}$ ampicillin and 34 $mg.L^{-1}$ chloramphenicol) over 4 h (OD_{600}=2) at 37°C with stirring at 140 rpm. A second preculture of 5.2 L was inoculated from the previous one in the same medium at 37°C over 4 h (OD_{600}=2) with stirring at 180 rpm. Five liters of preculture was used to inoculate 495 L of ZYP-5052 medium complemented with ampicillin (100 $mg.L^{-1}$) and chloramphenicol (34 $mg.L^{-1}$)[410]. The culture was performed at 37°C with pO_2 of 20%, aeration of 100-500 slpm (standard liter per minute) and stirring at 60-160 rpm. When the OD_{600} reached 0.8-1, protein production was induced by decreasing the temperature to 23°C with addition of $CoCl_2$ (200 µM) and L(+)-arabinose (0.2% w/v). After 12 h, cells were harvested by centrifugation at 11,000 g (clarifier Clara 15 Alfa Laval, flow rate 1 $L.min^{-1}$). The cell pellet was resuspended in lysis buffer (50 mM HEPES, 150 mM NaCl, 0.2 mM $CoCl_2$, 10 $mg.L^{-1}$ DNase I , 250 $mg.L^{-1}$ Lysosyme, 0.1 mM PMSF and pH=8.0) and frozen at -80°C for 48 h. The extract was then sonicated (Sonifier 450 with 902R needle, Branson) and centrifuged for 5 min at 4°C and 18,000 g. The supernatant was heated 30 min at 80°C followed by another centrifugation at 18,000 g and 4°C for 25 min to remove precipitate. Supernatant was ultrafiltrated (PES 10 kDa, Synder) and atomized (Mini Spray dryer B290, Buchi). Unless otherwise specified, 1.5 U of *Sso*Pox-W263I in 0.5 mL were used for the experiments.

Paraoxonase activity measurement

The phosphotriesterase activity was measured with ethyl paraoxon (Sigma Aldrich) as previously described [29]. Briefly, in a 96-well plate, 2 µL of enzymatic solution were added to 98 µL of activity buffer (HEPES 50 mM, NaCl 150 mM and pH=8.0). 100 µL of 2 mM ethyl paraoxon in activity buffer were added to each well for starting the reaction and the OD_{405nm} was followed with a microplate reader (Synergy HT, BioTek, USA) for 10 min.

Heat shock resistance

The heat shock was performed using a dry bath (Isotemp, Fisher Scientific). Solid (i.e. atomized) or liquid (i.e. atomized enzyme resuspended in ultrapure water) enzyme was aliquoted in 1.5 mL Eppendorf tubes. The temperature was set from 40°C to 120°C for the resuspended samples and from 40°C to 150°C for the powder samples during 5 min. After the heat shock, the samples were cooled 5 min at ambient temperature (23°C) and then, for the powder samples, resuspended in ultrapure water. As control, samples were left at ambient temperature (23°C).

Tolerance to solvents

The test was performed using atomized enzyme aliquoted in 3 mL glass vials (Wheaton, USA). 16 solvents were used: acetone, acetonitrile, butanone, butyl acetate, chloroform, dichloromethane, diethyl ether, ethanol, ethyl acetate, isopropanol, methanol, methoxypropanol, methylcyclohexane, petroleum ether, toluene, xylene (all purchased from Sigma Aldrich). To vials containing 1.5 U of *Sso*Pox-W263I, 500 µL of pure solvent was added and was followed by 30 s stirring with a vortex. Vials were let open under a chemical hood for 1h50min followed by a 10-15 min evaporation under a nitrogen gas flux. Finally, the dry residue obtained was resuspended in 500 µL ultrapure water. As control, samples directly resuspended in water with a 2 h evaporation under a nitrogen gas flux (group used for relative activity calculation) and without. The last condition was used to check the impact of evaporation on *Sso*Pox-W263I activity.

Reactivation by culture supernatants

The reactivation was investigated with four bacterial strains: *P. aeruginosa* PAO1*, A. baumannii* AYE*, B. cereus* CIP6624T (clinical strain), and *S. aureus* ATCC 29213. Strains were grown on 5% sheep blood Columbia agar plate and incubated at 37°C overnight. From one colony, 15 mL LB medium was inoculated and incubated 24 h at 37°C with stirring (600 rpm). The culture was then centrifuged at 8,000 g for 10 min to pellet down bacterial cells. The supernatants were collected and filtered over a 0.22 µm-filter. Aliquots of filtered supernatants were heated in a dry bath at 100°C for 10 min to remove the majority of thermolabile compounds.

Unheated supernatants were used to resuspend solid enzyme heated or not at 150°C for 5 min in a dry bath. Heated enzyme was also resuspended in heated supernatants. The samples were incubated at room temperature (23°C) for 1 h. As control, the enzyme was resuspended in sterile LB medium. Furthermore, the potential activity on paraoxon of LB, with supernatants heated or not, was controlled using 2 µL and the resulting value was subtracted to each category of samples.

Statistical analyses

Statistical analyses were performed using SPSS v22 software. The type I error, or α, was set at 0.05. First, the Shapiro-Wilk's test and the Levene's test were used in order to check the normality and equality of variance assumptions for each group containing LB controls and supernatant treated samples. One-way ANOVA was then performed on each group. In case of unequal variance, the Welch's Test and Brown-Forsythe's test were used to confirm the significant difference observed with the one-way ANOVA. Five orthogonal contrasts were analyzed to evaluate the significance of the results. Following the Levene's test results, the p-value of contrasts was calculated considering the equivalence or lack of equivalence of variances. Furthermore, according to the Bonferroni correction, the p-value of each contrast was compared to α=0.01 to give a global type I error of 0.05. In total, five contrasts comparing two conditions were used: A versus B, A versus C, B versus D, B versus E and D versus E.

Sterilization processes

Autoclaving was performed in a 50-mL glass bottle (Duran, Germany) with 60 U of *Sso*Pox-W263I in powder directly or resuspended in 20 mL of ultrapure water. The autoclaving cycle was adapted to small volume in order to have 15 min at 121°C with a quick temperature increase and decrease. After samples were cooled down, 20 mL of water were added to the autoclaved powder sample. As control, a freshly made 3 $U.mL^{-1}$ (equivalent to 1 mg of enzyme by mL) water solution was used.

β-radiation sterilization was performed in 3 mL glass vials on 12 U of *Sso*Pox-W263I in powder directly or resuspended in 1 mL of ultrapure water. The samples were put under an electron beam (frequency of 520 Hz) 2.6 mm wide with a speed of 0.85 $m.min^{-1}$. One, two and four passages through the beam were enough to apply a dose of 25, 50 and 100 kGy. As control, samples without β-radiation treatment but with the same environmental conditions were used.

Ethylene oxide sterilization was performed on 12 U of *Sso*Pox-W263I in powder contained in flat pouches (SPS, France). The samples were submitted to from one to three consecutive cycles of sterilization by ethylene oxide. Briefly, a cycle consisted of a preheating time (from 51 to 67 min) followed by a conditioning time (longer than 180 min) at the end of which, the temperature was between 40-50°C and humidity longer than 50%. The sample was then exposed to ethylene oxide for 300-310 min with a temperature of 40-50°C and a weight of gas of 10.7-13.0 kg. As control, samples without treatment were used.

Room temperature storage

To maintain an anhydrous atmosphere, 1.5 mL Eppendorf tubes containing enzyme powder were stored with desiccant packets (Clariant, Switzerland). Every week aliquots were resuspended in 500 µL of ultrapure water and the activity was measured. The results were normalized as compared to the control at day 0.

Low temperature kinetics

The reaction was performed in activity buffer complemented with 50% of glycerol. Two separated solutions were prepared containing either 500 µM of ethyl paraoxon or 0.06 U.mL^{-1} of *Sso*Pox-W263I. Both solutions were cooled to -18°C. In a 2 mL Eppendorf tube at -18°C, 1 mL of each were mixed together to start the reaction. Every hour, 100 µL was removed and mixed with 100 µL of chloroform and vortexed. The organic phase was transferred into gas-chromatography vial. The same experiment was performed at room temperature as control.

GC analysis were performed as follow: 100 µL of reaction medium was extracted with 100 µL of chloroform. Organic extracts were analyzed by using a Clarus 500 gas chromatograph equipped with a SQ8S MS detector (Perkin Elmer, Courtaboeuf, France). 1 µL of organic extract was volatilized at 220 °C (split 15 mL/min) in a deactivated FocusLiner with quartz wool (SGE, Ringwood, Australia) and compounds separated on an Elite-5MS column (30 m, 0.25 mm i.d., 0.25 mm film thickness) during 12 minutes using a temperature gradient (80-280 °C at 30 °C/min, 5 minutes hold). Helium flowing at 2 mL/min was used as carrier gas. The MS inlet line was set at 280 °C and electron ionization source at 280 °C and 70 eV. Full scan monitoring was performed from 40 to 400 m/z in order to identify chemicals by spectral database search using MS Search 2.0 operated with the Standard Reference Database 1A (National Institute of Standards and Technology, Gaithersburg, MD, USA). Selected Ion Recording using base peaks ions was applied in order to specifically monitor pesticides and collect peak areas for kinetics. Peak areas were converted to percentage of initial concentration value. All samples were analyzed in short periods of time to avoid signal drift. All data were processed using Turbomass 6.1 (Perkin Elmer).

It was reasonably assumed that the substrate concentration was negligible as compared to K_M. k_{cat}/K_M was thus estimated by using the one phase decay function in GraphPad Prism v6. Curves were then fitted using One-Phase Decay non-linear regression with the equation:

$$Y = (Y0 - Plateau) \times e^{(-kt)} + Plateau$$

Where $Y0 = 0\%$ and $Plateau = 100\%$.The resulting rate constant K was divided by the enzyme molar concentration to estimate the k_{cat}/K_M in $M^{-1}.s^{-1}$.

Temperature dependence of pH for HEPES was previously described with a ΔpKa value of -0.14 every 10°C [411]. So by varying the temperature from -18°C up to 70°C, pH may vary within a range of 1.5 unit. However, the tolerance of *Sso*Pox to pH changes was previously reported and no significant modification of the specific activity was observed from pH 7.0 to 9.0 [23].

Immobilization

The immobilization was performed with 15 U.mL^{-1} of enzyme mixed in a 3% (w/v) solution of sodium alginate. Glutaraldehyde was added to the solution at a final concentration of 0.5%. Alginate beads were obtained by dropping the enzymatic solution with a 1 mL syringe connected to a 35 g needle, in 40 mL of 0.2 M $CaCl_2$ solution. After 1 h with stirring at room

temperature, the beads were washed for 30 min with 30 mL of the activity buffer complemented with 0.2 M $CaCl_2$.

Activity of the beads was evaluated in 5 mL solution of 1 mM ethyl in activity buffer complemented with 0.2 M $CaCl_2$. After 3 min of reaction with upside down agitation, 200 µL of the solution was transferred into a 96-well plate and the OD_{405nm} was followed for 10 min. For the recycling test, after each cycle of catalysis, the beads were washed twice for 10 min with 30 mL of $CaCl_2$ complemented activity buffer. As negative control, empty beads were made and no activity was detected. For yield determination, a positive control was made with 15 U of free enzyme resuspended in 1 mL of water and diluted to 1/10 with complemented activity buffer. Afterward, 1 mL of the diluted solution was added to 4 mL of ethyl paraoxon solution (final concentration 1 mM).

Acknowledgements

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Author Contributions

Benjamin Rémy, David Daudé and Eric Chabrière designed the study; Benjamin Rémy, Laetitia Poirier and David Daudé performed the experiments; Benjamin Rémy, Laure Plener, Mikael Elias, David Daudé and Eric Chabrière analyzed the data; Benjamin Rémy, Laure Plener, Mikael Elias, David Daudé and Eric Chabrière wrote the manuscript.

2. Effet du variant W263I de *Sso*Pox sur des souches cliniques de *Pseudomonas aeruginosa*

L'ulcère du pied diabétique est la conséquence d'une plaie chronique liée à un défaut du processus de cicatrisation rencontré chez les personnes atteintes de diabète [412–415]. Cette pathologie est associée à une hyperglycémie pouvant entrainer, entre autres, une réduction du flux sanguin (ischémie) et un endommagement des tissus nerveux périphériques (neuropathie). La plaie chronique constitue un terrain propice à la croissance microbienne et favorise le développement des infections qui ralentissent le processus de cicatrisation et peuvent entrainer de sérieuses complications pour la santé du patient [414,415]. En effet, si l'infection n'est pas efficacement combattue, les tissus se détériorent jusqu'à une nécrose irréversible (gangrène) qui nécessite une amputation du membre touché [414,416].

Le nombre de personnes atteintes de diabète était estimé à 415 millions en 2015 et devrait atteindre 642 millions en 2040 [417]. Les ulcères du pied touchent 15 à 20% des patients diabétiques et sont à l'origine de 85% des cas d'amputation d'un membre inférieur [412,418]. Les diabétiques contractant un ulcère ont une mortalité 2,5 fois plus élevée sur cinq ans et la mortalité à cinq ans après amputation peut atteindre 70% [412]. Par ailleurs, des études ont largement souligné l'impact économique lié au traitement de ce type de plaie. Le montant de la prise en charge de l'ulcère s'élève à environ 40 000 dollars, l'amputation génère quant à elle un coût compris entre 35 et 45 000 dollars [419–421]. Aux Etats-Unis, le surcoût de la prise en charge d'un ulcère chez un diabétique lié notamment à un nombre plus important de jours d'hospitalisation, de soins à domicile, d'urgences et de suivis médicaux serait compris entre 9 et 13 milliards de dollars par an [422]. Ces chiffres mettent en évidence l'ampleur du problème tant au niveau sanitaire, que sociétal et économique et soulignent l'intérêt de développer de nouvelles approches pour lutter contre les infections de ce type de plaies.

P. aeruginosa est une bactérie fréquemment impliquée dans les infections des ulcères du pied diabétique. Elle est généralement trouvée en biofilm au niveau de la plaie et peut être responsable de graves complications [24,423,424]. De nombreux isolats résistants, à un voire plusieurs antibiotiques, ont été rapportés et peuvent limiter l'efficacité des soins [423,425].

P. aeruginosa est peu sensible à certains antibiotiques comme les tétracyclines et les macrolides grâce notamment à un système performant de pompes à efflux [24]. De plus, elle possède une grande capacité d'adaptation aux agents antimicrobiens via l'acquisition de plasmides portant des gènes de résistance ou par modification de sa perméabilité membranaire [24,426,427]. Ainsi, *P. aeruginosa* est classée par l'OMS dans la liste des pathogènes prioritaires pour la recherche et le développement de nouveaux antibiotiques [428].

Outre sa capacité à s'adapter aux traitements antibiotiques, *P. aeruginosa* dispose d'un vaste arsenal de facteurs contribuant à sa virulence, parmi lesquels un biofilm complexe, des toxines, des protéases, des sidérophores (e.g. pyoverdine) et des agents oxydants (e.g. pyocyanine) [24,52]. Beaucoup de ces facteurs sont finement régulés par le QS qui joue un

rôle déterminant pour leur mise en place à haute densité cellulaire [52]. Chez *P. aeruginosa*, quatre systèmes sont interconnectés dont deux impliquent l'utilisation d'un AHL (Las et Rhl). Le système Las a été décrit comme jouant un rôle principal dans la régulation des autres systèmes ainsi que dans la synthèse des facteurs liés à la virulence et du biofilm [52]. Ce système fait intervenir l'autoinducteur 3-oxo-C_{12} HSL qui est efficacement hydrolysé par *Sso*Pox. L'utilisation de cette enzyme permettrait d'empêcher la synchronisation des cellules et ainsi limiter la production des facteurs de virulence associés. Des travaux antérieurs ont montré que l'activité des exoprotéases et la quantité de pyocyanine sécrétée par la souche modèle de *P. aeruginosa* PAO1 sont drastiquement diminuées avec l'utilisation de *Sso*Pox (sauvage) adsorbée sur une membrane [26]. Le variant *Sso*Pox W263I, présentant une plus grande efficacité pour la dégradation du 3-oxo-C_{12} HSL, s'est avéré efficace *in vitro* et *in vivo* contre *P. aeruginosa* [30]. Dans la continuité de ces premières observations sur une souche modèle, la capacité de l'enzyme à réduire la virulence chez des isolats cliniques de *P. aeruginosa* a été étudiée.

Ainsi, en collaboration avec le professeur Jean-Philippe Lavigne du CHU de Nîmes, 51 souches cliniques et deux souches modèles (PAO1 et PA14) ont été testées dans un milieu enrichi en sang afin de mimer un environnement de plaie. La quantité de biofilm formé ainsi que la sécrétion de protéases et de pyocyanine ont été mesurées. Pour pouvoir mettre ces résultats en perspectives, *Sso*Pox W263I a été comparée à des inhibiteurs du QS (le C-30, une furanone bromé, et le 5-fluorouracile, un analogue de l'uracile) qui ont déjà été décrits dans la littérature [153,216]. Alors que *Sso*Pox W263I est une enzyme catalysant l'hydrolyse des 3-oxo-C_{12} HSL depuis l'extérieur des cellules, les inhibiteurs doivent eux entrer en contact direct avec ces dernières afin de se fixer sur leur(s) cible(s).

L'ajout de *Sso*Pox W263I en solution a permis de réduire efficacement la formation du biofilm et la sécrétion des facteurs de virulence pour la majorité des souches cliniques testées. D'autre part, *Sso*Pox W263I s'est montrée plus efficace que les inhibiteurs chimiques du QS utilisés seuls ou combinés. Par ailleurs, contrairement à l'enzyme, les inhibiteurs ont entrainé, dans certains cas, une augmentation de la sécrétion de facteurs de virulence ou du biofilm. Enfin, l'enzyme conserve son activité même après immobilisation dans du polyuréthane et est capable de réduire le biofilm et les facteurs sécrétés chez la souche modèle PAO1. Cette preuve de concept s'avère donc particulièrement intéressante pour le développement de dispositifs médicaux innovants. Cette technologie a été retenue pour la fonctionnalisation des pansements antivirulents. L'idée consiste en l'application d'un revêtement à base de polyuréthane réticulé par du glutaraldéhyde sur un des textiles médicaux composant le pansement **(Figure 17)**. L'enzyme se retrouvera piégée dans cette couche qui sera en contact avec les exsudats de la plaie contenant les AHL.

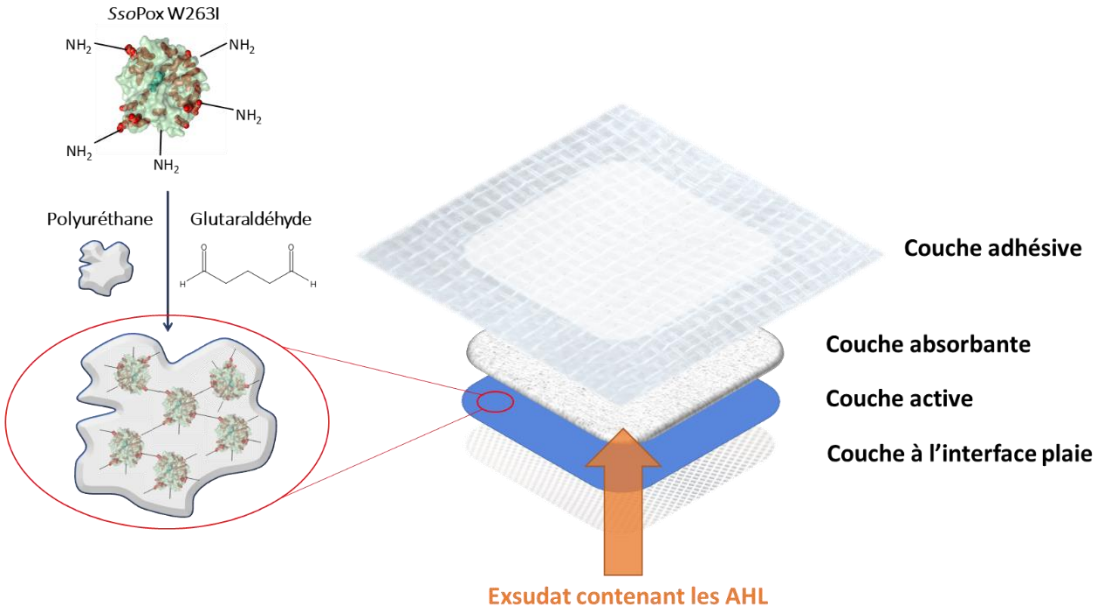


Figure 17 : Schéma du mode de fixation de l'enzyme et du prototype de pansement.

Pour ces travaux, j'ai réalisé la fixation de *Sso*Pox W263I dans le mélange polyuréthane/glutaraldéhyde et les tests microbiologiques qui ont suivi sur PAO1. J'ai participé à l'écriture et à la relecture du manuscrit. Ce deuxième article a été publié dans *Frontiers in microbiology* [359].

2.1 Introduction

P. aeruginosa is a human opportunistic pathogen involved in many infection types and which causes serious health complications [24,429]. In 2006/2007, this Gram negative bacterium alone was responsible for 8 % of general healthcare associated infections in the USA [128]. *P. aeruginosa* is involved in both community-acquired and hospital-acquired infections including otitis, keratitis, wound and burn infections, pneumonia and urinary tract infections [24]. Furthermore, *P. aeruginosa* is, along with *S. aureus*, the most common pathogen isolated from diabetic foot infections notably in South East Asia, and is also highly involved in infections when an alteration of the skin occurs [24].

Many bacteria, including *P. aeruginosa*, use a molecular communication system, referred to as quorum sensing (QS), to synchronize the expression of certain genes and adopt a group behaviour. Among QS-regulated traits, virulence factors production such as pyocyanin and proteases [153], motility [430] and biofilm formation [431] are involved in the development of infections.

QS in *P. aeruginosa* depends on four different hierarchically organised systems: Las, IQS, Rhl and PQS. The first system to be activated is the Las system which depends on the production and perception of an acyl-homoserine lactone (AHL): *N*-(3-oxododecanoyl)-homoserine lactone (3-oxo-C_{12} HSL) [432,433]. Induction of the Las system triggers the expression of the Las protease and elastase and activates the other QS systems. Despite its dominant role in the QS circuitry, eliminating LasR activation only results in a delayed activation of the *Pseudomonas* quinolone signal (PQS) system but does not completely eliminate QS [434]. In addition, the QS system of *P. aeruginosa* is interconnected with other regulatory networks involved in environmental cues such as phosphate, iron and oxygen sensing [52].

Regarding the importance of bacterial communication in the development of virulence, strategies for QS disruption, known as quorum quenching (QQ), have emerged to maintain bacteria in a commensal lifestyle. To this end, quorum sensing inhibitors (QSI) and QQ enzymes have been particularly considered [207,212,220,435,436]. QSI, such as brominated furanones, aim to prevent bacteria from perceiving endogenous QS molecules. Pyrimidine analogue has also been reported as a QS disruptor [216]. QQ enzymes such as acylases or lactonases degrade AHL signals [47,237]. Among these, the enzyme *Sso*Pox, isolated from the archaea *S. solfataricus,* has been considered based on both its lactonase activity and tremendous stability due to its extremophile origin [358].

Many QQ examples have been reported and describe the reduction of virulence factor secretion (e.g. siderophores, proteases, rhamnolipids, etc.) [437,438] and/or biofilm formation, especially using QSI molecules [153]. However a large proportion of these reports is dedicated to investigating model strains *P. aeruginosa* PAO1 and PA14, and only a few reports have described the response of clinical isolates to QS disruption, whereas natural isolates frequently harbour mutations in QS genes [439].

In this article, we investigated the effectiveness of the QQ enzyme *Sso*Pox-W263I, a variant of *Sso*Pox with increased catalytic effectiveness against 3-oxo-C_{12} AHL [29,358],

previously reported as being efficient to drastically reduce the mortality in a rat pneumonia model [30], to modulate virulence factors in 51 clinical *P. aeruginosa* isolates collected from diabetic foot ulcers. We also compared its QQ potential to the most common QSI, the brominated furanone C-30 and the pyrimidine analogue 5-FU, by quantifying three virulence factors: pyocyanin production, protease secretion and biofilm formation [216,440]. Finally, the lactonase was immobilised to assess its ability to functionalise medical devices and was proved to maintain sufficient activity for QQ.

2.2 Materials and Methods

Bacterial strains and growth conditions

Experiments were performed with *P. aeruginosa* strains from samples held by the Department of Microbiology of the Nîmes University Hospital. The strains were isolated from diabetic patients with a suspected newly presenting episode of diabetic foot infection for a period of one year (2014). All the patients received an oral information, were anonymized and gave a non opposition statement to bacterial storage. This study was approved by the local ethics committee (South Mediterranean III) and was carried out in accordance with the Declaration of Helsinki as revised in 2008. The samples were frozen at -80 °C. Bacterial strains were cultivated on Luria Bertani (LB) agar plates at 37 °C.

The model strains *P. aeruginosa* PAO1 and PA14 (Taxonomy ID: 208964 and 652611) and the clinical isolates were inoculated from a single colony and pre-cultivated in LB (10 g l^{-1} NaCl, 10 g.L^{-1} tryptone, 5 g.L^{-1} yeast extract) for 6 h at 37 °C with shaking at 650 rpm. Subsequently, 3 ml of LB supplemented with 2 % sheep blood (Biomérieux, France) was inoculated with 3 µl pre-culture and incubated at 37 °C with shaking at 650 rpm. Pyocyanin production and protease activity were measured 24 h post-inoculation. Biofilm weight was determined 48 h post-inoculation.

The enzyme *Sso*Pox was added at 0.5 mg.mL^{-1} and the QSI 5-FU and C-30 (Sigma) were used at 60 and 30 µM respectively as determined by a dose response experiment (**Supplementary figure 1 to Supplementary figure 3** in the "Annexes" section).

Bacterial identification

All strains were checked using Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) as previously described [441]. In brief, isolates were grown overnight on blood agar at 37 °C under aerobic conditions. Single colonies were applied as a thin film to a 96-spot steel plate (BrukerDaltonics) and allowed to visibly dry at room temperature. Subsequently, 2 µl of MALDI matrix (saturated solution of alpha-cyano-4-hydroxy-cinnamic acid, 500 µl HPLC-grade acetonitrile, 475 µl HPLC-grade water, 25 µl trifluoacetic acid) was applied on the spots and dried at room temperature. Isolates were tested in duplicate by MALDI-TOF mass spectrometry. The MALDI target plate was introduced into a microflex LT MALDI-TOF mass spectrometer for automated measurement and controlled by the

FlexControl 3.3 (Bruker®) program. The spectra were collected in a mass range between 2,000-20,000 m/z then analyzed using the Bruker Biotyper 3.0 software package and compared to reference spectra for identification.

Protein production and purification

Enzyme production was performed using *E. coli* BL21(DE3)-pGro7/GroEL strain (TaKaRa) carrying plasmid pET22b-*Sso*Pox-W263I as previously described [28,29]. In brief, cells were grown in ZYP medium supplemented with 100 µg.mL^{-1} ampicillin and 34 µg.mL^{-1} chloramphenicol at 37 °C until OD_{600nm} reached 0.8-1. L-arabinose was added to a final concentration of 0.2 % (w/v) in order to induce chaperones expression. Subsequently, 0.2 mM $CoCl_2$ was added and the temperature was reduced to 23 °C for an additional 20 h. Cells were harvested by centrifugation (4,400 g, 4 °C, 20 min), the supernatant was discarded and the pellet was resuspended in lysis buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 0.2 mM $CoCl_2$, 0.25 mg.mL^{-1} lysozyme, 0.1 mM Phenylmethylsulfonyl fluoride (PMSF) and 10 µg.mL^{-1} DNaseI) and stored at -80 °C overnight. Frozen cells were thawed at 37 °C for 15 min and disrupted by three 30 seconds sonication steps (QSonica sonicator Q700; amplitude at 45). Cell debris were removed by centrifugation (21,000 g, 4 °C, 15 min). Crude extract was incubated for 30 min at 80 °C and was further centrifuged to precipitate *E. coli* proteins (21,000 g, 4 °C, 30 min). *Sso*Pox-W263I was concentrated by overnight ammonium sulfate precipitation (75 % saturation), and resuspended in activity buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 0.2 mM $CoCl_2$). Remaining ammonium sulfate was eliminated via desalting (HiPrep 26/10 desalting, GE Healthcare; ÄKTA Avant). The obtained protein sample was concentrated to 2 ml and subsequently loaded onto a size-exclusion chromatography column and purified to homogeneity (HiLoad 16/600 Superdex^{TM} 75pg, GE Healthcare; ÄKTA Avant). The purity of the protein was checked by 10 % SDS-PAGE (**Supplementary figure 4** in the "Annexes" section) separation and protein concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

Proteolytic activity

Cell-free culture supernatants were prepared by centrifugation for 5 min at 12,000 g. Protease activity was determined using azocasein (Sigma, St. Louis, USA) as a substrate [442]. The reaction was performed in Phosphate-buffered saline (PBS) solution pH 7.0 with 50 µl of azocasein (30 mg.mL^{-1} in water) and with 25 µl of culture supernatant for a final volume of 750 µl. The reaction was incubated at 37 °C for 1 h and stopped by adding 125 µl of 20 % (w/v) trichloroacetic acid. The blank assay was realised using 25 µl of culture medium with and without 0.5 mg.mL^{-1} of *Sso*Pox. After centrifugation at 12,000 g for 5 min, the absorbance of the supernatant was measured at OD_{366nm} using a plate reader (Synergy HT, BioTek, USA).

Pyocyanin production

Pyocyanin was extracted from 500 µL of cell-free supernatant using 250 µL of chloroform. The mix was vortexed for 20 sec, and centrifuged at 12,000 g for 5 min. The absorbance of the lower organic phase was measured at OD_{690nm} using a plate reader (Synergy HT, BioTek, USA) [443].

Biofilm weight measurement

After 48 h, each culture was sieved through a 100 µm pore-size cell strainer (Corning, New York, USA) to separate biofilm from planktonic cells. The biofilm was washed with 2 mL PBS and centrifuged at 600 g for 5 min. Biofilms were weighed directly in the cell strainers using a precision scale (**Supplementary figure 5** in the "Annexes" section).

Immobilisation

In a 25 cm^2 culture flask (Corning, New York, USA), 1 mL of 5 % Impranil® DLU polyurethane (Covestro, Leverkusen, Germany) mixed with 20 $mg.mL^{-1}$ of *Sso*Pox-W263I and 0.5 % Glutaraldehyde (Sigma) in purified water was dried over 12 h at 37 °C. As a control, the same volume of activity buffer was added instead of *Sso*Pox-W263I. Before being used for culture, the flask was rinsed with 3 mL of purified water and then 3 mL of LB.

Enzymatic activity measurement

Enzymatic activities were measured after 24 h based on the paraoxonase activity of *Sso*Pox using ethyl paraoxon as a substrate and the apparition of para-nitrophenol, paraoxon degradation product, was followed at OD_{405nm}.

Released enzyme was measured after 24 h from culture supernatants. 5 µl of cell-free culture supernatant was transferred into a 96 well plate containing 95 µl of activity buffer (50 mM HEPES, 150 mM NaCl, pH 8). Then 100 µl of 2 mM ethyl paraoxon (Sigma) in activity buffer was added and OD_{405nm} was monitored during 10 min with a plate reader (Synergy HT, BioTek, USA) and the slope corresponding to paraoxon degradation kinetics was calculated.

Fixed enzyme was determined by adding 3 mL of a 1 mM ethyl paraoxon solution. After 3 min of incubation with shaking (300 rpm), 200 µl of paraoxon solution was transferred into a 96-well plate and OD_{405nm} was measured. The slope (OD $unit.min^{-1}$) was calculated and compared to a standard with a known enzyme concentration to calculate the amount of enzyme immobilised in the PU coating.

2.3 Results

Dose-response determination

P. aeruginosa model strains PAO1 and PA14 were used to determine the optimal concentrations of the QQ enzyme *Sso*Pox and the QSI 5-FU and C-30 to decrease virulence factor production. Time points for sampling were determined by performing dose response experiments at different concentrations (**Supplementary figure 1 to Supplementary figure 3** in the "Annexes" section). Under our conditions and in the absence of quenchers, protease and pyocyanin levels were similar for both strains (data not shown). The use of *Sso*Pox did not induce any delay in growth and maximal quenching was obtained for a concentration of 0.5 $mg.mL^{-1}$ of enzyme corresponding to 14.5 µM after 16 h of cultivation (**Supplementary figure 1** in the "Annexes" section). At this time point, pyocyanin production was almost completely abolished for both strains and protease production was reduced to 10 % as compared to the control (untreated sample) for PAO1 and 43 % for PA14. Biofilm formation was measured after 48 h to allow complete biofilm formation and direct weighing of cell aggregates (**Supplementary figure 5** in the "Annexes" section). The addition of *Sso*Pox (0.5 $mg.mL^{-1}$) reduced biofilm formation by 90 % for PAO1 and 60 % for PA14 and increasing *Sso*Pox concentration did not enhance quenching. The QSI molecule 5-FU only weakly impacted production of pyocyanin and protease in PAO1 but had a greater influence on PA14, with pyocyanin and protease productions reduced to 10 and 50 % respectively compared to the untreated culture. Biofilm formation was reduced in both strains when concentrations of 5-FU of 60 µM or higher were used. According to these results, quorum quenching of the clinical isolates was tested using 0.5 $mg.mL^{-1}$ *Sso*Pox or 60 µM 5-FU and 30 µM C-30. The same values were also reported in previous studies [362,444]. Pyocyanin and protease productions were determined after 24 h and biofilm formation 48 h post-inoculation.

Importantly, the addition of 5-FU to the culture led to a delay in growth, but this delay was recovered after 24 h for PAO1 (**Supplementary figure 6** in the "Annexes" section). The second QSI molecule, C-30, had much less impact in our experiment set-up as protease level remained up to 90 % of the control for both PAO1 and PA14, and pyocyanin production was not reduced.

Quenching of virulence factors by SsoPox

In the experimental conditions used here, the two model strains PAO1 and PA14 both produced the three virulence factors tested. Among the 51 clinical isolates of *P. aeruginosa*, 16 strains produced the three virulence factors studied, 13 produced two virulence factors and 22 produced only one virulence factor **(Figure 18)**. Proteolytic activities ranged from 0.47 to 1.37 with a median value at 1.08 **(Figure 19A)**. Median pyocyanin production, among the 26 strains producing pyocyanin, was measured at 0.17 and this virulence factor showed the highest variations ranging from 0.01 to 1.00 **(Figure 19B)**. Finally, the most common feature between the clinical isolates was biofilm formation as 42 strains out of 51 produced biofilm. Among these strains, the median value for wet biofilm was 73 mg with values ranging from 15

mg to 289 mg **(Figure 19C)**. *Sso*Pox was used at a concentration of 0.5 mg.mL^{-1} and pyocyanin and protease quantities were determined after 24 h. In these conditions, *Sso*Pox reduced protease activity of 26 out of 28 strains, with 20 of them being reduced by more than 50 % and complete quenching was achieved for 16 of them. No clinical isolate harboured a higher value upon treatment with *Sso*Pox than without **(Figure 20A)**. Pyocyanin production was reduced in 25 strains out of 26 and 20 of them were reduced by more than half the control level. Furthermore, pyocyanin production was completely eliminated in six strains. As noticed for protease production, no strain had increased pyocyanin production when the QQ enzyme *Sso*Pox was added **(Figure 20B)**. Biofilm was reduced in 37 strains out of 42, 36 of them were reduced by more than half and 20 of them completely lost their ability to grow in biofilms. Unlike pyocyanin and protease production, one clinical isolate, A12, had a slightly thicker biofilm upon treatment with *Sso*Pox than without but this difference was not statistically significant **(Figure 20C)**.

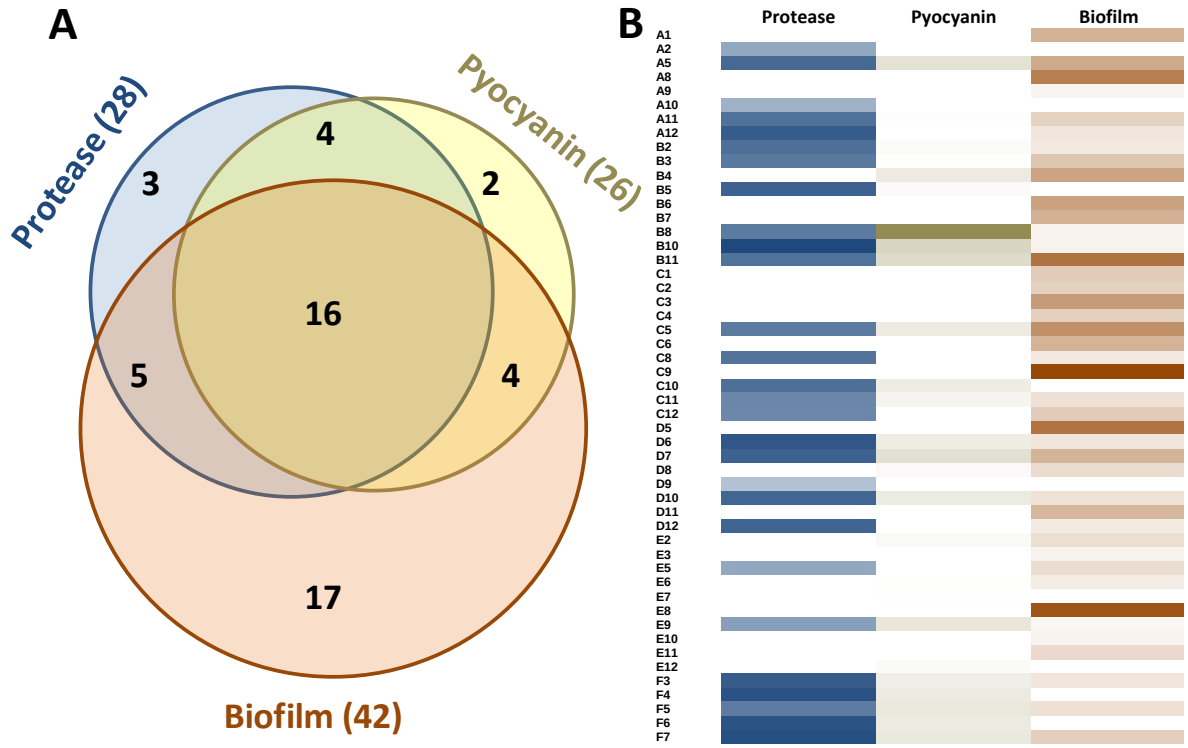


Figure 18 : Measurable virulence factor expression among the 51 clinical isolates of *P. aeruginosa*. (A) Venn diagram describing the distribution of the 51 strains according to their production of virulence factors. (B) Proteolytic activities are presented according to OD_{366nm} value ranging from 0 to 1.37. Pyocyanin values represent the OD_{690nm} and range from 0 to 1. Biofilm values correspond to the weight of biofilm formed after 48 h for 3 ml of culture and range from 0 to 289 mg.

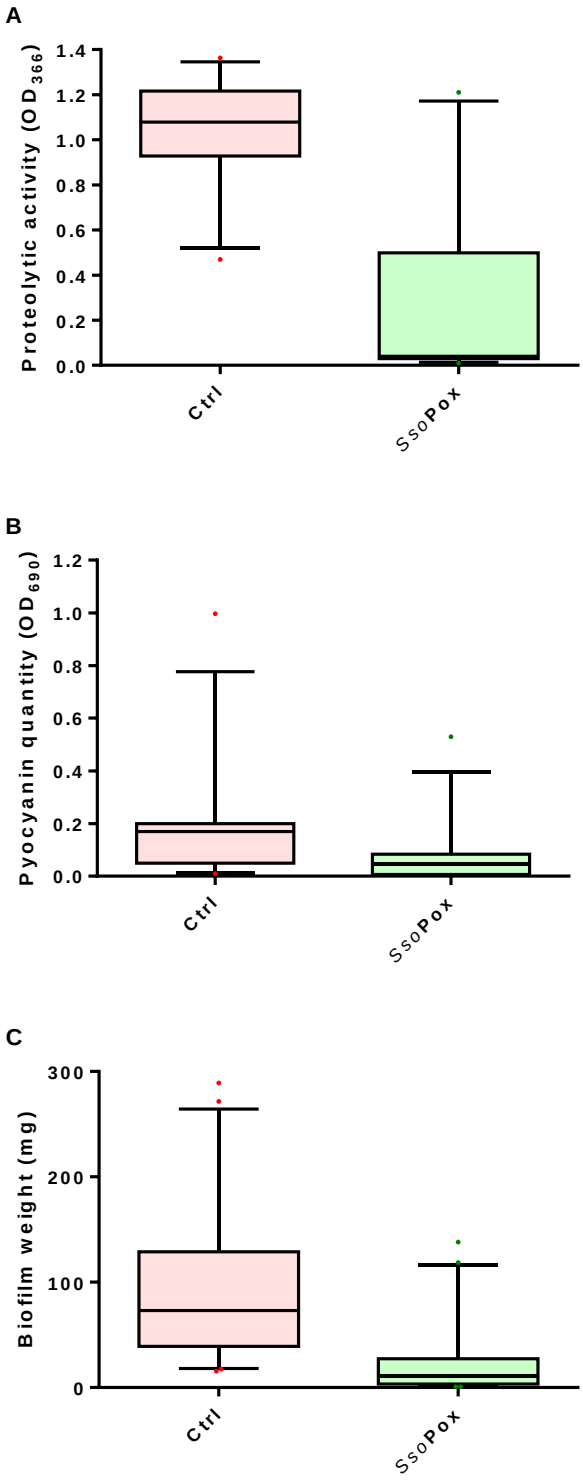
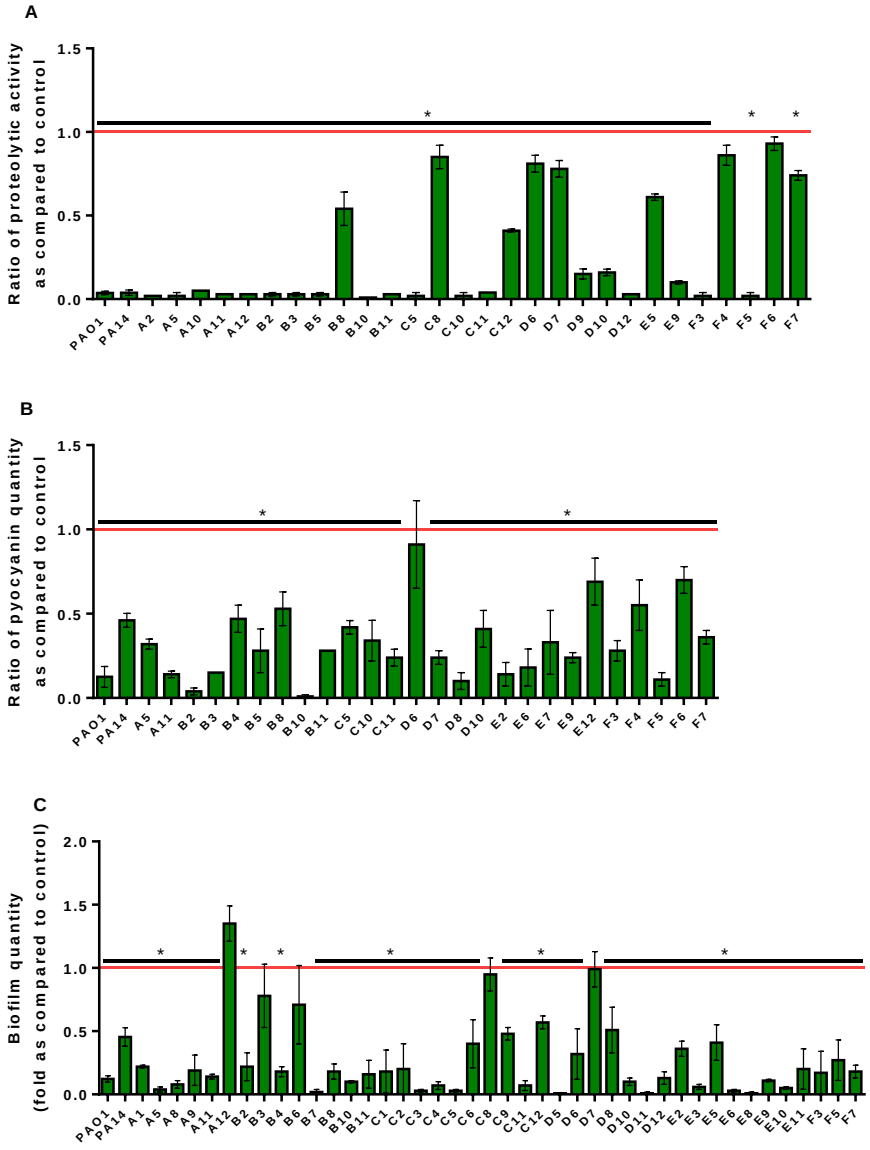


Figure 19 : Distribution plots of virulence factor values with and without quenching treatment of the 51 clinical isolates. Values of proteolytic activity (A), pyocyanin quantity (B) and biofilm formation (C) are shown as whiskers plots down to the 5th percentile and up to the 95th. Control samples (without enzyme) are shown in red while *Sso*Pox treated samples are in green. *Sso*Pox was added to cell cultures at a concentration of 0.5 mg.mL^{-1}. Points below and above the whiskers represent outlier values.



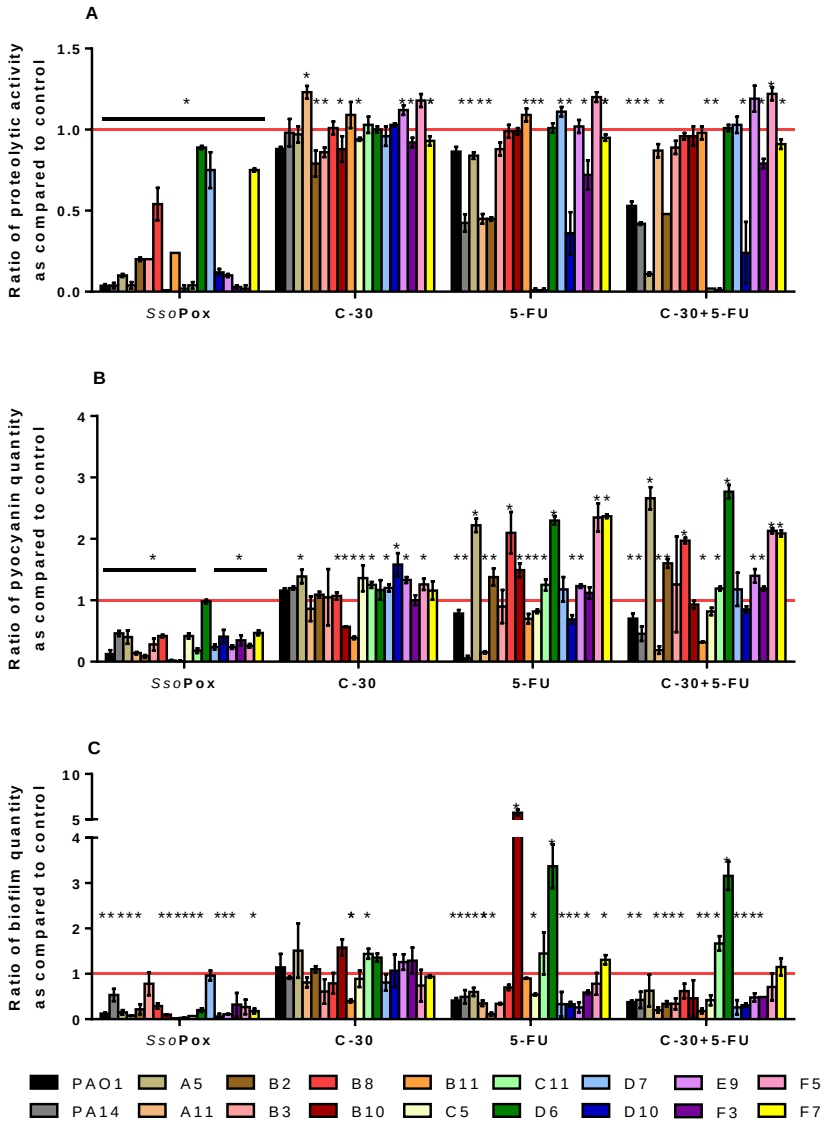
<u>Figure 20</u> : Quenching of virulence factors using *Sso*Pox. For each strain, bars represent the mean ratios of protease (A), pyocyanin (B) and biofilm (C) levels between the treated culture with 0.5 mg.ml^{-1} *Sso*Pox versus the untreated culture of three experiments. Error bars represent the standard deviations of three replicated experiments. Stars indicate *p-values*<0.05 according to Student's t-test.

Comparison between SsoPox and the QSI 5-FU and C-30

To compare quorum quenching potentials between *Sso*Pox and QSI molecules, the 16 clinical strains producing the three virulence factors were tested with 0.5 mg.mL^{-1} *Sso*Pox, 30 µM C-30, 60 µM 5-FU and both QSI. Under our conditions, C-30 showed very little quorum quenching potential either with the model strains PAO1 and PA14 or with the clinical isolates (**Figure 21** and **Supplementary figure 3** in the "Annexes" section). Only five strains showed a slight decrease in protease production, the maximum decrease measured was 20 % for strain B2. Pyocyanin production was only reduced for two strains, B10 and B11, with ratios of 0.57 and 0.39 respectively. Concerning biofilm formation only one strain showed a statistically

significant decrease with a ratio of 0.4 as compared to the control. More importantly, many strains showed significantly higher virulence factor production after treatment with C-30.

The results obtained using 5-FU indicated more quorum quenching activity than C-30 in these experimental conditions. Indeed, 7 strains out of 16 presented decreased proteolytic activity, two strains had decreased pyocyanin production and 10 strains formed less biofilm upon treatment with 5-FU. The combination of both QSI molecules did not lead to an increase in quenching potential as compared to the use of 5-FU alone. *Sso*Pox was the most active quorum quenching agent, reducing proteolytic activities in every strain tested, reducing pyocyanin production in all but one strain and reducing biofilm formation in 12 strains out of 16.



<u>Figure 21</u> : Comparison of quenching by SsoPox and QSI C-30 and 5-FU. For each strain, bars represent the mean ratios of protease (A), pyocyanin (B) and biofilm (C) levels between the treated culture with optimal concentrations of quorum quenching agents (0.5 mg.mL^{-1} SsoPox, or 30 µM C-30 and/or 60 µM 5-FU) versus the untreated culture of three experiments. Error bars represent the standard deviations of three replicated experiments. Stars indicate p-values<0.05 according to Student's t-test.

Immobilisation

To prove the effectiveness of *Sso*Pox-W263I in medical device-like conditions, the enzyme was immobilised in a waterborne polyurethane coating with the cross-linking agent glutaraldehyde. The influence of the PU coating without enzyme on growth and virulence factor production of PAO1 model strain was assessed to ensure that PU did not have a major impact on bacterial metabolism. Neither growth nor protease production differed. Pyocyanin and biofilm productions were slightly impacted with a decrease of pyocyanin production and an increase in biofilm formation. Despite these differences inherent to PU coating the impact of enzyme could be measured and quenching was successfully achieved **(Figure 22)**. Indeed, in the same culture conditions as free enzymes, the immobilised *Sso*Pox was able to decrease pyocyanin production by 2.1, protease activity by 24 and biofilm quantity by 6.5 as compared to the control with polyurethane coating. Enzyme release was determined by measuring enzymatic activity of culture supernatants after 24 h of growth. No enzyme activity was detected in culture supernatants indicating that the immobilisation is effective and no enzyme is released after fixation. About 200 µg of active enzyme were detected in 25 cm² flask corresponding to a final concentration of 8-10 µg.cm^{-2}. In these conditions, the *Sso*Pox coating was effective in reducing significantly the production of all tested virulence factors.

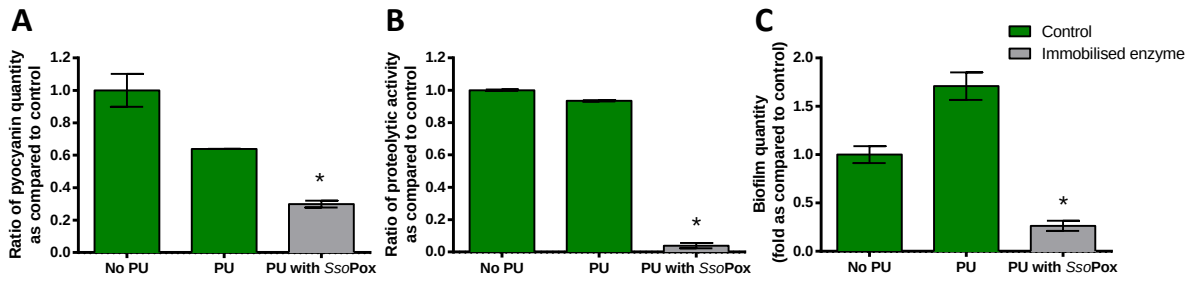


Figure 22 : Quenching *P. aeruginosa* PAO1 with enzyme immobilised in polyurethane (PU). Bars represent the mean ratios of pyocyanin (A), protease (B) and biofilm (C) levels between the treated culture with immobilised *Sso*Pox (PU with *Sso*Pox) versus the untreated culture containing a polyurethane coating (PU) or without coating (No PU). Error bars represent the standard deviations of two replicate experiments. Stars indicate *p-values*<0.05 according to Student's t-test using control with PU as reference.

2.4 Discussion

Foot ulcers are common in diabetic patients mainly due to arteriopathy and neuropathy. The risk of lower-extremity amputation for patients suffering from diabetes is up to 155 times higher than for non-diabetic people [445,446]. Diabetic foot ulcers are often polymicrobial, bacteria from various types can maintain a chronic infection on their own, or act synergistically in a pathogenic biofilm to cause infection. *P. aeruginosa* is one of the most frequent pathogens isolated in diabetic foot ulcers, particularly in warmer countries (Asia and Africa) where Gram-negative bacilli are more prevalent [423]. This bacterium has developed

many strategies to counteract antimicrobial treatments such as antibiotics [447]. Biofilm formation [431], β-lactamases [448] and increased efflux rates [449] are part of the molecular arsenal that *P. aeruginosa* deploys to resist antibacterial agents, resulting in severe economic and health outcomes [450]. The quest for new therapeutic strategies to fight *P. aeruginosa* infections is highly challenging.

Remarkably, among the 51 strains assayed in this study, the clinical isolates of *P. aeruginosa* harboured distinct phenotypes with only 16 strains producing the three virulence factors as the model strains PAO1 and PA14. Most strains produced only one or two of the studied virulence factors in the conditions tested. In addition, the levels of each virulence factor were highly variable from one isolate to another. Such phenotypic variations have already been reported and underline the necessity to test potential therapeutic molecules on clinical isolates [451].

In this study, the ability of the lactonase variant *Sso*Pox-W263I to decrease virulence in clinical isolates of *P. aeruginosa* was evaluated. This variant was selected for an increased catalytic efficiency towards 3-oxo-C_{12} HSL (45-fold increase as compared to the wild-type enzyme) while maintaining a high thermostability (T_m=88 °C). Three virulence factors were assayed: pyocyanin production, protease secretion and biofilm formation. *Sso*Pox-W263I was shown to drastically affect the synthesis of both virulence factors and biofilm while the QSI C-30 and 5-FU only showed moderate abilities, at their optimum dose, in the same conditions. These results were obtained *in vitro* using a rich medium in which bacterial growth is fast and without pre-treatment of the cultures with the QSI which could explain their poor activity. Notably, the use of 5-FU on PAO1 cultures led to a reduced growth rate of bacteria, possibly indicating some level of toxicity.

Even though *Sso*Pox targets, in principle, only one QS system used by *P. aeruginosa* its QQ potential was very effective. Proteolytic activity was decreased by at least half in 67 % of the strains, pyocyanin production in 68 % and biofilm in 82 %. The use of lactonases as biocontrol agents in bacterial infection therefore seems very promising. Moreover, upon lactonase treatment, no increase in proteolytic activity or pyocyanin production was noticed, only biofilm formation of one strain was slightly enhanced upon treatment with *Sso*Pox but was not statistically relevant. Conversely, the increase of virulence factor production after treatment with QSI molecules has already been reported, when clinical strains of *P. aeruginosa* isolated from cystic fibrosis patients were treated with C-30 and 5-FU and when isolates from diverse origins (urinary tract, wound infection, blood and respiratory tract) were exposed to natural inhibitors such as catechin, caffeine, curcumin and salicylic acid [362,444,452]. Similar observations are made in this study with several tested isolates after treatment with C-30 or 5-FU but never after treatment with *Sso*Pox, showing that *Sso*Pox does not induce any deleterious effect *in vitro* as compared to the QSI under the conditions used here.

Resistance of *P. aeruginosa* clinical isolates to QSI has previously been described [161,362,444]. The reported resistance mechanisms involved increased activity of antibiotic efflux pumps to move C-30 more efficiently out of the bacterial cell. Such mutations were

obtained *in vitro* by evolving a model strain in the presence of C-30. They were also identified in natural isolates that had never been challenged with C-30. Other reported mutations induce a reduced uptake of C-30 [46]. Nevertheless, such mutant strains would not show any resistance to *Sso*Pox quenching potential as the enzyme neither enters the bacteria nor binds to a cellular receptor. Even though no growth delay was observed using *Sso*Pox, suggesting no or very low selection pressure, other resistance mechanisms could appear. Bacteria could increase AHL production, produce an enzyme inhibitor, or modify the AHL molecule to prevent its recognition by the enzyme. However, AHL overproduction would represent a significant metabolic cost that would decrease the bacterial fitness in non selective environments. Modifying the AHL molecules would involve several mutations, first at the level of the synthase then at the level of the receptor. Furthermore, such resistance mechanisms could be easily circumvented by increasing the amount of enzyme or by broadening its spectrum to target more diverse AHL molecules.

Lastly, *Sso*Pox was successfully immobilised in the cultivation flasks using polyurethane and glutaraldehyde. Under these conditions the enzyme was still active and quorum quenching was achieved with a reduction of each virulence factor tested. In addition, no release of the enzyme was measured. Other enzymes have already been described and successfully used to quench *P. aeruginosa*. For example acylases from *Pectobacterium atrosepticum* and *Agrobacterium tumefaciens* have been used to degrade AHL from *P. aeruginosa* [453]. Even though the acylases efficiently decreased PAO1 virulence factor production, the stability of these enzymes at high temperatures (above 60°C) was really poor offering a limited industrial potential. The acylase from *Aspergillus meleus* was immobilized in coatings and decreased *P. aeruginosa* biofilm formation [135,357]. Nevertheless, these reports do not mention the industrial potential of such enzymes regarding their stability and tolerance to solvents, temperature and bacterial secretions. Regarding the use of lactonases as QQ agents, *Mom*L, a lactonase produced by the marine bacterium *Muricauda olearia* was showed to degrade AHL *in vitro* and to decrease virulence of *P. aeruginosa* PAO1 [320]. To our knowledge, unlike QSI no QQ enzyme was tested on a wide range of clinical isolates but only on model strains which behaviours differ from natural isolates. The biochemical characteristics of *Sso*Pox-W263I have already been assessed and reported [29,358]. The data presented here, together with the high resistance of *Sso*Pox and its potential to meet industrial constraints, open the way to incorporating *Sso*Pox in medical devices such as functionalised catheters and anti-virulence dressings. To fully assess the quenching potential of *Sso*Pox and its effect on virulence, infection models should be used.

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Conflict of Interest Statement

Mikael Elias and Eric Chabrière have a patent WO2014167140 A1 licensed to Gene&GreenTK. Laure Plener, David Daudé, Mikael Elias and Eric Chabrière report personal fees from Gene&GreenTK during the conduct of the study.

3. Etude comparative de l'impact de lactonases aux spectres d'action différents sur *P. aeruginosa*

Le QS de *P. aeruginosa* a fait l'objet de nombreuses études afin de définir son rôle dans la mise en place de la virulence. Quatre systèmes de communication ont été identifiés et semblent interconnectés : Las, Rhl, IQS, PQS. Le système Las, utilisant la 3-oxo-C_{12} HSL, apparait comme le système prédominant et active l'ensemble des quatre systèmes **(Figure 23)** [11,52,454]. Le système basé sur l'IQS, ou 2-(2-hydroxyphenyl)-thiazole-4-carbaldéhyde, est capable d'activer le système Rhl et la synthèse de PQS dépendamment de Las et de la concentration en phosphate. Le système basé sur le PQS, ou 2-heptyl-3-hydroxy-4-quinolone, est capable d'activer le système Rhl et semble aussi nécessaire à l'expression maximale du système Las [238]. Enfin, le système Rhl, utilisant la C_4 HSL, est capable de réprimer le PQS. Le QS intègre aussi de nombreuses régulations extérieures ce qui souligne le fait que le QS chez *P. aeruginosa* n'est pas isolé mais s'intègre dans un mécanisme de régulation globale. De nombreux stimuli environnementaux parmi lesquels, la limitation en certains nutriments (e.g. phosphate), les faibles ressources du milieu (« starvation ») et l'interaction avec l'hôte (e.g. interféron gamma ou IFN γ, le peptide antimicrobien LL-37, l'hormone peptide natriurétique de type C ou CNP, ou encore la dynorphine) peuvent ainsi impacter le QS **(Figure 23)** [52].

L'impact de l'inactivation des gènes du QS ou l'utilisation de QSI sur *P. aeruginosa* a été largement étudié au niveau phénotypique (biofilm, facteurs sécrétés, virulence *in vivo*) et moléculaire avec des approches globales comme la transcriptomique ou la protéomique [226,288,452,455]. Ceci a permis de mettre en évidence le rôle des différentes voies et de souligner les liens entre ces systèmes. Devant la complexité de ces interactions, l'intérêt de bloquer plusieurs voies a été souligné. L'effet additif ou synergique d'inhibiteurs agissant sur LasR, RhlR ou PqsR a notamment été mis en évidence [438]. Néanmoins, ces inhibiteurs peuvent potentiellement agir sur des protéines non impliquées dans le QS et leur spécificité reste difficile à évaluer. La combinaison d'un QSI avec une enzyme capable d'inhiber le QS, la lactonase AiiA, a par ailleurs pu montrer des effets synergiques. [456]. Cependant les résultats de cette étude se sont axés sur l'expression d'un nombre réduit de gènes et la contribution des deux approches reste difficile à estimer.

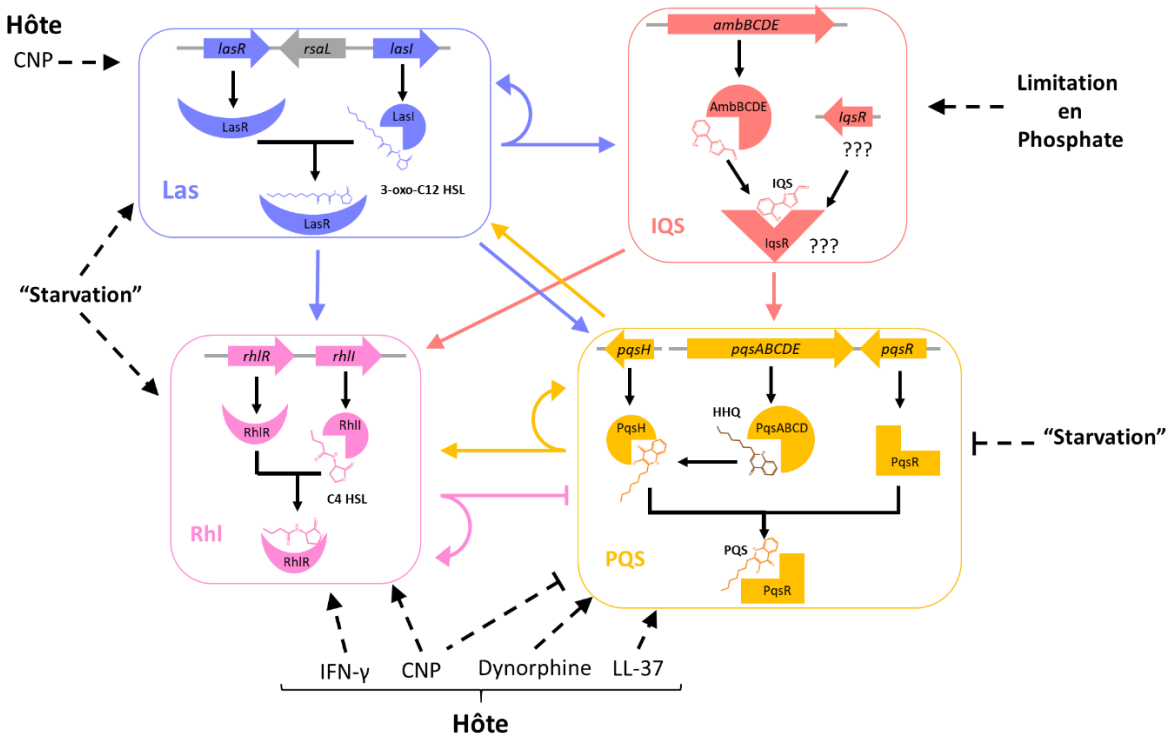
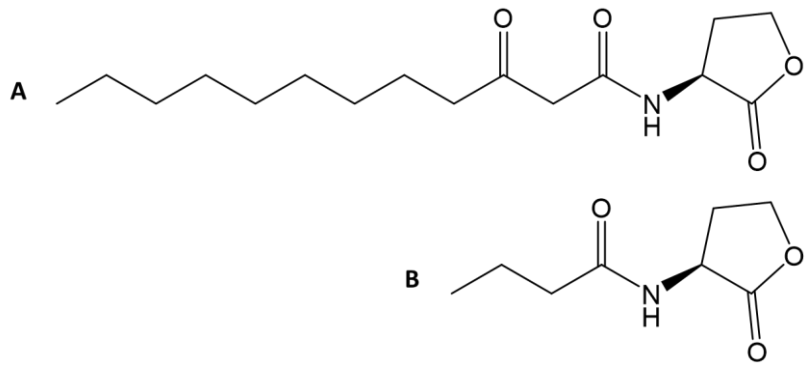


Figure 23 : Schéma simplifié des interactions entre les systèmes du QS ainsi que des influences environnementales chez *P. aeruginosa*. Les systèmes de QS sont en couleurs avec quelques stimuli environnementaux les influençant en noir. Une flèche ou un « T » représente respectivement une activation et une répression. Une ligne pleine ou en pointillé représente respectivement une interaction directe ou indirecte. Les points d'interrogation indiquent que IqsR n'est pas encore connu. Le schéma se base sur les informations issues de [11,52,238,457].

Les enzymes QQ constituent des outils extrêmement intéressants pour étudier précisément la contribution des différents systèmes de QS car elles sont capables d'agir de manière spécifique sur certaines molécules de communication sans autre impact direct sur la bactérie. De nombreuses enzymes ont aujourd'hui été décrites pour leur capacité à bloquer le QS, notamment en agissant sur les AHL, mais peu d'études se sont intéressées aux répercussions moléculaires de ces enzymes ni au rôle de leur spécificité dans la modulation du comportement de la bactérie ciblée. Pour *P. aeruginosa*, qui utilise deux HSL très distinctes dans leur chaine aliphatique (une à 4 carbones et une autre à 12 carbones et une fonction cétone en plus) **(Figure 24)**, l'efficacité catalytique et la spécificité de ces enzymes envers ces molécules n'ont pas été clairement prises en compte.

En collaboration avec l'équipe du Dr. Mikael Elias de l'Université du Minnesota, qui a récemment caractérisé la lactonase thermostable (*Gc*L) ayant un spectre différent de *Sso*Pox W263I, une étude comparative a été engagée afin de d'évaluer l'impact de la spécificité des enzymes sur la régulation du QS. *Gc*L est capable de dégrader efficacement, à la fois, les C_4 et 3-oxo-C_{12} HSL utilisés par *P. aeruginosa* contrairement à *Sso*Pox W263I qui est faiblement active sur la C_4 HSL.



<u>Figure 24</u> : Structure de la N-(3-oxo-dodécanoyl)-L-homosérine lactone (A) et N-butyryl-L-homosérine lactone (B).

L'impact des deux enzymes aux niveaux phénotypique et moléculaire sur la souche modèle PA14 a été étudié. D'abord, l'action distincte des deux lactonases a pu être mise en évidence à la fois dans leur dose efficace et dans l'impact *in vitro* sur les facteurs de virulence et la formation du biofilm. Une très nette différence a aussi été observée *in vivo* dans un modèle d'amibes, *Sso*Pox W263I se montrant capable de réduire la virulence de PA14 contrairement à *Gc*L qui a été inefficace. *Sso*Pox W263I s'est par ailleurs avérée capable, contrairement à *Gc*L, d'augmenter la sensibilité de la souche aux trois traitements antimicrobiens utilisés (antibiotiques et peroxyde d'hydrogène). De manière surprenante la combinaison des deux enzymes a montré une efficacité comparable à *Sso*Pox W263I utilisée seule, soit l'enzyme ayant le spectre d'action le moins large.

Pour aller plus loin dans l'interprétation, l'impact global des deux lactonases a été évalué à la fois au niveau de l'expression des gènes des quatre systèmes du QS et du protéome. L'analyse comparative a permis de montrer des variations similaires dans l'expression des gènes du QS entre les deux enzymes avec néanmoins une différence notable au niveau du PQS. Cette différence a été confirmée par une analyse protéomique qui a aussi révélé d'autres changements. Entre autres, des effets divergents ont été observés sur l'abondance de protéines impliquées dans la synthèse du biofilm, la résistance aux antimicrobiens, ainsi que dans la virulence confirmant certaines observations phénotypiques. Enfin, une fois encore, la combinaison des deux enzymes semble impacter le profil protéique de la bactérie de manière similaire à *Sso*Pox W263I seule.

Pour cette étude en cours, j'ai réalisé l'ensemble des tests phénotypiques *in vitro* à l'exception des tests de sensibilité aux antimicrobiens. J'ai mis au point et réalisé le test de virulence sur amibes en collaboration avec l'équipe du Professeur La Scola. De plus, j'ai préparé et analysé les échantillons de RT-qPCR et de protéomique (avec l'aide de la plateforme de spectrométrie de masse du laboratoire). Enfin, j'ai participé à l'écriture, la mise en forme et l'analyse des résultats. Des expériences complémentaires sont actuellement en cours afin de finaliser le manuscrit en vue de sa soumission.

3.1 Introduction

The human pathogen *Pseudomonas aeruginosa* is frequently isolated in Healthcare Associated Infections (HAI) and frequently displays drug or multidrug resistances [128,458]. This latter constitutes a serious therapeutic threat and the quest for new alternatives to fight bacterial infections is highly challenging. The inhibition of bacterial communication, referred to as quorum sensing (QS), has emerged as a non-bactericide approach to address virulence [199]. To this day, four QS systems have been described in *P. aeruginosa* relying for two of them on acyl-homoserine lactones (AHL) (Las, Rhl), one on quinolone(s) (PQS) and a last one on a carbaldehyde (IQS) as autoinducers [11,52]. Each system is composed by one or several enzymes to synthesize the autoinducer and a transcriptional regulator. These four systems are interconnected and the Las system (LasI/LasR), relying on N-(3-oxo-dodecanoyl)-homoserine lactone (3-oxo-C_{12} HSL), plays a role of global activator for all four systems. Under Las, the recently described IQS system (AmbBCDE/receptor yet unknown) can activate both PQS and Rhl through Las-dependent or independent pathways influenced by phosphate availability. The PQS system (PqsABCDH/PqsR) is able of autoinduction and can activate Rhl. PqsR was also proved to be involved in full expression of the Las pathway [238]. Rhl (RhlI/RhlR), the second AHL-based system, using butyryl homoserine lactone (C_4 HSL), is self-activating and inhibits PQS pathway. In addition, the QS systems are also interconnected with a large number of other transcriptional factors which, for a part of them, are known to integrate environmental stimuli [52]. For *P. aeruginosa,* QS is a complex network allowing a fine tuning of virulence factors production, biofilm formation and antimicrobial tolerance and therefore represents a suitable target for next generation of *P. aeruginosa* treatments [47,199].

Quorum quenching (QQ) represents all approaches relying on QS disruption using either chemical inhibitors (QSI) or autoinducer degrading enzymes [10,199]. QSI require close contact to fix their target, whereas QQ enzymes act remotely by inactivating QS signals. Both QSI and QQ enzymes have been well documented for their capacity to reduce phenotypic traits such as biofilm and virulence factors production of *P. aeruginosa* [199]. QSI effects have also been studied at the molecular level using proteomic or transcriptomic approaches [153,226,288,452]. Moreover, QQ approach targeting multiple QS pathways have been investigated using a combination of QSI or QSI plus QQ enzyme and reported some synergistic effects [438,456]. In this article, we investigated on *P. aeruginosa* PA14 the impact of two QQ lactonases with distinct AHL specificities. On one hand, we used *Sso*Pox W263I variant of the phosphotriesterase like lactonase (PLL) *Sso*Pox isolated from the thermophilic archaea *S. solfataricus*, with a proficient activity for the degradation of 3-oxo-C_{12} HSL [29]. Its QQ efficiency for biofilm and virulence factors reduction was previously demonstrated on two model strains (PAO1 and PA14) and 51 clinical isolates [30,359]. Furthermore, it was also able to reduce mortality in a rat pneumonia model either used as an immediate or deferred treatment [30]. Its extremophile origin is valuable for biotechnological applications as it confers high resistance to numerous denaturating conditions [28,358]. On the other hand, we used the newly described thermostable metallo-β-lactamase like lactonase (MLL) *Gc*L isolated from *Geobacillus caldoxylosilyticus* which is a proficient lactonase on a wide range of

AHL [459]. *Sso*Pox W263I and *Gc*L were used, independently or in combination, to quench *P. aeruginosa* PA14 and phenotypic and molecular impacts of quenching were evaluated. This work constitutes the first investigation on the role of enzyme specificity for quenching bacterial communication.

3.2 Materials and methods:

Bacterial strains

For enzyme production, *Escherichia coli* BL21 $(DE)_3$-carrying plasmids pGro7/GroEL for chaperones and pET22b with either *Sso*Pox W263I, *Sso*Pox 5A8 (V27G / P67Q / L72C / Y97S / Y99A / T177D / R223L / L226Q / L228M / W263H) or N-terminal strep tagged *Gc*L wild type were used [359,459]. For all the phenotypic and proteomic study, *Pseudomonas aeruginosa* PA14 was used.

Enzyme production and purification

*Sso*Pox W263I and 5A8 were produced as previously described [359]. Overnight precultures were incubated at 37°C in Luria Bertani (LB) medium (10 $g.L^{-1}$ NaCl, 10 $g.L^{-1}$ tryptone and 5 $g.L^{-1}$ yeast extract) complemented with chloramphenicol (34 $\mu g.mL^{-1}$) and ampicillin (100 $\mu g.mL^{-1}$). Then, cultures in ZYP-5052 medium complemented with the same antibiotics were inoculated and incubated at 37°C until optical density at 600 nm reached 0.8-1 [410]. At this state, $CoCl_2$ and L-arabinose were added at a final concentration of 0.2 mM and 0.2% (w/v). Cultures were further incubated at 23°C for another 20 h. Afterward, cells were pelleted down by centrifugation (4,400 g, 4°C, 20 min) and resuspended in HEPES lysis buffer (50 mM HEPES, 150 mM NaCl, 0.25 $mg.mL^{-1}$ lysozyme, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 $mg.m^{-1}$ DNaseI and pH 8.0). After being stored overnight at -80°C, resuspended cells were thawed and sonicated three times for 30 s with an amplitude of 45 % (QSonica sonicator Q700). Cell debris were pelleted down by centrifugation (12,000 g, 4°C, 30 min) and discarded. The supernatant was heated at 80°C over 30 min to precipitate *E. coli* proteins which were removed afterward by centrifugation (12,000 g, 4°C, 15 min). The remaining proteins were incubated overnight at 4°C in 75% ammonium sulfate in order to precipitate and concentrate *Sso*Pox. After resuspension in HEPES buffer (50 mM HEPES, 150 mM NaCl and pH 8.0), ammonium sulfate was eliminated via desalting (HiPrep 26/10 desalting, GE Healthcare; ÄKTA Avant). The resulting fractions were pooled and concentrated with 10 kDA centricon (Millipore). The proteins were then loaded onto a size exclusion chromatography column (HiLoad 16/600 SuperdexTM 75pg, GE Healthcare; ÄKTA Avant) and were eluted in HEPES buffer.

For G*cL*, cultures were realized in the same conditions, but cells were resuspended in Tris-HCl lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 0.25 $mg.mL^{-1}$ lysozyme, 0.1 mM PMSF and 10 $mg.mL^{-1}$ DNaseI and pH 8.0). After being stored overnight at -80°C, resuspended cells were thawed and sonicated twice for 30 s with an amplitude of 45%. Cell debris were pelleted down

by centrifugation (12,000 g, 4°C, 30 min). The crude extract was then loaded onto a Strep-tag column (5mL StrepTrap HP, GE Healthcare; ÄKTA Avant). The elution was performed in Tris-HCl buffer (50 mM Tris-HCl, 300 mM NaCl and pH 8.0) complemented with 2.5 mM of desthiobiotin (Sigma Aldrich).

After purification on chromatography column, the *Gc*L or *Sso*Pox containing fractions were pooled and concentrated with 30 kDa centricon (Millipore). The purity of each protein was checked by 12.5% SDS-PAGE separation and the concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

Lactonase activity measurement

The activity was measured on 3-oxo-C_{12} and C_4 HSL at ambient temperature using a colorimetric pH based assay [459]. For specific activity determination, the degradation of 1 mM of lactones in a cresol buffered solution (2.5 mM Bicine, 150 mM NaCl, 0.2 mM CoCl2, 0.2 mM cresol purple, 0.5% DMSO and pH 8.3) by 5-20 µg of enzyme was followed in 200 µL at 577 nm using a plate reader (SynergyHT, BioTek). For *Sso*Pox W263I activity on C_4 HSL, 140 µg were used as little activity was detected. For *Sso*Pox 5A8, up to 2 mg of this protein were used and it did not prove to be active toward neither 1 mM of C_4 or 3-oxo-C_{12} HSL.

Culture media and conditions

Selected medium and culture conditions were adapted from Welsh et al. [438]. Briefly, *P. aeruginosa* PA14 was cultivated in LB in 25 cm^2 culture flask (Corning) and incubated over 5-6h at 37°C with a 300 rpm agitation (Titramax 3000, Heidolph) to inoculate cultures at 1/1,000. Cultures were realized in MOPS minimal medium complemented with nitrogen (15 mM NH_4Cl), iron (5 µM Fe_2SO_4), phosphate (4 mM K_2HPO_4) and glutamate (25 mM) as carbon source. Cultures received either *Sso*Pox W263I, *Gc*L or a mix of both enzymes (50/50) to have the same activity ($U.mL^{-1}$) on 3-oxo-C_{12} HSL in all conditions. The inactive variant *Sso*Pox 5A8 was added in equivalent quantity than *Sso*Pox W263I.

For growth measurement, cells were grown over 26 h at 37°C and 300 rpm agitation. Cell density was estimated by measuring the optical density at 600 nm with a plate reader (SynergyHT, BioTek) and 200 µL of planktonic cells.

Pyocyanin assay

Pyocyanin was extracted by mixing 250 µL of chloroform in 500 µL of cell-free supernatant. After centrifugation at 10,000 g for 1 min, 200 µL of the lower chloroform phase was transferred into a quartz 96 wells plate. As blank, 200 µL of chloroform were used. The absorbance was measured at 690 nm [443]. Results for each condition and the blank absorbance were plotted directly.

Proteolytic activity assay

Protease activity was measured by using azocasein (Sigma Aldrich) degradation assay [442]. Briefly, 25 µL of cell-free supernatant were mixed to 675 µL of phosphate saline buffer pH 7.0 and 50 µL of azocasein solution (30 $mg.mL^{-1}$ in water). After 2 h at 37°C with agitation (300 rpm), 125 µL of 20% (w/v) trichloroacetic acid were added. Then, undegraded azocasein was pelleted down by centrifugation (10 000 g, 5 min). Afterward, 200 µL of supernatant were used to measure the optical density at 366 nm. As blank, an equivalent of volume of sterile MOPS medium was used. Results for each condition and the blank absorbance were plotted directly.

Elastolytic activity assay

Elastase B activity was measured by using elastin-Congo red conjugate (Sigma Aldrich) degradation assay [460]. In a 96 wells plastic plate (Greiner), 50 µL of cell-free supernatant were mixed to 150 µL of elastin-Congo red solution (5 $mg.mL^{-1}$ in 10 mM Tris-HCl and 1 mM $CaCl_2$ buffer at pH 7.2). After 24h incubation at 37°C with agitation (300 rpm), the plate was let to rest 10 min at ambient temperature in order to pellet undigested elastin-Congo red. Afterwards, 100 µL was carefully transferred into an empty well and then absorbance was measured at 490 nm. As blank, an equivalent of volume of sterile MOPS medium was used. Results for each condition and the blank absorbance were plotted directly.

Biofilm formation measurement

Biofilm was measured using crystal violet (Sigma Aldrich) biomass staining [461]. After culture in 12 well plates (Nunc™, Thermo Scientific), planktonic cells were removed. Wells were washed with 3 mL of phosphate buffered saline (PBS) solution (Biomérieux) and stained with 3 mL of 0.05% (w/v) crystal violet solution After removing crystal violet, wells were washed with 4 mL of PBS and dissolved with 3 mL of pure Ethanol. Absorbance at 595 nm was measured. As blank, sterile MOPS medium was used in the same culture conditions. Results for each condition and the blank absorbance were plotted directly.

Amoeba virulence assay

Acanthamoeba polyphaga Linc AP1 was routinely cultivated into peptone yeast extract glucose (20 $g.L^{-1}$ proteose peptone, 2 $g.L^{-1}$ yeast extract, 0.1 M glucose, 4 mM $MgSO_4$, 0.53 mM $CaCl_2$, 3.4 mM sodium citrate, 50 µM $(NH_4)_2Fe(SO_4)_2$, 2.5 mM KH_2PO_4, 1.3 mM Na_2HPO_4, pH 6.8) medium [462]. After 2-3 days of cultivation at 28°C, the cells were pelleted down at 750 g and resuspended into Page's amoeba saline (PAS) buffer (2 mM NaCl, 16 µM $MgSO_4$, 27 µM $CaCl_2$, 0.53 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 6.9). The volume was adjusted to obtain a 10^5 $cells.µL^{-1}$ final concentration. On the other side, after culture in 6 well plates (Nunc™, Thermo Scientific), 3 mL of bacterial culture were pelleted down and resuspended into a minimum of 1 mL of PAS buffer. Accordingly, the buffer volume was adjusted to have the same concentration of bacteria in each condition. Then a PAS agar plate was flooded with 1 mL of

bacterial suspension. After drying at ambient temperature, 5 µL of *A. polyphaga* were spotted at the center and let to dry. Afterwards, the plate was incubated at 30°C over 7 days. Each day, amoeba propagation was followed by directly measuring the central spot with a ruler. The results were plotted directly for each condition from day 0 to 7.

Antimicrobial sensitivity assay

The effect of tobramycin, gentamicin and hydrogen peroxide on PA14 was evaluated using the MBEC™ assay (Innovotech) [463]. As previously described, PA14 was grown in 180 µL MOPS medium in the 96 wells plate of the MBEC™ assay device for 24 h at 37°C under orbital agitation (110 RPM). Afterward, the lid was transferred, first to a 96 well plate containing 190 µL of fresh non-complemented MOPS medium to wash the planktonic cells and then transferred to another plate containing antimicrobial agents. Tobramycin and gentamicin were used at concentration ranging from 0 to 20 $\mu g.mL^{-1}$ and H_2O_2 from 0 to 500 mM. PA14 was exposed for 1h30 and 3h to respectively H_2O_2 and antibiotics under agitation (110 RPM). The cells were washed in 200 µL of non-complemented MOPS medium and then transferred into 200 µL of recovery LB (LB supplemented with 20.0 $g.L^{-1}$ saponin and 10.0 $g.L^{-1}$ Tween-80). After 1 h, cells were diluted in non-complemented MOPS medium and plated on LB agar. CFU were counted after 56 h growth at 20°C.

RNA extraction and QS gene expression measurement

From 200 µL of culture cells pellet, RNA was extracted and purified with PureLink™ RNA Mini Kit (Invitrogen) and residual DNA were digested with TURBO DNA-*Free*™ kit (Invitrogen). Then cDNA was synthesized using TaqMan™ Reverse Transcription Reagents (Applied Biosystems) and provided random hexamer. Eventually, qPCR was realized using LightCycler® 480 SYBR Green I Master (Roche), and a CFX96 Touch™ Real Time PCR Detection System (Biorad). Primers used are indicated in **Supplementary Table 1** (see the "Annexes" section). The resulting data for each gene were normalized using housekeeping gene *recA* expression and analyzed using the $2^{-\Delta Ct}$ method [464]. The results were plotted as relative expression level by dividing each $2^{-\Delta Ct}$ values by the mean of the control without added protein.

Protein extraction

Cells were harvested by centrifugation (10 000 g, 5 min) and washed with 2 mL of PBS and centrifuged again (10 000 g, 5 min). Pellets were resuspended in 100 µL of UTSTS buffer [8 M Urea, 2 M Thiourea, 100 mM NaCl, 25 mM Tris-HCl, pH 8.2 and protease inhibitor (complete, Roche)] and sonicated on ice for 30 sec with an amplitude of 15% (Vibra cells) until it became clear. Cell debris were removed by centrifugation (16 000 g, 20 min) and the supernatant was carefully transferred into a dialysis cassette (Slides Alyzer dialysis cassette 2K MWCO, Thermo scientific). The cassette was incubated for 4 h in 2 L of Urea/Ambic buffer (1 M Urea, 50 mM ammonium bicarbonate, pH 7.4) and overnight in 2 L of fresh Urea/Ambic

buffer. Protein quantity was estimated with Braford assay (BioRad) and 50 µg of proteins were mixed to Urea/Ambic buffer to a final volume of 50 µL. 1 µL of 0.5 M dithiothreitol in Urea/Ambic buffer was added for disulphide bonds reduction and the reaction was driven over 1 h at 37°C. For alkylation, 2 µL of 0.5 M iodoacetamide in Urea/Ambic buffer were added and let to react over 1 h protected from light. Afterwards, pH was checked to be above 7. Protein digestion was performed by adding 2 µL of 1 $\mu g.mL^{-1}$ trypsin (Agilent) and samples were incubated overnight at 37°C. Digestion efficiency was checked on 10 % SDS-Page gel. Finally, detergent removal spin column (Pierce™, Thermo Fisher) and C18 spin column (Pierce™, Thermo Fisher) were used to clean the samples.

Label-free quantitative nano-LC-MS/MS proteomics analysis

Protein digests were in a first step separated by Ultra Performance liquid chromatography (UPLC) using the NanoAcquity UPLC System (Waters) connected to a Synapt G2Si Q-TOF ion mobility hybrid mass spectrometer (Waters). The chromatographic system was used in 1D configuration with an analytical column (ACQUITY UPLC M-Class Trap Column Reversed-Phase 1.7 µm spherical Hybrid, CSH, 75 µm x 150 mm, Waters) after a trapping column (ACQUITY UPLC M-Class Trap Column Reversed-Phase 5µm spherical silica, 180 µm x 20 mm, Waters). Eluted peptides were separated using a 100 min gradient (300 $nL.min^{-1}$; 0.5 to 40% acetonitrile–0.1% formic acid). Data-independent MS/MS analysis was performed with the ion mobility feature (HDMSe method). The ion source parameters were capillary voltage 3 kV, sampling cone voltage 40 V, ion source temperature 90°C, cone gas flow 50 $L.h^{-1}$. Transfer collision low energy was set to 5 V, trap collision low energy was set to 4 V. The high energy ramp was applied from 4 V to 5 V for the trap collision and from 19 V to 45 V for the transfer collision enabling fragmentation of the ions after the ion mobility cell and before the time-of-flight (TOF) MS. On-column sample load was 800 ng (2µL injected). Each sample was injected in duplicate.

Proteomic data processing and analysis

The acquired files were imported into Progenesis QI software Version 2.0 (Nonlinear Dynamics, Newcastle, UK) for label-free quantification analysis. The data were aligned automatically and normalized. Processing parameters were 150 counts for the low energy threshold, 30 counts for the elevated energy threshold. The database used to identify peptides contains the protein sequences of *Pseudomonas aeruginosa* PA14 (TrembL, 25/04/2017, 5886 sequences). Search tolerance parameters were: peptide and fragment tolerance, 15 ppm, FDR < 1%; Minimum Ion matching requirements were three fragments per peptide, seven fragments per protein and two peptides per protein. The enzyme specificity was trypsin allowing 1 missed cleavage, the accepted modifications were carbamidomethyl of cysteine (fixed), oxidation of methionine (variable), carbamyl of lysine and N-terminal (variable), deamidation (variable) of asparagine and glutamine. The protein normalization was performed according to the relative quantitation using non-conflicting peptides. To determine

the significance of changes between samples, a significant ANOVA (P_{value}<0.05) and a fold change superior to 2 were used as the thresholds to define differently expressed protein.

The principal component analysis (PCA) was performed on normalized data using SIMCA 14. The data were Pareto scaled, autofitted for principal components and the Hotelling's T^2 was used to assess the possible presence of outlier.

For the heat map, the logarithm with base 10 (log_{10}) of the fold change was calculated. According to the reference condition, either log_{10} (higher expressed) or – log_{10} (lower expressed) was used in the representation. For non-significant level modification, the fold change was considered of 1 and thus was represented by a zero in the heat map.

Statistical analyses

For virulence factors, biofilm and QS gene expression measurement data, statistical analyses were performed using GraphPad Prism 7. The type I error, or α, was set at 0.05. For all these data, normality distribution was checked with the D'Agostino and Pearson omnibus normality test. For virulence factors and biofilm, statistical analyses were performed on raw optical density data. A two-way ANOVA was performed according to enzyme treatment and concentration. Then when ANOVA P_{value} was inferior to 0.05, the Holm-Sidak's multiple comparisons test was used to assess difference between: *Sso*Pox W263I and GcL or *Sso*Pox W263I+GcL; *Gc*L and *Sso*Pox W263I+*Gc*L for each concentration. For the QS gene expression, statistical analyses were performed on $2^{-\Delta Ct}$ [464]. A one-way ANOVA was used and if the ANOVA P_{value} was inferior to 0.05, the Sidak's multiple comparison test was used to assess the difference between: without protein and *Sso*Pox 5A8 (inactive); *Sso*Pox 5A8 (inactive) and *Sso*Pox W263I, *Gc*L or *Sso*Pox W263I + *Gc*L; *Sso*Pox W263I and *Gc*L or *Sso*Pox W263I + *Gc*L; *Gc*L and *Sso*Pox W263I + *Gc*L.

3.3 Results

Lactonases specificities on P. aeruginosa AHL

To study the impact of enzymatic QQ on PA14, two lactonases were used, namely *Sso*Pox W263I and *Gc*L, harboring distinct activities **(Table 6)**. *Gc*L efficiently hydrolyzes both C_4 and 3-oxo-C_{12} HSL produced and sensed by *P. aeruginosa* whereas *Sso*Pox W263I mainly degrades 3-oxo-C_{12} HSL with a very low activity on C_4 HSL. The specific activity ratio between *Gc*L/*Sso*Pox on 3-oxo-C_{12} AHL was around 2.7 in this condition and was used to adapt the enzyme quantity in the following experiments. The variant *Sso*Pox 5A8 demonstrated no detected activity on neither AHL and was used as one of the negative controls.

Table 6 : Catalytic efficiency of the proteins used in this study.

Enzyme	*Sso*Pox W263I		*Gc*L		*Sso*Pox 5A8	
AHL	3-oxo-C_{12} HSL	C_4 HSL	3-oxo-C_{12} HSL	C_4 HSL	3-oxo-C_{12} HSL	C_4 HSL
Specific activity ($U.mg^{-1}$)	1.7±0.2	0.054±0.001	4.7±0.3	5.7±0.6	N.D.	N.D.

N.D.: Not Detected

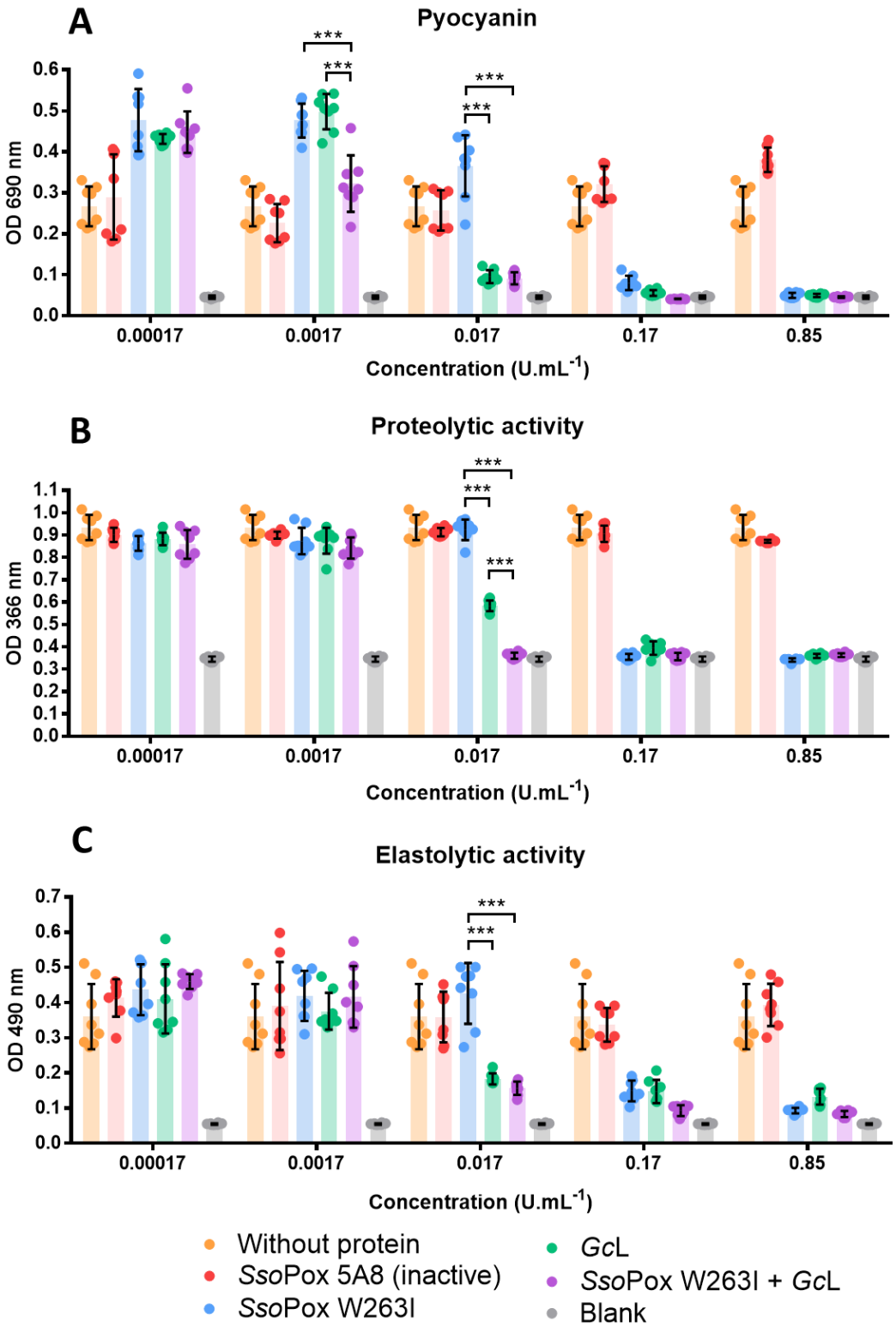
U or enzymatic unit: $\mu mol.min^{-1}$

Impact of quorum quenching lactonases on in vitro virulence factors and biofilm formation

In order to compare their action, the lactonases were used at equivalent activity on 3-oxo-C_{12} HSL, the common substrate efficiently hydrolyzed by both enzymes, on PA14 growing in a MOPS minimal medium which allows the simultaneous measurements of several QS regulated phenotypes in one unique condition [438,465]. To check the impact of enzyme addition in a minimal medium, control samples without protein and an inactive variant (*Sso*Pox 5A8) were included. Based on previously known QQ concentrations, the growth of PA14 in MOPS medium over 26h was followed, cells entered in stationary phase after 20h in all tested conditions, thereby this time point was chosen for further experiments [359] (**Supplementary figure 7** in the "Annexes" section).

Pyocyanin, protease and elastase productions were measured after culture with different enzyme concentrations and combinations. In all cases, their production decreased when increasing lactonase concentrations while the addition of inactive variant had no effect on the production of these factors **(Figure 25)**. The highest concentration of enzymes reduced by more than 75% the production of elastase and totally prevented pyocyanin and protease production in all conditions.

Importantly *Gc*L reduced virulence factor production at lower concentrations than *Sso*Pox W263I (0.017 $U.mL^{-1}$ versus 0.17 $U.mL^{-1}$ for all three factors). Furthermore, the combination of the two lactonases showed synergistic effects. Indeed, pyocyanin and protease production were further decreased at 0.0017 and 0.017 $U.mL^{-1}$ respectively as compared to enzymes alone **(Figure 25)**.



<u>Figure 25</u> : Pyocyanin (A), proteases (B), elastase B (C) measurements after lactonase treatment. For each condition, all n=8 independent samples are plotted with mean and standard deviation in colored histogram and black bars. For difference between *Sso*Pox W263I, *Gc*L and the combined treatment, statistical significance according to Holm-Sidak's multiple comparison test was highlighted by black stars (multiplicity adjusted P_{value} ≤0.05 *, ≤0.01 **, ≤0.001 ***).

Biofilm formation was also reduced in a dose-dependent manner in every condition and a difference was observed from a low enzyme concentration (0.0017 U.mL^{-1}) **(Figure 26)**. As reported for the other virulence factors, the combination of enzymes showed a synergistic effect at 0.0017 and 0.017 U.mL^{-1} as compared to enzymes alone. *Gc*L was again more efficient at 0.0017 U.mL^{-1} than *Sso*Pox W263I with a stronger impact on biofilm formation. Interestingly, *Sso*Pox W263I suppressed completely biofilm formation from 0.17 U.mL^{-1} whereas small bacterial aggregates were still observable with *Gc*L even at higher concentration (2.3 U.mL^{-1}) **(Figure 26)**. Moreover, this phenomenon was not observed when *Gc*L was used in combination with *Sso*Pox W263I.

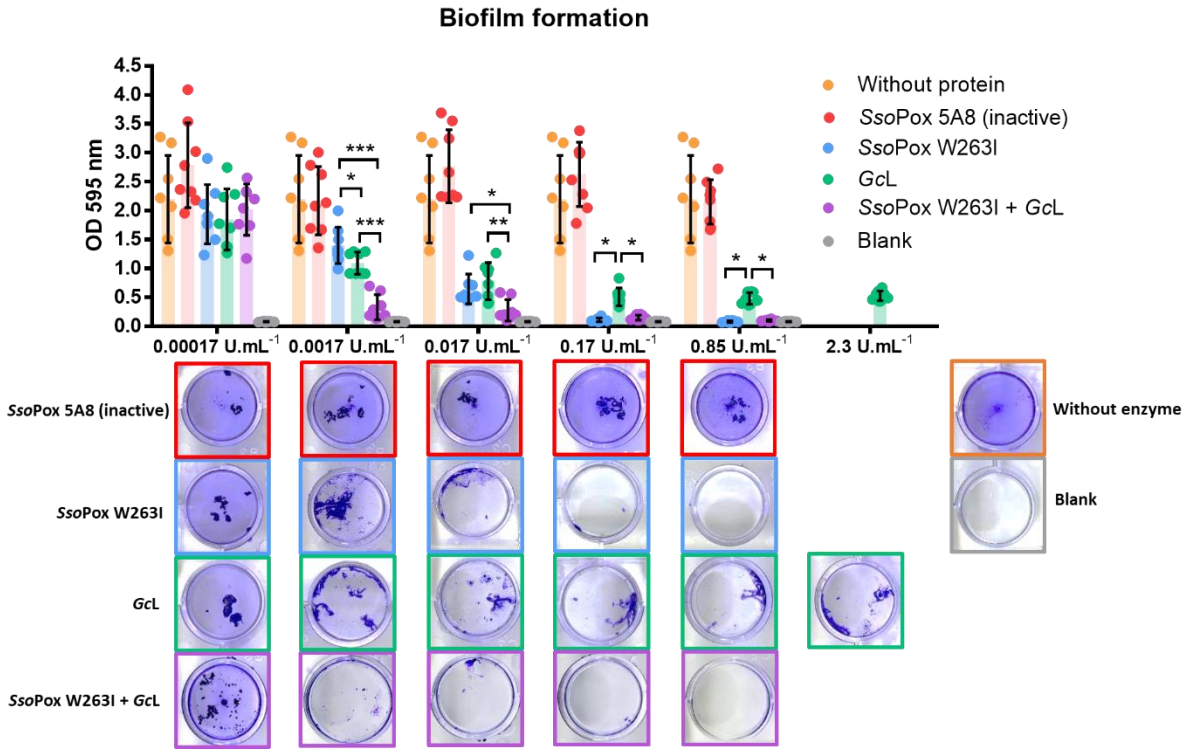


Figure 26 : Biofilm formation measurements by crystal violet staining and corresponding pictures of well plates after lactonase treatment. All n=8 independent samples are plotted with mean and standard deviation in colored histogram and black bars. For *Sso*Pox W263I, *Gc*L and the combined treatment, statistical significance according to Holm-Sidak's multiple comparison test was highlighted by black stars (multiplicity adjusted P_{value} ≤0.05 *, ≤0.01 **, ≤0.001 ***).

In all assays, *Gc*L quenching was observed at lower doses than for *Sso*Pox W263I, even though *Gc*L did not fully suppress biofilm formation unlike *Sso*Pox W263I. Furthermore, the use of both enzymes showed a synergistic effect as compared to the use of enzymes alone. Those results suggested potential difference(s) between *Sso*Pox W263I and *Gc*L impact on *P. aeruginosa* which could be more than just a cumulative effect. To further evaluate this aspect and not restrict our results to specific phenotypic traits, an *in vivo* virulence assay was developed.

Impact of quorum quenching lactonases on in vivo virulence toward Acanthamoeba polyphaga

A previously described *in vivo* assay based on *A. polyphaga* Linc AP1 was adapted to clearly assess QQ effect on PA14 virulence [462]. Virulence of PA14 was evaluated by the ability of *A. polyphaga* to grow or not in the presence of the bacteria. In both controls, amoebas were not able to grow in presence of PA14 demonstrating the virulence of the strain on *A. polyphaga* **(Figure 27)**. After quenching with *Sso*Pox W263I, virulence towards amoebas was reduced in a dose-response manner and *A. polyphaga* was able to propagate even at the lowest enzyme concentration **(Figure 27A)**. However, using *Gc*L, virulence was not obviously impacted even at the highest concentrations **(Figure 27B)**. Finally, when both enzymes were mixed, virulence was reduced with a comparable tendency as *Sso*Pox W263I alone and unlike *Gc*L **(Figure 27C)**.

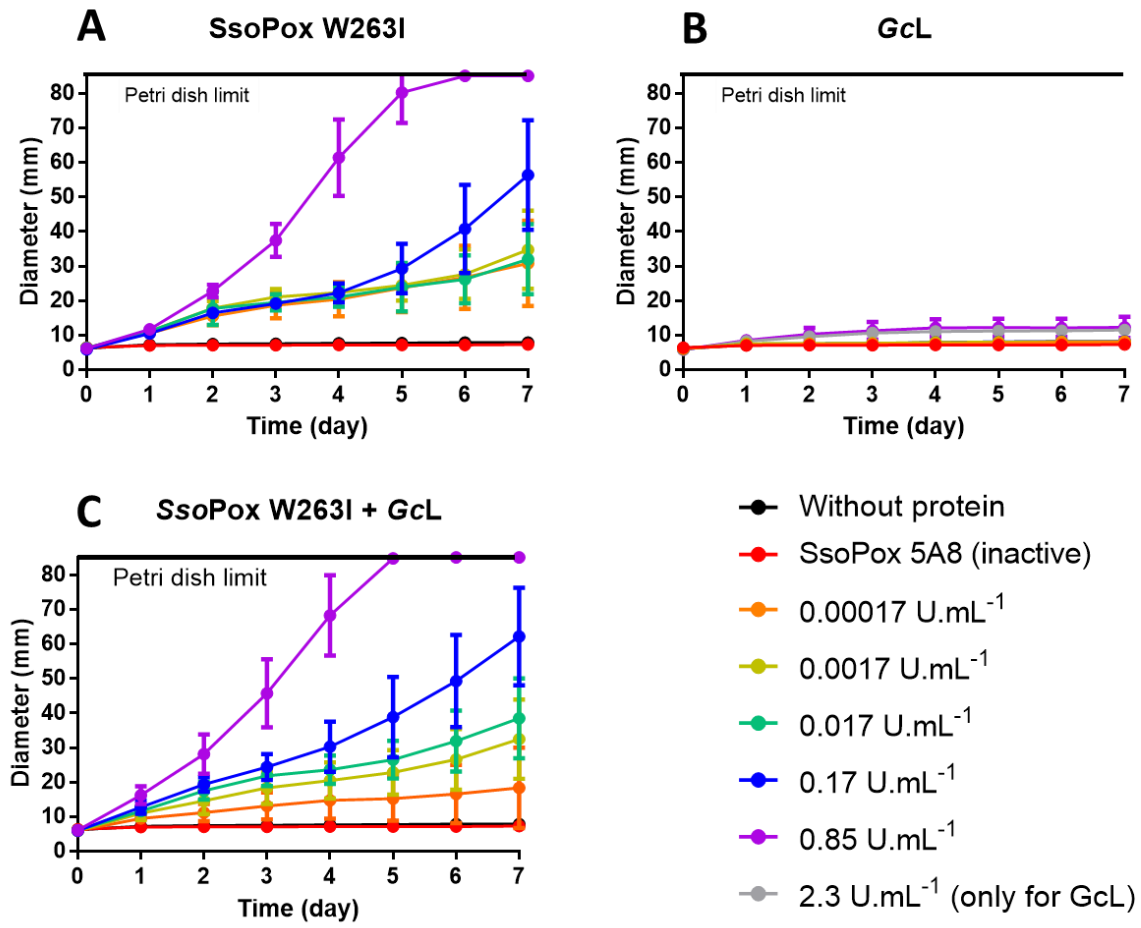


Figure 27 : Propagation of *A. polyphaga* Linc AP1 in virulence plate assay after treatment of *P. aeruginosa* PA14 by A) *Sso*Pox W263I, B) *Gc*L, C) *Sso*Pox W263I and *Gc*L. All n=8 independent samples are represented by the mean and standard deviation in corresponding colored bars.

Impact of quorum quenching lactonases on in vitro antimicrobials tolerance

As QQ have been reported to increase antimicrobial susceptibility [199], two aminoglycoside antibiotics (tobramycin and gentamicin) and one antiseptic (H_2O_2) were tested to determine the minimal biofilm eradication concentration (MBEC) of PA14 after lactonase exposure [463]. PA14 was grown on peg lids in presence of each lactonase alone or combined (0.85 $U.mL^{-1}$) and the inactive variant was used as control. Without antimicrobial application, similar cell quantities were recovered from the peg lids **(Figure 28)**. After antimicrobial exposure, PA14 was more sensitive to any of the three agents used when pretreated with *Sso*Pox W263I **(Table 7 and Figure 28)**. The MBEC values were reduced by 10-fold for a relatively short exposure of 1 h 30 and 3 h with respectively H_2O_2 and antibiotics. *Gc*L, on the other side, only impacted the H_2O_2 sensitivity with a 2-fold reduction of the MBEC **(Table 7)**. The combined pretreatment with both lactonases induced an increased sensitivity for all three antimicrobials with a similar fold change as *Sso*Pox W263I alone **(Table 7 and Figure 28)**.

Taken together these results confirm that quenching PA14 has different outcomes depending on the specificity of the enzymes used.

Table 7 : MBEC values for the three antimicrobials tested.

MBEC	SsoPox 5A8 (Inactive)	SsoPox W263I	GcL	SsoPox W263I + GcL
H_2O_2 [mM]	100	10	50	10
Tobramycin [µg.mL^{-1}]	10	1	10	1
Gentamicin [µg.mL^{-1}]	20	2	20	2

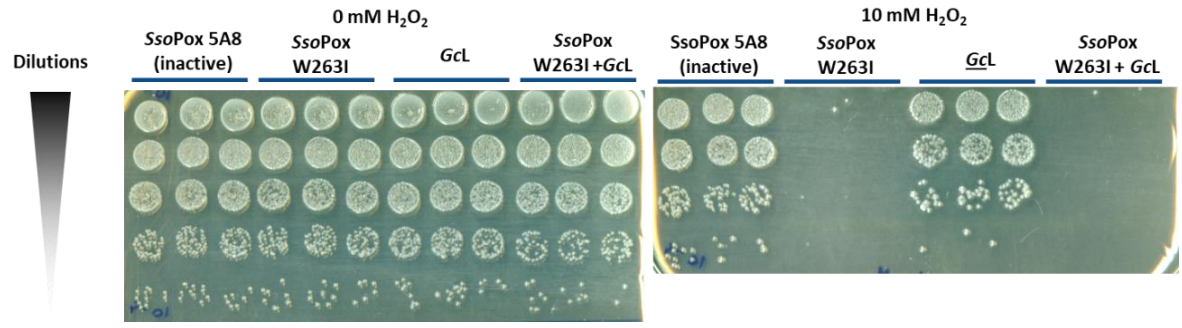


Figure 28 : Hydrogen peroxide efficiency on PA14 after QQ treatment with each lactonase alone or combined. Representative results of the CFU counting on agar plate with and without H_2O_2 treatment.

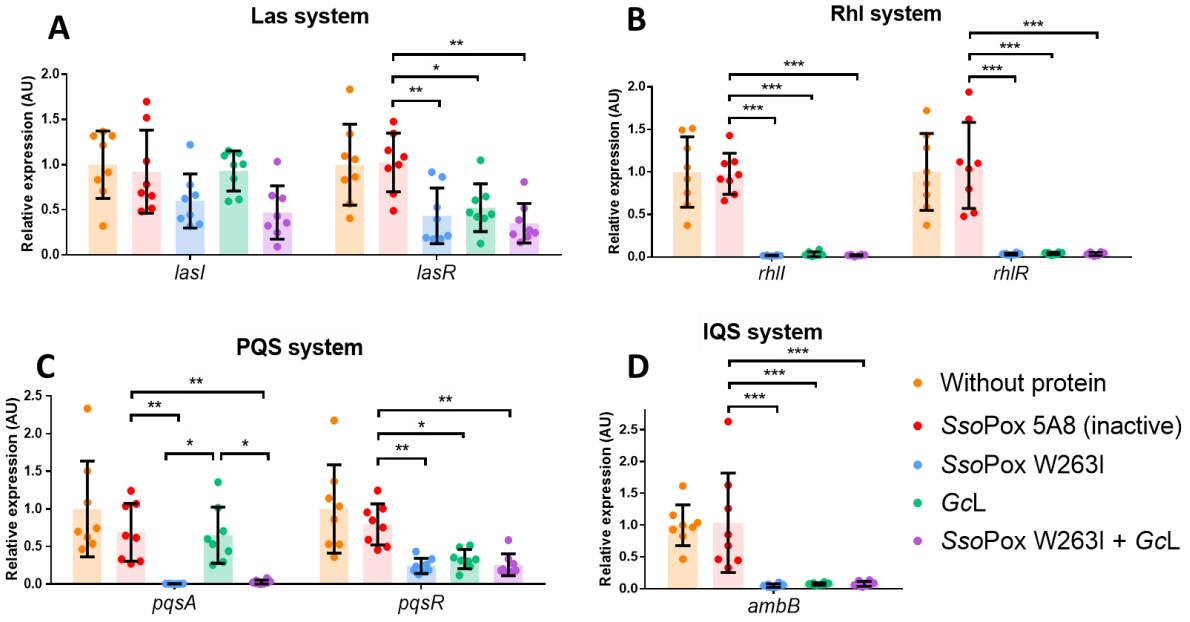
Impact of QQ lactonases on QS genes expression

In order to decipher the regulation mechanisms governing the phenotypic differences between quenching with *Gc*L and *Sso*Pox W263I, the expression of major QS genes after treatment with the highest enzyme concentration (0.85 $U.mL^{-1}$) alone and in combination was investigated. Enzymatic treatment with either lactonase decreased significantly the expression of *lasR* but did not impact *lasI* **(Figure 29A)**. The Rhl system was similarly impacted by *Gc*L or *Sso*Pox W263I without distinct differences **(Figure 29B)**. The *ambB* gene expression

which is involved in IQS signal molecule synthesis, a non AHL autoinducer, was also strongly reduced with either enzyme **(Figure 29D)**. For the PQS system, *pqsR* expression followed the same tendency and the expression was significantly reduced with both lactonases alone **(Figure 29C)**. However, a major difference arose for *pqsA* expression level, a gene involved in the synthesis of PQS autoinducer. The expression was strongly reduced with *Sso*Pox W263I, whereas *Gc*L did not show any statistically significant impact on *pqsA* transcript level as compared to the control.

Regarding the combination of both lactonases, the results were similar to each enzyme alone for *lasI/R, rhlI/R, ambB* and *pqsR* expression and no significant difference was detected. However, for *pqsA*, the enzyme mix behaved as *Sso*Pox W263I alone with a strong reduction of *pqsA* expression statistically similar to *Sso*Pox W263I but significantly different from the level observed with *Gc*L **(Figure 29C)**.

In order to confirm discrepancies observed in the PQS regulation and get a broader picture of the differences between the impact of *Sso*Pox W263I and *Gc*L treatments on PA14, a proteomic study in those conditions was conducted.



<u>Figure 29</u> : Relative expression of genes involved in Las (A), Rhl (B), PQS (C) and IQS (D) systems after lactonase treatment at 0.85 U.mL^{-1}. For each condition, all n=8 independent samples are plotted with mean and standard deviation in colored histogram and black bars. Statistical significance according to Sidak's multiple comparison test was highlighted by black stars (multiplicity adjusted P_{value} ≤0.05 *, ≤0.01 **, ≤0.001 ***).

Global proteomic profile is clearly distinct between lactonase treatments

The proteomic profiles of PA14 treated with the inactive lactonase *Sso*Pox 5A8 as control, the active lactonases *Sso*Pox W263I or *Gc*L and the enzymatic mix were compared. Pooling all the conditions together, we identified 515 (8.7%) out of the 5,886 proteins of PA14 (Uniprot database) among which 210 (3.6%) were significantly changed (fold change ≥ 2 and $P_{value} \leq 0.05$) in at least one of the six comparisons (**Supplementary table 2** in the "Annexes" section). Similar values were previously obtained for the study of QSI effects with proteomic and transcriptomic studies [153,226,288,452]. Taking the 210 modified proteins, the resulting principal component analysis (PCA) resulted in 11 principal components with a cumulative explained variation (R^2X) of 98% and predicted variation (Q^2) of 86% (**Supplementary figure 8A, B and C** in the "Annexes" section). The two first components were enough to distinguish three distinct groups and explained the majority of the samples variation (cumulated R^2X of 70%) (**Supplementary figure 8A and C** in the "Annexes" section). The first PCA clearly separated the control (*Sso*Pox 5A8) from the three other QQ treatments. The second PCA distinguished the *Sso*Pox W263I and combined treatment from *Gc*L alone.

Comparing each treatment with the control, a distinct profile was observed between *Gc*L and *Sso*Pox W263I **(Figure 30A and B)**. Respectively 74 and 42 proteins were identified as less expressed after enzymatic treatment with *Sso*Pox W263I and *Gc*L as compared to the control, among which 35 were in common. Conversely, *Sso*Pox W263I and *Gc*L increased the level of 60 and 64 proteins as compared to the control, only 29 being common to both enzymes. By directly comparing *Sso*Pox W263I to *Gc*L, 53 and 22 proteins were less and more abundant with *Sso*Pox W263I treatment. Yet again, the proteomic profile of cells treated with both enzymes is closer to treatment with *Sso*Pox W263I alone than treatment with *Gc*L **(Figure 30A)**. Even though some distinctions were noticed when they were compared to the control, only 6 proteins significantly differed for the dual treatment compared to *Sso*Pox W263I directly. All 6 proteins were reduced upon combined treatment.

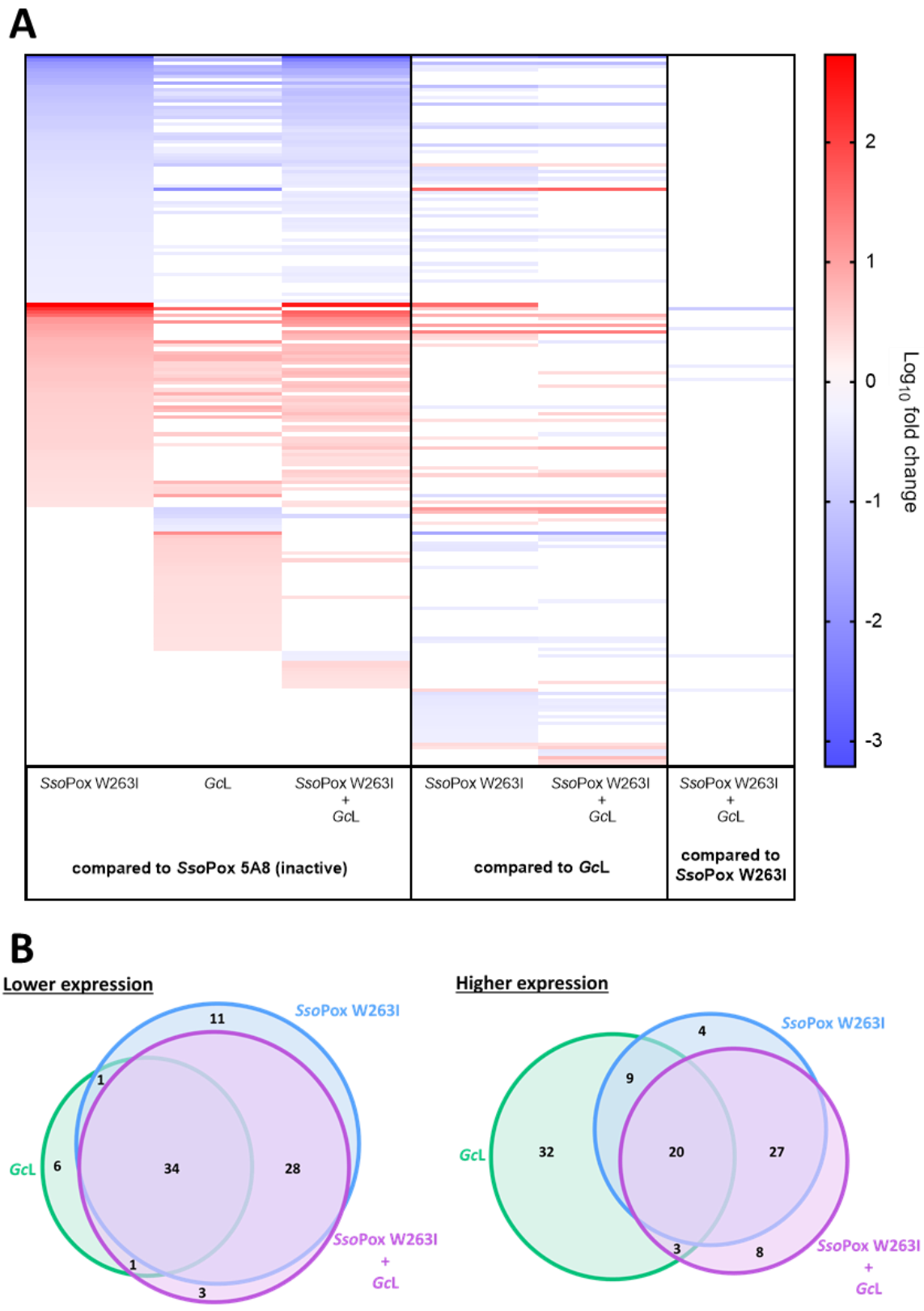


Figure 30 : Global modifications of PA14 proteome in response to lactonase treatment. A) Heatmap of all proteins represented by the $\log_{10}$ relative fold change mean for the six comparisons. B) Venn's diagrams of proteins with significant reduced or increased expression upon lactonase treatment as compared to the control.

Similar and specific trends of proteomic profiles between lactonase treatments

Thirty-five proteins were found less expressed after *Sso*Pox W263I or *Gc*L treatment than control. Among these, proteins involved in antimicrobials resistance, oxidative stress protection, virulence and in biofilm formation were detected **(Figure 31)**. For instance MexG, MexH and OpmD are part of an efflux pump complex known to transport, among others, fluoroquinolones, phenazines and PQS molecules [466–468]. The glutathione peroxidase PA14_47550 and the GTPase ObgE were also reduced and are known to be involved respectively in oxidative stress response and ofloxacin persistence [469,470]. The reduced protein PA14_61190 (PA4624/CdrB in PAO1) is part of a two partner secretion system that exports an adhesin involved in adherence and biofilm formation [471]. PA14_48590 and PA14_05510 (PA0423/PasP in PAO1) are associated to virulence and were also decreased by both treatments [472,473]. Following the same trend, the operon (PA14_18810/60), the transporter PA14_13170 (PA3920/CueA) and DsbG can be pointed out as they are linked to copper homeostasis and most of them contribute to its tolerance [474,475].

Among the proteins commonly increased in either treatment as compared to the control, PhoB, a regulator involved in IQS activation, biofilm inhibition, phosphate stress response and uptake, was found altered **(Figure 31)**. A significant increase was also observed for PA14_18690 (PA3529/TsaA in PAO1) involved in oxidative stress response and the transporters OpdB and OprG which are related to antimicrobial sensitivity or uptake [474,476–478].

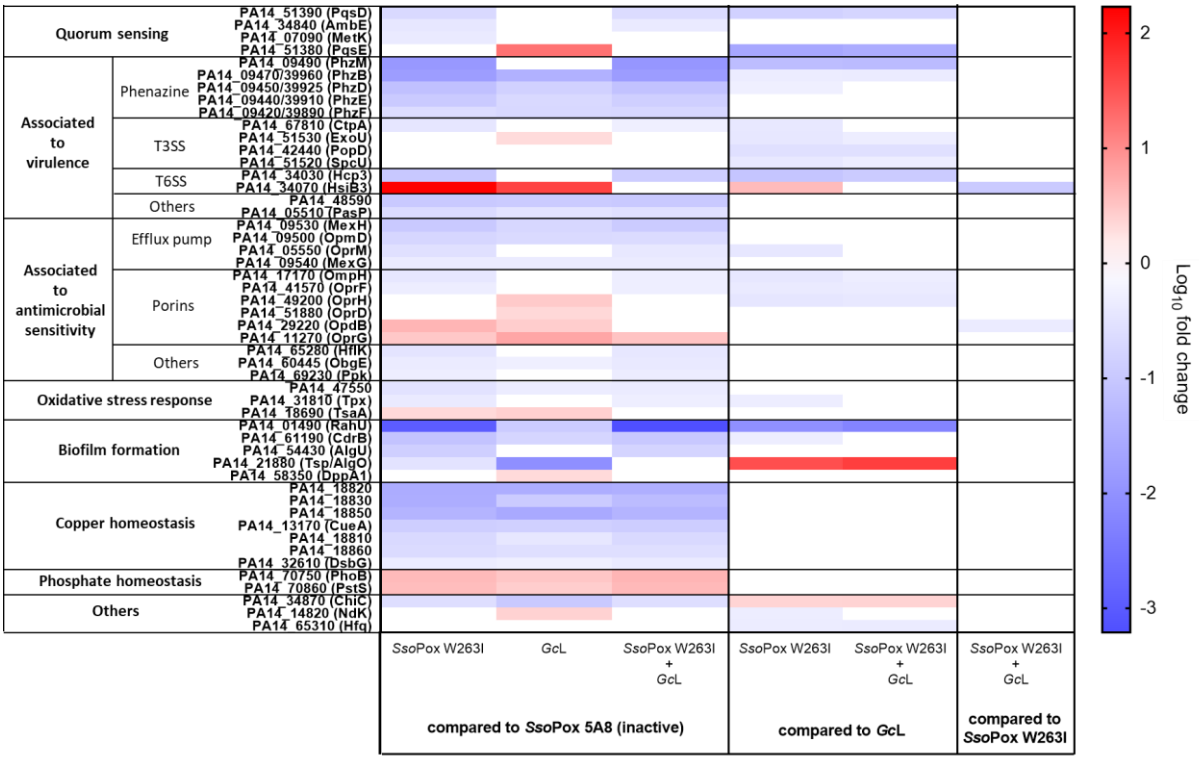
Comparing both lactonases, quenching by *Sso*Pox W263I or *Gc*L also resulted in different expression levels for some proteins **(Figure 31)**. The Rhl-dependent chitinase ChiC and alginate synthesis pathway related Tsp (or AlgO) were found to be less abundant upon treatment with *Gc*L than *Sso*Pox W263I, even though it was reduced in both profiles as compared to control [294,479,480]. Conversely, enzymes responsible for phenazine synthesis, PhzB, D, E and F, were reduced by both enzymes but PhzB and PhzD were more importantly decreased by *Sso*Pox W263I. The same trend was followed by the putative hemolysin RahU involved in biofilm modulation for which *Sso*Pox W263I induced reduction was much more important than *Gc*L [481].

Specifically, some protein levels were only changed with *Sso*Pox W263I but not with *Gc*L as compared to the control **(Figure 31)**. Among them, PhzM, PqsD, AmbE, AlgU and MetK are known to be involved in QS signal synthesis, pyocyanin production, biofilm formation and virulence [52,467,480,482]. Of note, the thiolperoxidase Tpx, the protease HflK, the transporters OprF, OprM, OmpH and the kinase Ppk are associated with oxidative stress protection or antibiotic susceptibility and were found reduced with *Sso*Pox W263I only [477,478,483–486]. Finally, the protease CtpA, involved in T3SS, was also less expressed only with *Sso*Pox W263I [487].

On the other hand *Gc*L treatment specifically increased several protein levels such as PqsE, which is part of PQS synthesis operon but is not directly involved in PQS synthesis, the AHL induced peptides transporter DppA1 involved in biofilm formation [488,489], the porin OprD [477] and the global regulator Hfq [490,491] **(Figure 31)**. Remarkably, ExoU, PopD and

SpcU, three proteins linked to the T3SS, were found more abundant upon *Gc*L treatment than *Sso*Pox W263I treatment.

Among all these modifications, the combined treatment using both enzymes had a proteomic profile very similar to *Sso*Pox W263I with very few exceptions **(Figure 31)**.



<u>Figure 31</u> : Highlighted modifications of PA14 proteome in response to lactonase treatment. Heat map of the $\log_{10}$ relative fold change mean for the six comparisons.

3.4 Discussion

These phenotypic and molecular studies provided the first evidence that enzyme specificity plays a crucial role in triggering QQ effects.

Overall, *Gc*L was first shown to have a broader lactonase activity and was found more efficient for reducing virulence factors at low concentrations than *Sso*Pox W263I. Conversely, *Sso*Pox W263I, with a poor lactonase activity toward C_4 HSL, was proved to completely remove biofilm while aggregates were still observable with *Gc*L even at higher concentrations. A second major difference was observed in the amoeba virulence assay where *Sso*Pox W263I was able to drastically reduce virulence whereas *Gc*L had little, if any, effect. In the antimicrobial sensitivity assay, *Sso*Pox W263I was also more efficient in increasing H_2O_2 and aminoglycosides effectiveness compared to *Gc*L. These observations were consistent with modifications observed at the molecular level.

For pyocyanin, lactonase treatments reduced the abundance of phenazine synthesis pathway enzymes PhzB, D, E, F and the known phenazine transporter MexGHI-OpmD as

compared to the control [467]. Interestingly, a higher reduction was observed with *Sso*Pox W263I than with *Gc*L on PhzB and D with a drastic decrease of PhzM (fold change of -77). This observation could be related to the difference in the PQS system as it participates to the activation of the phenazine synthesis pathway [492,493].

For biofilm formation, several proteins involved in this process were modified but only CdrB was decreased with similar fold change by both lactonases. *Gc*L induced a stronger decrease in AlgO (or Tsp) abundance as compared to *Sso*Pox W263I. This periplasmic protease is involved in the cleavage of truncated antisigma factor MucA which sequesters AlgU, a major alginate synthesis regulator. However, this truncated MucA was reported in mucoid isolates and its role in non-mucoid strain like PA14 or PAO1 is still unclear [480,494,495]. The opposite trend was observed for RahU which has been reported to be Rhl activated, binding to lipids and to modulate biofilm formation [481]. AlgU, the alginate regulator, and OprF, a pleiotropic porin also involved in biofilm formation, seemed to be reduced with *Sso*Pox W263I [477,480]. Ndk, which is related to alginate synthesis, and DppA1 related to aggregation were both increased with *Gc*L (Kim et al., 1998; Lee et al., 2018; Sundin et al., 1996). These observations could explain both the global biofilm reduction by lactonase treatment and the remaining biofilm observed with *Gc*L. Moreover, the PQS is known to be involved in biofilm formation partially through extracellular DNA release and the lower impact of *Gc*L could participate to the presence of remaining aggregates [52,250].

Regarding virulence in amoeba, higher abundance of T3SS structural and effector proteins with *Gc*L could explain the inefficiency of this lactonase to alleviate virulence in this assay. Indeed, T3SS and the T3SS-secreted toxin ExoU were reported to be highly important in *P. aeruginosa* virulence toward amoeba [280,281]. QS, more precisely Rhl and PQS systems, has been reported to have a repressing action on T3SS expression [498–501]. However, in a recent study PQS was reported as an activator of T3SS [502]. Then the differential impact on PQS could explain the modification of T3SS expression according to the lactonase used. As T3SS was not affected, or slightly, with *Sso*Pox W263I as compared to the control, other virulence mechanisms could be potentially responsible for the drastic virulence reduction observed with this lactonase. PA14_48590, PA14_05510, OprF and the phenazine associated proteins might also be involved in the drastic virulence reduction induced by *Sso*Pox W263I on amoeba, however such evidence would need to be further confirmed.

Several proteins involved in oxidative stress response, copper toxicity protection and antibiotic sensitivity harbored a modified expression level. In common, two proteins (OpdB and OprG) were increased and six others (PA14_47550, PA14_18690, ObgE, MexG, MexH and OmpD) were reduced by both treatments as compared to the control. A similar reduction was observed on the proteins involved in copper homeostasis which are related to copper toxicity protection and, for some of them, to ciprofloxacin susceptibility [474,503]. However, several protein levels differ between the two lactonases. Five proteins (OprF, OprM, HflK, OmpH and Ppk) were reduced by *Sso*Pox W263I as compared to the control and two others (OprD and OprH) were increased by *Gc*L. Furthermore, PQS was reported to modulate both oxidative

stress protection and antibiotic sensitivity and then could also participate to the difference observed in aminoglycosides and H_2O_2 sensitivity [238,252,504].

Related to QS, each lactonase induced a decreased expression of at least one component of all four QS systems according to RT-qPCR **(Figure 32)**. This confirms the interconnection of QS systems and that interfering only with AHL affect the whole four systems. Surprisingly, the Las system registered the smallest impact with only a 2-fold decrease in *lasR* expression by either single lactonase treatment. In this experimental setting, no autoinduction on *lasI* was observed. As Las is usually described to play a major role in QS hierarchy, *lasI* was maybe activated by other factors as downstream response was hijacked. Indeed, the central Rhl system was strongly impacted by both lactonases even though *Sso*Pox W263I poorly hydrolyses C_4 HSL. The IQS synthesis pathway followed the same fate and was strongly reduced by both lactonases. This is in agreement with the hierarchical regulation of the Las system on the IQS one [505]. Interestingly, reduction of AmbE was only confirmed for *Sso*Pox W263I with a significant fold change of -2.8 at the proteomic level. In the PQS system, a major difference arose: even if *pqsR* was reduced by each single lactonase, *pqsA* was only significantly and strongly reduced by *Sso*Pox W263I. This tendency was confirmed by proteomic results as PqsD protein level, present in the same operon as *pqsA*, was only reduced by *Sso*Pox W263I. Moreover, PqsE, also in encoded by *pqsABCDE* operon but not involved in PQS synthesis, was found more abundant in *Gc*L treated PA14 proteomic profile.

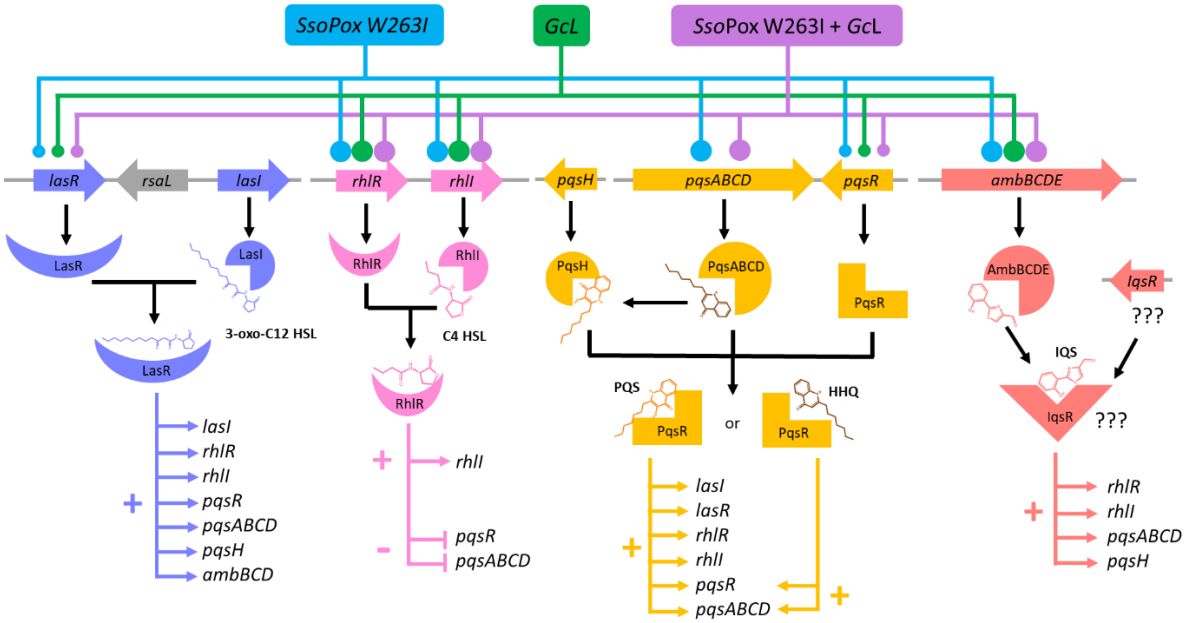


Figure 32 : Schematic representation of the four QS systems, their interconnections and the QQ lactonases impacts on them. For each enzyme treatment, a colored line with a small (fold change < 5) or big (fold change > 5) round end represents the significant induced expression reduction. The question mark indicates that the IQS receptor is not yet known. The gene *pqsH* and *rsaL* were represented but their expression level was not measured. The descriptions of interconnection between QS systems were based on information reported by [11,238,505,506].

This study indicates that the outcome of the QQ treatment is strongly driven by the QQ lactonase catalytic efficiency. Importantly, the effect on several phenotypes and thus the potency of the treatment is not equivalent depending on the enzyme used.

The impact of combined treatment demonstrated interesting effects both at the phenotypic and molecular levels. The synergistic effect observed with the combination of the two lactonases on pyocyanin, protease and biofilm could result from a higher degradation efficiency of C_4 and 3-oxo-C_{12} HSL when the enzymes work together. At lower concentrations, both Las and Rhl systems would be quenched and these phenotypes would be impacted as they are regulated by both systems [52,457]. In contrast, it is more difficult to fully explain the similar response of the combined treatment to single *Sso*Pox W263I one at high concentration. Indeed, combining the enzymes results in a degradation of C_4 and 3-oxo-C_{12} HSL closer to *Gc*L condition. Thus, the global response would have been expected to be closer to single *Gc*L treatment. However, the opposite was observed on phenotypes (full biofilm disappearance and *in vivo* virulence reduction), QS gene expression (*pqsA* level decreased) and on the proteome (closer profile to *Sso*Pox W263I one). Even if the exact mechanism remains elusive, two hypotheses seem possible to explain the *Sso*Pox W263I behavior of the combined treatment. First, a fine balance between C_4 and 3-oxo-C_{12} HSL degradation would be required for optimum QQ and would be in favor of a higher 3-oxo-C_{12} HSL hydrolysis provided by both lactonases in the combined treatment. The second possibility is that *Sso*Pox W263I, a broadly promiscuous enzyme displaying various latent activities, might interact with another unknown target involved in *P. aeruginosa* regulation and which *Gc*L is unable to act on.

Even though QS regulation varies depending on growth conditions and strains, this study provided evidences that enzymatic QQ is not equivalent depending on the enzyme specificities. More than the dose, the choice of enzyme spectrum is crucial to obtain the highest QQ efficiency on multiple phenotypes. In our conditions, *Sso*Pox W263I, a PLL, clearly stands out as the most efficient of both lactonases for biofilm, virulence and antimicrobials tolerance reduction of PA14. Moreover, the combined treatment highlighted the complexity of *P. aeruginosa* QS system and requires further investigations.

IV. Conclusions et perspectives

1. Conclusions

Ces travaux de recherche ont démontré le potentiel fondamental et appliqué de *Sso*Pox W263I pour le QQ notamment en vue d'une incorporation dans des dispositifs médicaux antivirulents comme les pansements.

L'utilisation des enzymes en biotechnologie est souvent limitée par leur faible stabilité dans leur production, stockage et utilisation. Ceci peut entrainer des coûts importants pour des applications à grandes échelles et donc bloquer leur développement. En vue du développement de pansements antivirulents, l'enzyme hyperthermostable *Sso*Pox W263I a été soumise à de nombreuses conditions extrêmes pouvant être retrouvées dans des procédés industriels. Elle a prouvé être résistante à de hautes températures, comme son origine le laissait suggérer, jusqu'à environ 90°C en liquide et 120°C en solide. Elle conserve même 10% d'activité à 150°C lorsqu'elle est atomisée. *Sso*Pox W263I a pu être stérilisée par deux méthodes industrielles avec des pertes acceptables voire nulles. L'enzyme sous forme solide a aussi gardé environ 30% d'activité après passage à l'autoclave ce qui est assez remarquable. Le stockage à long terme pendant plus de 300 jours à température ambiante n'a montré que peu d'effets sur son activité. De plus, 14 des 15 solvants organiques purs utilisés se sont montrés inoffensifs pour l'enzyme. L'effet des secrétions bactériennes auxquelles elle pourrait faire face n'impacte pas voire améliore l'activité de l'enzyme. L'amélioration est même systématique pour l'enzyme préalablement chauffée à 150°C. Une méthode simple de fixation dans des billes d'alginate permet de retenir 15% d'activité et d'avoir ainsi un système réutilisable de nombreuses fois. Enfin, l'enzyme est capable de fonctionner à -18°C ce qui élargit son spectre d'activité de -18 à 85°C [28]. Toutes ces caractéristiques démontrent la remarquable capacité de *Sso*Pox W263I à résister et fonctionner dans des conditions extrêmes. Ces résultats ont permis de lever des verrous technologiques importants quant à son utilisation dans un pansement et son incorporation dans le procédé de fabrication.

Après avoir montré sa résistance, l'efficacité de *Sso*Pox W263I sur *P. aeruginosa* a été évaluée sur deux souches modèles et 51 isolats cliniques afin de pouvoir généraliser l'effet du QQ enzymatique sur le biofilm et les facteurs de virulence (pyocyanine et protéases). *Sso*Pox W263I s'est avérée capable de réduire au moins un phénotype chez 50 souches cliniques sur les 51 utilisées. La réduction était de plus de moitié pour 71%, 77% et 86% des souches productrices, respectivement, de protéases, pyocyanine et biofilm. Aucune augmentation significative à la suite du traitement n'a été observée. Sur les 16 souches qui produisaient les trois facteurs, deux QSI, identifiés dans la littérature et utilisés seuls ou combinés, se sont montrés moins efficaces que *Sso*Pox W263I. Pour 15 des 16 souches, les QSI ont même induit une augmentation significative d'au moins un des trois facteurs mesurés. Par contraste, *Sso*Pox W263I a permis la diminution de ces phénotypes chez 13 souches, les trois autres isolats cliniques ne répondant pas pour un facteur seulement (deux sur le biofilm et une sur

la pyocyanine). Enfin, l'immobilisation de l'enzyme dans un revêtement en polyuréthane réticulé par du glutaraldéhyde a servi de preuve de concept pour le futur pansement. Sur PAO1, *Sso*Pox W263I fixée s'est montrée efficace pour diminuer de plus de 50% les trois facteurs. Ces résultats ont prouvé le potentiel de *Sso*Pox W263I pour une utilisation clinique avec un effet réducteur des facteurs de virulence et du biofilm plus prononcé que des QSI. L'immobilisation de l'enzyme a conforté l'idée d'un QQ à distance confirmant l'intérêt d'une utilisation dans un pansement.

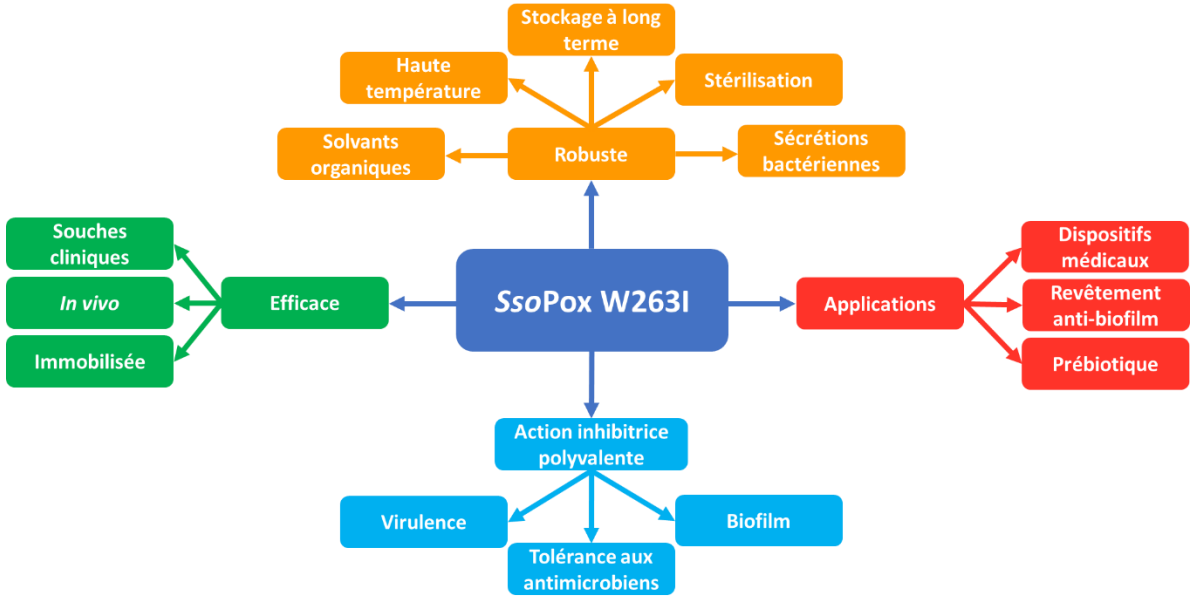


Figure 33 : **Schéma récapitulatif des caractéristiques, des effets sur *P. aeruginosa* et des applications possibles de *Sso*Pox W263I.**

Fort de ces résultats, un travail plus fondamental a été entrepris pour analyser en profondeur les effets du QQ enzymatique sur la souche PA14 de *P. aeruginosa*. Une étude comparative sur l'impact de *Sso*Pox W263I et d'une deuxième lactonase (*Gc*L), au spectre d'action distinct de la première, a permis de soulever des différences notables aux niveaux phénotypique et moléculaire. Sur la réduction des facteurs de virulence (pyocyanine, élastase, protéases), *Gc*L s'est montrée plus efficace à basse concentration. Cette différence n'était pas observable avec une dose plus élevée où les deux enzymes ont le même effet. En revanche, *Sso*Pox W263I s'est révélée capable d'inhiber complètement la formation de biofilm contrairement à *Gc*L. L'effet de *Sso*Pox W263I sur la virulence a été également démontré *in vivo* sur un modèle d'amibes, *Gc*L se montrant quant à elle inefficace. Sur la tolérance aux antimicrobiens, *Sso*Pox W263I a été plus efficace que *Gc*L et a permis de diminuer d'un facteur 10 la MBEC sur les trois antimicrobiens testés.

Au niveau protéique, les différences de phénotype ont pu être expliquées par une plus faible abondance de protéines impliquées dans la virulence, le biofilm et la résistance aux antimicrobiens par *Sso*Pox W263I. A la fois au niveau transcriptionnel et protéique, la voie de synthèse du PQS n'est pas impactée par *Gc*L alors que des effets notables ont été observés avec *Sso*Pox W263I. Comme ce système intervient dans la régulation de la virulence, du

biofilm et de la sensibilité aux antimicrobiens, cette différence est probablement liée à l'observation de phénotypes et profils protéiques distincts entre les deux lactonases [52,252]. Enfin, l'utilisation combinée des enzymes a permis de mettre en évidence un effet synergique sur la production de biofilm, de pyocyanine et des protéases. De plus, le mélange se comporte comme *Sso*Pox W263I seule en réduisant la virulence et en inhibant complètement le biofilm. Au niveau moléculaire, les profils protéiques et transcriptionnels sur les gènes du QS sont très proches de ceux obtenus avec *Sso*Pox W263I. Les raisons d'une telle similitude restent encore à définir.

L'efficacité et les propriétés physico-chimiques de *Sso*Pox W263I sont très avantageuses pour une utilisation du QQ dans des dispositifs médicaux tels que les pansements **(Figure 33)**. Elle semble se distinguer vis-à-vis des QSI avec une plus grande efficacité sur des souches modèles et cliniques. Son spectre d'action s'est révélé plus performant pour le QQ de *P. aeruginosa*, dans les conditions testées, qu'une autre lactonase thermostable plus efficace sur les AHL à chaine courte.

2. Perspectives

Suite à ces travaux, de nombreuses voies sont encore ouvertes pour des avancées applicatives et fondamentales pour l'utilisation de *Sso*Pox W263I pour inhiber le QS.

La raison qui explique la différence entre l'efficacité de *Sso*Pox W263I et *Gc*L pour le QQ reste encore inconnue et requiert donc des études supplémentaires. D'une part, il faudrait tester le mélange avec différents ratios de *Sso*Pox W263I/*Gc*L afin de mieux discerner la contribution de chaque enzyme. De plus, *Sso*Pox possède une activité de promiscuité phosphotriestérase ainsi que d'autres plus marginales (arylestérase, phosphodiestérase) [22,23]. L'utilisation de l'enzyme sauvage ou d'autres variants de *Sso*Pox, spécialisés sur son activité de promiscuité (phosphotriesterase) et inactifs sur les C_4 et 3-oxo-C_{12} HSL, pourrait permettre de savoir si l'effet n'est pas spécifique au variant W263I ou si son activité de promiscuité serait la cause de cette différence. L'utilisation d'autres lactonases appartenant à la famille des PLL ou MLL permettrait de voir si l'effet se généralise avec d'autres enzymes du même type que *Sso*Pox ou *Gc*L. Enfin, une approche globale comme de la métabolomique pourrait permettre de définir la modification de la composition du surnageant et éventuellement de déterminer la ou les molécules qui pourraient être substrat de *Sso*Pox W263I et non de *Gc*L.

Dans le cadre du développement des pansements antivirulents, l'innocuité cutanée a été démontrée chez le rat et sur de la peau humaine reconstituée lors de tests d'irritation réalisés par un laboratoire indépendant. Par la suite, il reste à démontrer l'efficacité de *Sso*Pox W263I libre et surtout fixée dans un pansement dans un modèle d'infection de plaie à *P. aeruginosa in vivo* d'infection (e.g. murin). En plus des pansements, d'autres dispositifs médicaux pourraient bénéficier de l'utilisation de *Sso*Pox W263I pour prévenir les infections à cette bactérie qui représente un fardeau sanitaire et économique. Parmi les IAS, *P. aeruginosa* est la 2 ou 3ème bactérie la plus fréquemment retrouvée lors d'infections urinaires liées à l'utilisation d'un cathéter aux Etats-Unis sur la période de 2009 à 2014 avec plus de 10% du total des infections dont environ 15% de souches multirésistantes [128,458]. Ainsi l'utilisation de cathéters fonctionnalisés avec *Sso*Pox pourrait permettre de réduire la colonisation de cette surface par la bactérie et ainsi prévenir des infections et donc limiter le recourt aux antibiotiques.

P. aeruginosa est aussi la bactérie pathogène la plus fréquemment retrouvée lors infections pulmonaires chez les patients atteints de mucoviscidose [507]. Comme il a été précédemment démontré, *Sso*Pox W263I est capable *in vivo* chez le rat de réduire la mortalité en prévenant l'infection pulmonaire à *P. aeruginosa* [30]. Ainsi le développement d'un médicament à base *Sso*Pox W263I, sous forme d'aérosol par exemple, serait un complément thérapeutique aux antibiotiques pour traiter les infections pulmonaires chez ces patients. En plus, ces travaux de thèse renforcent l'idée d'une synergie entre les deux thérapies ce qui

pourrait permettre de réduire les doses d'antibiotiques utilisées. Néanmoins le développement d'un médicament requiert des phases cliniques plus longues et coûteuses que celui d'un dispositif médical qui présente la solution actuelle la plus rapide pour une mise sur le marché par la start up Gene&GreenTK.

En parallèle des antibiotiques, la phagothérapie pourrait bénéficier des effets de *Sso*Pox W263I sur le QS bactérien. En effet, le lien entre la régulation du système de défense contre les phages, CRISPR/Cas, et le QS a été mis en évidence chez *P. aeruginosa* et *S. marcescens* [191,271]. Une étude a également montré l'influence globale du QS sur la sensibilité aux bactériophages [265]. Comme l'utilisation de phages, dans le domaine médical, connait un regain d'intérêt pour faire face aux antibiorésistances, *Sso*Pox W263I pourrait permettre à la fois de réduire le biofilm, la tolérance aux antimicrobiens et aussi sensibiliser aux attaques de phages. L'effet sensibilisateur du QQ enzymatique au traitement par des phages isolés ou des cocktails commerciaux mériterait donc d'être étudié pour valider si une synergie entre phagothérapie et QQ existe.

Outre l'action sur *P. aeruginosa*, *Sso*Pox pourrait être utilisée pour inhiber la virulence d'autres bactéries pathogènes de l'homme utilisant des AHL. Par exemple, *A. baumannii, S. marsescens* ou *B. cepacia* sont aussi des bactéries représentant un potentiel infectieux non négligeable et dont certaines sont concernées par un haut taux de résistance [199,428]. Le QQ pourrait également avoir d'autres applications dans d'autres domaines comme l'agriculture et l'élevage. En effet, de nombreuses bactéries pathogènes des plantes ou des animaux utilisent des systèmes de communication basés sur des AHL ou des lactones (e.g. *Vibrio spp., Aeromonas spp, A. tumefaciens, Pectobacterium spp., …*) [47]. Récemment, un article a également montré l'efficacité d'une lactonase sur une bactérie pathogène de plante à Gram positif (*Streptomyces scabies*) utilisant une γ-butyrolactone pour son système de QS [508]. Pour déterminer tout le potentiel applicatif du QQ par *Sso*Pox W263I il serait nécessaire de définir le spectre d'action sur les bactéries de cette lactonase. La mise en place de tests phénotypiques pour chacune de ces bactéries est envisageable mais peut se révéler très fastidieux pour déterminer les bonnes conditions de culture et les tests phénotypiques peuvent ne pas être révélateurs de la virulence. Des approches moléculaires telles que la RT-qPCR sur certains gènes de la virulence pourrait également constituer une alternative intéressante. Cependant, cette approche est limitée aux bactéries pour lesquelles le QS a déjà été rapporté et étudié pour pouvoir identifier des gènes candidats. Des approches globales comme la protéomique différentielle pourrait permettre justement de déterminer rapidement un effet sur la bactérie. L'utilisation de *P. aeruginosa,* une bactérie très bien caractérisée, a prouvé que cette technique permet de distinguer aisément l'impact du traitement et de distinguer les différences de QQ liées à l'enzyme utilisée. Ceci en fait un outil très puissant pour l'utilisation de ces enzymes sur des espèces bactériennes dont le système de QS n'est pas aussi bien défini que chez *P. aeruginosa*. Ainsi la protéomique différentielle

pourrait permettre d'évaluer l'impact d'une enzyme sur le comportement bactérien et d'orienter le choix vers l'enzyme la plus appropriée pour le QQ.

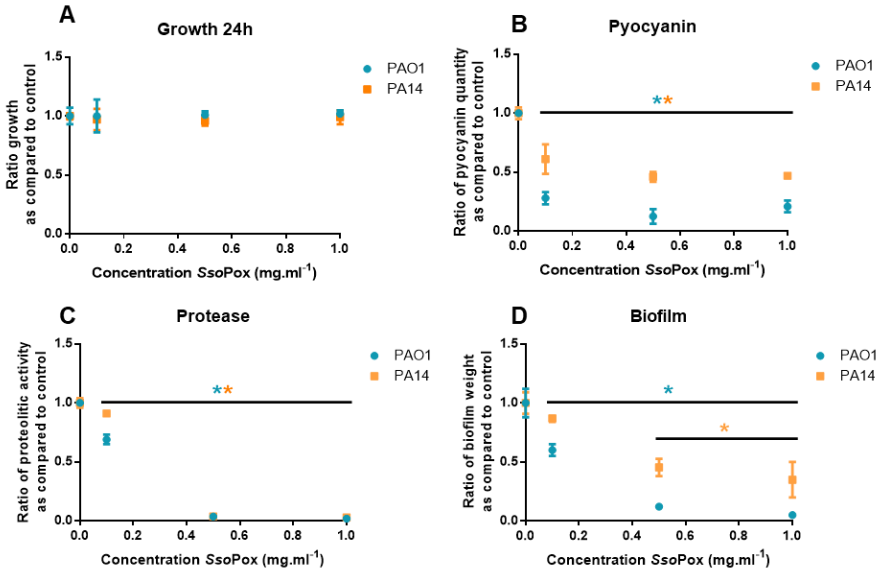
Pour se rapprocher encore plus des conditions naturelles, une autre perspective est de l'étude et l'utilisation du QS et QQ dans des interactions plus complexes comme celles retrouvées dans les microbiotes. Des études récentes semblent indiquer les effets bénéfiques de la modulation du QS sur la distribution des communautés bactériennes complexes [19,20,509]. Le QQ pourrait être ainsi utilisé comme pré ou probiotique pour prévenir l'installation et le développement de bactérie(s) pathogène(s). Cependant, les données disponibles sur le sujet sont limitées et il reste encore beaucoup à déterminer sur le rôle du QS, mais aussi du QQ, dans des écosystèmes aussi complexes. Ainsi des expériences sur des *inocula* complexes de bactéries (e.g. écouvillons de plaies infectées) ou directement sur l'utilisation de modèles *in vivo* permettrait de gagner en connaissance sur ce domaine.

Des points limitant et verrous technologiques restent à élucider quant à l'utilisation du QQ enzymatique. Comme abordé dans ce travail de thèse, toutes les lactonases ne sont pas équivalentes pour obtenir un QQ optimal. Ainsi l'efficacité et la spécificité de la ou les enzymes utilisées seraient donc des paramètres à choisir avec attention afin d'obtenir les effets les plus importants. Le potentiel anti-virulence du QQ est aujourd'hui bien établi mais son potentiel anti-infectieux doit être encore étudié lors d'infections aigues ou chroniques déjà établies. Enfin l'apparition, la fréquence et les mécanismes de résistance à des traitements de type QQ restent encore à comprendre surtout suite à l'utilisation d'enzyme comme les lactonases. L'apparition et le maintien de bactéries qui ne répondent plus aux QS ou « social cheaters » sont des aspects qui sont encore mal estimés particulièrement *in vivo* et dans un contexte multi microbien. Le QQ enzymatique est décrit comme imposant une faible pression de sélection car il n'a pas d'effet bactéricide seul. En revanche, l'utilisation conjointe du QQ avec une antibiothérapie ou *in vivo* (système immunitaire) pourrait augmenter la sensibilité des bactéries à de telles attaques. Ainsi l'apparition de résistance au QQ pourrait apparaître et plusieurs mécanismes de résistance sont envisageables. Par exemple, une bactérie pourrait sécréter plus d'autoinducteurs, utiliser des autoinducteurs avec une structure chimique modifiée ou augmenter l'affinité des récepteurs. Cependant tous ces mécanismes pourraient être facilement contournés par l'utilisation de plus d'enzyme QQ ou avec des activités plus importantes et plus variées. En comparaison, l'apparition de résistance aux QSI peut être plus problématique notamment à cause de leur spécificité incertaine et leur potentiel effet bactéricide suivant la concentration. En plus, les mécanismes de résistance sont probablement plus fréquents et plus difficiles à contourner que ceux du QQ enzymatique : un QSI peut perdre de son efficacité suite à un efflux plus important ou à une modification de sa cible. Parmi ces mécanisme, l'efflux a déjà été rapporté chez *P. aeruginosa* [161]. Afin d'explorer ces phénomènes de résistance au QQ, des expériences *in vitro* sur plusieurs générations pourraient permettre de déterminer la fréquence d'apparition de tels mécanismes avec et sans agent ayant une forte pression de sélection comme un antibiotique.

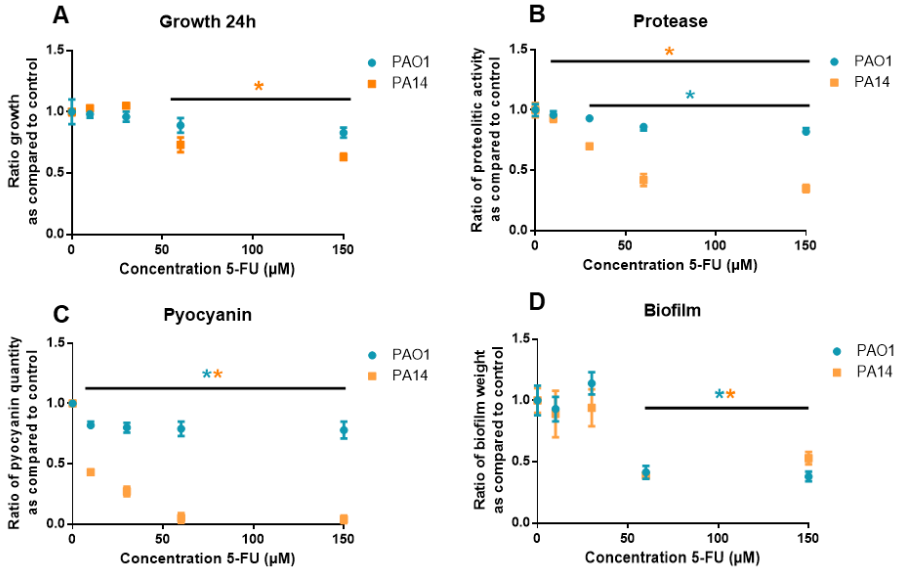
Le QQ enzymatique a de nombreuses applications potentielles dans des secteurs très variés en plus de la santé humaine [47] **(Figure 33)**. De la nutrition animale à l'agriculture en passant par l'antifouling, les possibilités sont nombreuses et le potentiel de l'approche reste encore à être exploité. *Sso*Pox est une candidate idéale pour le QQ par sa grande stabilité et résistance. De plus, c'est une enzyme aux possibilités multiples non seulement pour le QQ avec son activité lactonase mais aussi pour des applications liées à son activité de promiscuité, phosphotriestérase, dans le domaine de l'environnement (dégradation d'insecticides) et de la défense (dégradation d'agent chimique de guerre) [396].

V. Annexes

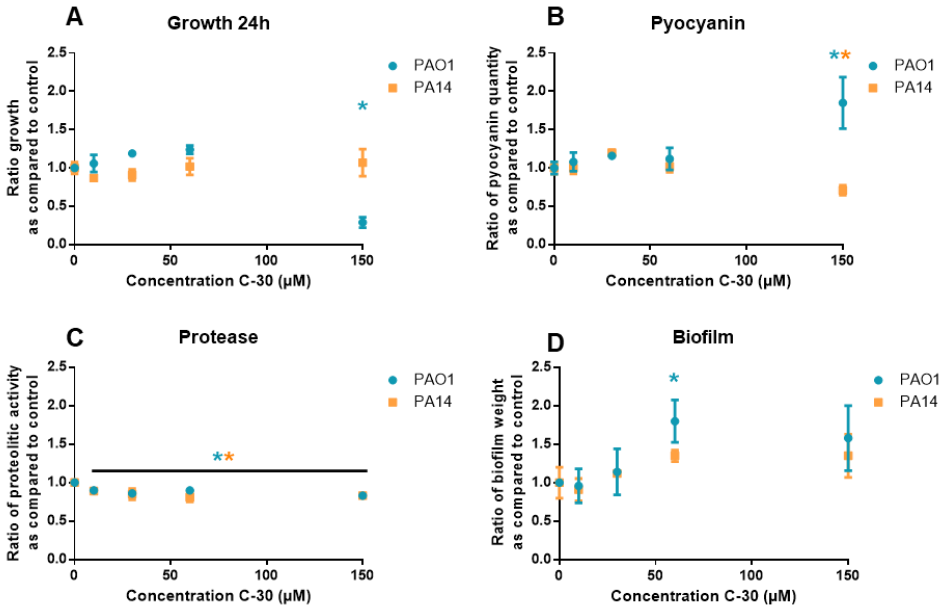
1.1 Projet n°2 : Effet du variant W263I de *Sso*Pox sur des souches cliniques de *Pseudomonas aeruginosa*



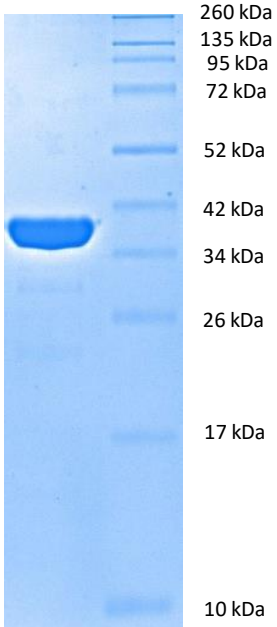
<u>Supplementary figure 1</u> : Dose response of SsoPox with the model strains PAO1 and PA14. Three SsoPox concentrations (0.1 mg/mL, 0.5 mg/mL, 1.0 mg/mL) were used. Values represent the mean ratios between treated and untreated samples of three experiments. The effect of the QQ enzyme on growth, pyocyanin secretion, protease activity and biofilm formation was evaluated. Stars indicate a p-value<0.05 according to Student's t-test.



<u>Supplementary figure 2</u> : Dose response of 5-FU with the model strains PAO1 and PA14. Four 5-FU concentrations (10 µM, 30 µM, 60 µM, 150 µM) were used. Values represent the mean ratios between treated and untreated samples of three experiments. The effect of QSI on growth, pyocyanin secretion, protease activity and biofilm formation was evaluated. Stars indicate a *p-value*<0.05 according to Student's t-test.

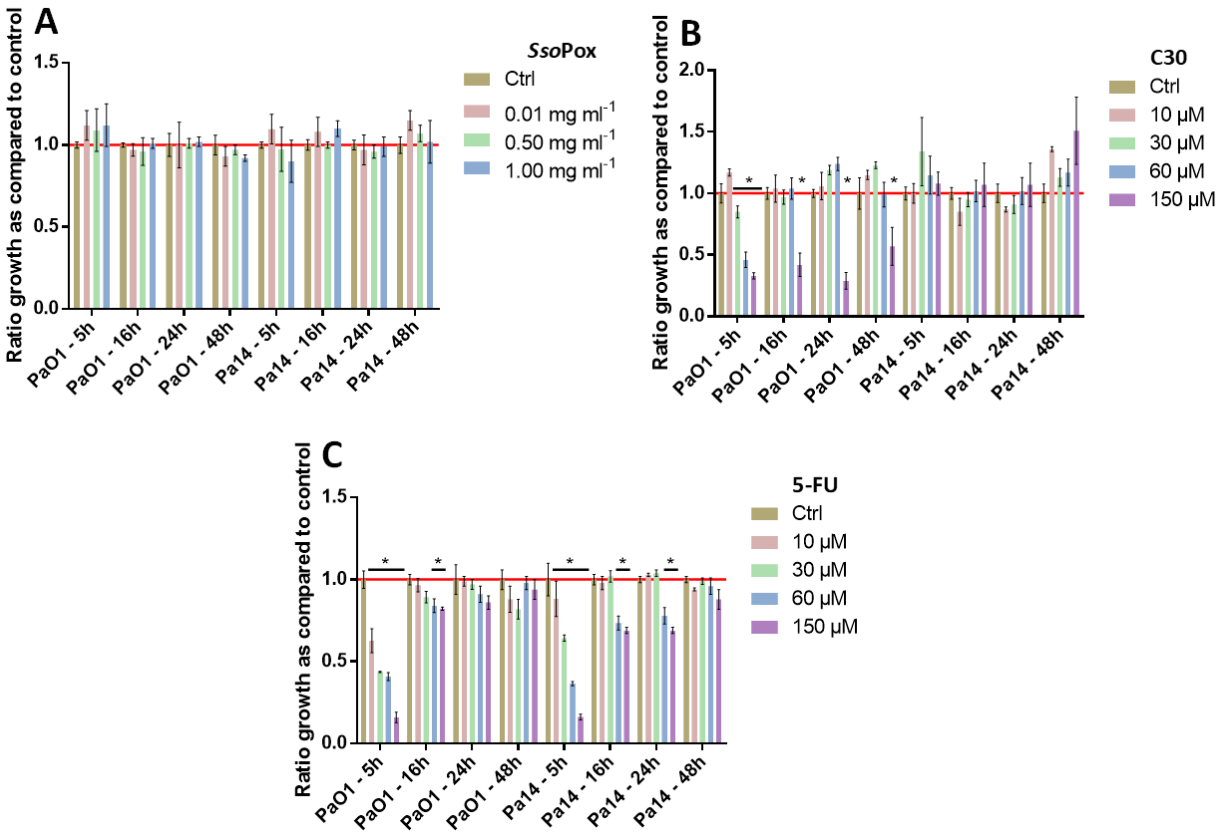


<u>Supplementary figure 3</u> : Dose response of C-30 with the model strains PAO1 and PA14. Four C-30 concentrations (10 µM, 30 µM, 60 µM, 150 µM) were used. Values represent the mean ratios between treated and untreated samples of three experiments. The effect of QSI on growth, pyocyanin secretion, protease activity and biofilm formation was evaluated. Stars indicate a *p-value*<0.05 according to Student's t-test.



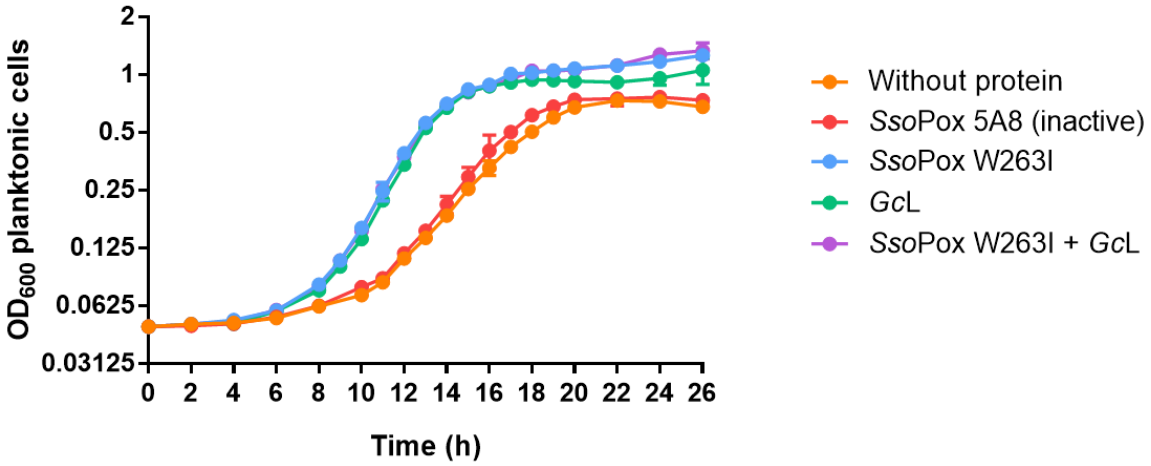
<u>Supplementary figure 4</u> : SDS-page of SsoPox-W263I obtained after gel filtration purification.

<u>Supplementary figure 5</u> : Direct measurement of biofilm by direct weighing of cell aggregates. After 48 h of growth, cells were filtered through a 100 µm cell strainer to separate planktonic cells from biofilms. Biofilms were directly weighed in the cell strainers. Left picture represents a culture without *Sso*Pox while right picture represents a culture treated with the enzyme.

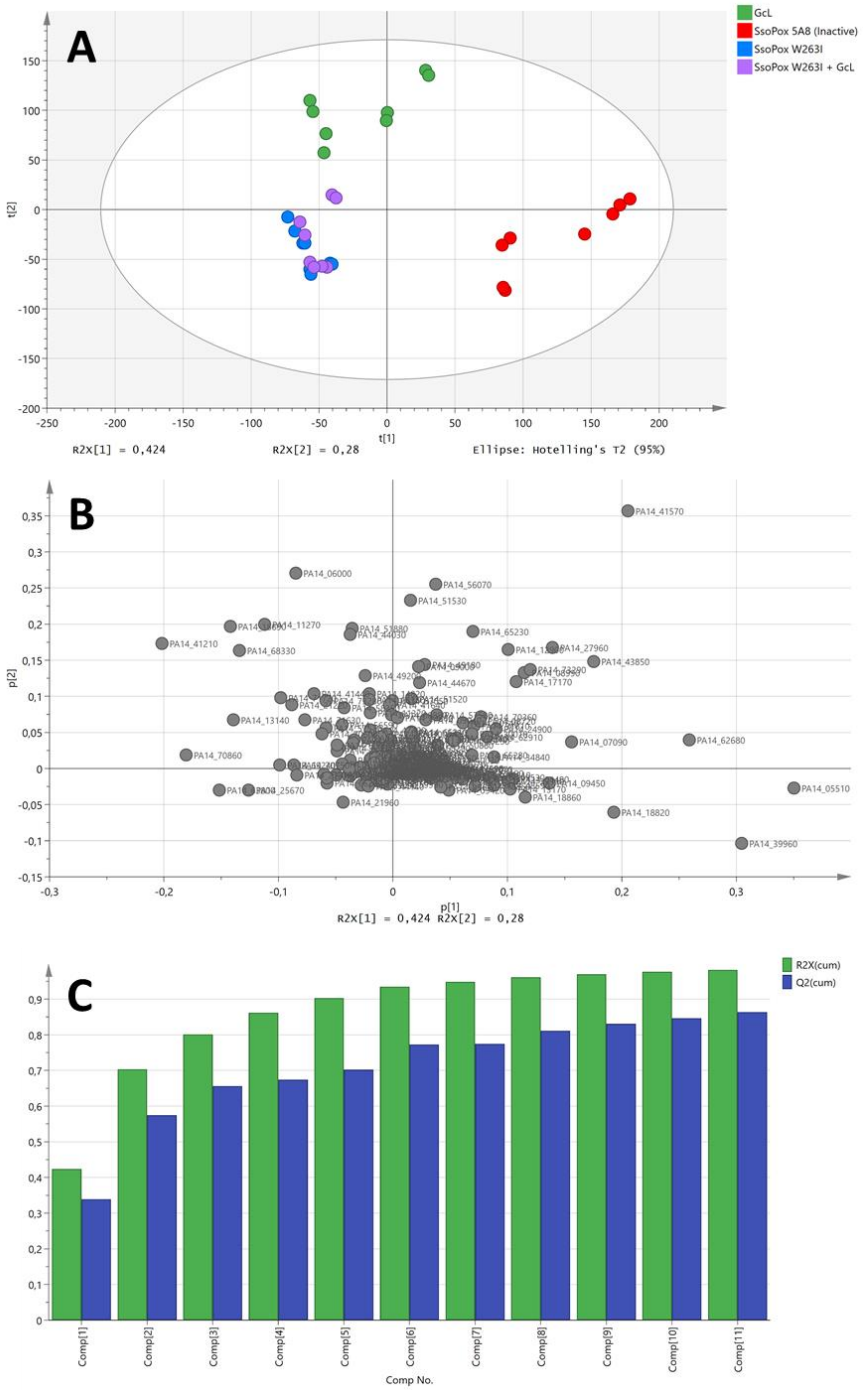


<u>Supplementary figure 6</u> : Impact of QQ enzyme and QSI on bacterial growth. The impact of *Sso*Pox (A), C30 (B) and 5-FU (C) on bacterial growth of PAO1 and PA14 for different concentrations.

1.2 Projet n°3 : Etude comparative de l'impact sur P. aeruginosa de lactonases aux spectres d'action différents



Supplementary figure 7 : Growth curve in Napierian logarithmic representation. All n=4 independent samples are represented by the mean and standard deviation in corresponding colored bars.



<u>Supplementary figure 8</u> : Principal component analysis (PCA) of each condition according to the 210 changed proteins. A) Score scatter plot and B) Loading scatter plot of the two first components. C) Histogram of the cumulative R^2X and Q^2 values of the 11 obtained components. The duplicate of the n=4 independent samples were used for the PCA analysis.

Supplementary Table 1 : Primers used in this study.

Primer	Sequence 5'-3'
lasI_F	AGTGTCATCGACGAGATGGA
lasI_R	CTGGAACAGGGTGGTGAAAT
lasR_F	TCGGTTATCTGCAACTGCTC
lasR_R	GACCCAAATTAACGGCCATA
pqsA_F	AACACGCTCGGATTCTGTCG
pqsA_R	GGGAATCGAATACAGCCGGT
pqsR_F	CAGCGTACTGCTCGACGATT
pqsR_R	TTCCGCGTTGTCCTGCTTGA
ambB_F	ACGAAGAGCGCCGTTTGCA
ambB_R	CCTCGAACAGATGGTGGAGT
recA_F	CGCAAGATCACCGGCAATATCA
recA_R	GGACCGAGGCGTAGAACTTC

Supplementary Table 2 : All proteomic data set. Gene ID and name, localization, operon and PseudoCAP function were reported from http://www.uniprot.org/ and http://www.pseudomonas.com/ [510].

https://drive.google.com/open?id=1PN7r7J0VvXr3KwxLf132ncHrd7iXnLk4

2.1 Revue française n°1 : Des enzymes pour bloquer la communication bactérienne, une alternative aux antibiotiques ?

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REVUE GÉNÉRALE

Des enzymes pour bloquer la communication bactérienne, une alternative aux antibiotiques ?

Enzymes for disrupting bacterial communication, an alternative to antibiotics?

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MOTS CLÉS
Quorum sensing ;
Virulence bactérienne ;
Quorum quenching ;
Biofilm ;
Lactonase

Résumé De nombreuses bactéries utilisent un système de communication, appelé *quorum sensing* (QS), pour échanger de l'information et synchroniser leur comportement proportionnellement à leur densité de population. Pour cela, elles utilisent des molécules médiatrices, les auto-inducteurs (AI), sécrétées dans l'environnement pour se signaler les unes aux autres et réguler l'expression de certains gènes. Les caractères phénotypiques régulés par le QS sont multiples mais certains tels que la pathogénicité, la formation de biofilm ou la résistance aux agents antibactériens sont particulièrement problématiques. Cibler le QS afin de bloquer la communication bactérienne constitue une approche prometteuse pour contrôler les bactéries. Cette stratégie, appelée *quorum quenching* (QQ), peut être réalisée en utilisant des molécules inhibitrices du QS ou des enzymes dégradant les AI. Le QQ présente un fort intérêt car, contrairement aux antibiotiques, il n'induit pas la mort de la bactérie mais l'empêche simplement d'adopter certains phénotypes comme la virulence. Même si la pression de sélection appliquée n'est pas nulle, elle n'en reste pas moins faible comparée aux méthodes bactéricides et devrait limiter l'apparition de phénomène de résistance. L'utilisation d'enzymes est notamment prometteuse car elles peuvent être utilisées, de manière extracellulaire et en quantité

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catalytique, pour dégrader les molécules médiatrices sécrétées dans l'environnement. Cette revue dresse un inventaire des différentes applications médicales du QQ par voie enzymatique avant de s'intéresser aux éventuels phénomènes de résistance qui pourraient émerger.

KEYWORDS
Quorum sensing;
Bacterial virulence;
Quorum quenching;
Biofilm;
Lactonase

Summary Quorum sensing (QS) is used by bacteria to communicate and synchronize their actions according to the cell density. In this way, they produce and secrete in the surrounding environment small molecules dubbed autoinducers (AIs) that regulate the expression of certain genes. The phenotypic traits regulated by QS are diverse and include pathogenicity, biofilm formation or resistance to anti-microbial treatments. The strategy, aiming at disrupting QS, known as quorum quenching (QQ), has emerged to counteract bacterial virulence and involves QS-inhibitors (QSI) or QQ-enzymes degrading AIs. Differently from antibiotics, QQ aims at blocking cell signaling and does not alter bacterial survival. This considerably decreases the selection pressure as compared to bactericide treatments and may reduce the occurrence of resistance mechanisms. QQ-enzymes are particularly appealing as they may disrupt molecular QS-signal without entering the cell and in a catalytic way. This review covers several aspects of QQ-based medical applications and the potential subsequent emergence of resistance is discussed.

Introduction

La communication bactérienne, appelée *quorum sensing* (QS), est un mécanisme moléculaire permettant aux bactéries d'adapter leur comportement en fonction de la densité de population [1]. Les bactéries produisent des molécules médiatrices, les auto-inducteurs (AI), qui sont sécrétées dans l'environnement et sont perçues par les récepteurs spécifiques des bactéries voisines. Ce mécanisme régule l'expression de nombreux gènes et a fait l'objet de plusieurs revues [2,3]. La réponse des bactéries au QS varie selon les microorganismes, mais certains traits comme la motilité, la production de divers facteurs (sidérophores, exo-polysaccharides, exo-enzymes, antibiotiques), les systèmes de sécrétion et la formation du biofilm semblent communs.

Différents AI du QS ont été identifiés. Les bactéries à Gram positif utilisent principalement des peptides AI, aussi nommés peptide-phéromones, spécifiques des différentes souches et espèces. Les bactéries à Gram négatif utilisent, quant à elles, différentes molécules régulant plusieurs types de QS :

- les acylhomosérines lactones (AHL) également désignées AI-1, composées d'un cycle lactone et d'une chaîne aliphatique dont la longueur varie selon les espèces bactériennes [4] ;
- l'auto-inducteur de type 2 (AI-2), rencontré chez de nombreuses bactéries à Gram négatif et positives principalement sous la forme d'un diester furanosyl-borate [5] ;

AI-3, l'adrénaline ou la noradrénaline sont fréquemment rencontrés chez les pathogènes opportunistes de l'homme (e.g. *Enterobacter* sp., *Escherichia* sp., *Klebsiella* sp., *Salmonella* sp.) [6] ;

- d'autres médiateurs incluant des quinolones (*Pseudomonas* spp.), des acides gras (*Xanthomonas* spp.), des esters (*Ralstonia* sp.), des hydroxycétones (*Legionella* spp., *Vibrio* spp.) ont également été décrits [7—10].

La plupart des bactéries à Gram négatif utilisent plusieurs systèmes de QS de manière additive [11,12], hiérarchisée [10], ou distincte [13]. Compte tenu de la grande diversité des réseaux de signalisation, le QS est probablement plus qu'un simple système de communication permettant également aux bactéries de percevoir et s'adapter à leur environnement [14].

Les bactéries pathogènes opportunistes de par leur rapidité de dissémination et leur capacité à acquérir des résistances aux traitements antibactériens classiques sont particulièrement problématiques. Elles sont notamment responsables d'une augmentation du risque de mortalité induisant des infections associées aux soins et engendrent d'importants surcoûts pour les systèmes de soins de santé [15]. Chez la plupart de ces bactéries, le QS est responsable de la transition du stade commensal/saprophyte au stade pathogène. C'est, par exemple, le cas du pathogène opportuniste *Pseudomonas aeruginosa*, naturellement présente dans l'eau et les environnements humides. *P. aeruginosa* est en effet capable, dans un environnement favorable telle qu'une plaie, de proliférer et d'adapter son comportement grâce à l'accumulation des AI et au QS, elle peut alors devenir pathogène et former un biofilm qui rend l'infection d'autant plus difficile à traiter [16].

Perturber le QS, pour limiter la pathogénicité des bactéries et leur capacité à former un biofilm, s'avère

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particulièrement prometteur. Cette stratégie, le *quorum quenching* (QQ), a émergé dans les années 2000 grâce à l'identification d'une enzyme capable de dégrader les AHL [17]. Deux approches peuvent être considérées, la première consistant à empêcher les bactéries de produire ou percevoir les AI par l'utilisation d'inhibiteurs, la seconde à dégrader ces molécules par voie enzymatique. Parmi les inhibiteurs de QS, les furanones halogénées, identifiées pour la première fois chez la macroalgue rouge *Delisea pulchra*, sont les plus étudiées [18]. Bien que de nombreux criblages aient été réalisés pour élargir la gamme des inhibiteurs disponibles, peu de composés ont dépassé la phase de laboratoire [19,20]. Seul le 5-fluorouracile a atteint la phase clinique et a été utilisé pour fonctionnaliser des cathéters [21]. Le QQ par voie enzymatique repose, quant à lui, sur l'utilisation de biocatalyseurs capables de dégrader les signaux moléculaires de la communication bactérienne [22]. À l'instar des inhibiteurs, la plupart des enzymes n'ont été testées qu'en laboratoire. Récemment des enzymes ont pu également être incorporées dans des cathéters pour empêcher la formation du biofilm de *P. aeruginosa* [23,24]. Pour l'instant les principales limitations de cette stratégie reposent sur la spécificité des enzymes pour dégrader un large spectre d'AI ainsi que leur piètre stabilité qui limite leur potentiel applicatif.

Cette revue recense les applications du QQ par voie enzymatique. Les avantages et les limitations de cette technique sont présentés à la lumière de résultats récents. Une attention particulière est dédiée aux enzymes de la famille des *Phosphotriesterase-like lactonases* (PLL). En effet, contrairement aux enzymes mésophiles peu stables, de nombreuses PLL sont issues de microorganismes extrêmophiles, ce qui leur confère une très grande robustesse et une compatibilité avec certaines contraintes industrielles qui leur ouvre un large champ d'applications possibles. Enfin, les éventuels mécanismes de résistance au QQ sont discutés.

Applications médicales du QQ

Les bactéries utilisant le QS pour déclencher la virulence sont particulièrement problématiques dans le secteur médical. Aux Etats-Unis, *P. aeruginosa* est responsable à elle seule de 7,5 % des infections nosocomiales. Les bactéries *Proteus* spp., *Serratia* spp. et *Acinetobacter baumannii* représentent 6,4 % de ces infections. Ces pathogènes sont fréquemment isolés dans les infections urinaires liées à l'utilisation de cathéters ou les infections pulmonaires [25]. Les enzymes capables de dégrader les AHL sont donc particulièrement pertinentes pour limiter les facteurs induits par le QS qui participe entre autres à la formation du biofilm, la sécrétion des facteurs de virulence, la compétence ou encore à la résistance aux agents antimicrobiens [26]. Les dispositifs médicaux fonctionnalisés avec des enzymes ont donc été considérés.

Les membranes fonctionnalisées

L'immobilisation de la PLL hyperthermostable *Sso*Pox issue de l'archée *Sulfolobus solfataricus* sur des membranes de nano-alumine a été étudiée pour bloquer la communication bactérienne [27]. La fixation s'effectue via les interactions électrostatique entre le support et l'enzyme. Peu de relargage a été détecté via des mesures d'activités et de quantité protéique dans les solutions de rinçage. Avec environ 25 % de l'activité initiale fixée, il est possible de diminuer drastiquement l'expression de la pyocyanine et l'activité élastase d'une culture de *P. aeruginosa* PAO1. Pour la première fois l'action d'une enzyme immobilisée pour le blocage de la communication bactérienne a ainsi été démontrée [27]. Ce résultat ouvre la porte à un large champ d'applications dans le secteur des dispositifs médicaux.

Les cathéters fonctionnalisés

La persistance des pathogènes dans les cathéters est extrêmement problématique pour la santé des patients et engendre des coûts importants [28]. Afin de limiter ces problèmes, un cathéter fonctionnalisé par le 5-fluorouracile a été développé et évalué cliniquement [21]. L'étude portant sur 960 adultes répartis dans 25 centres médicaux américains a montré que les cathéters basés sur le QQ constituent une alternative prometteuse aux dispositifs fonctionnalisés par la chlorhexidine ou la sulfadiazine d'argent. Fort de ces observations, de nouveaux cathéters à base d'enzymes ont été développés. Un cathéter en silicone, fonctionnalisé par l'acylase d'*Aspergillus melleus*, a été développé et a permis de réduire l'adhérence de *P. aeruginosa* ATCC 10145 [23]. La quantité de biofilm a également été diminuée dans des modèles statiques et dynamiques. L'innocuité du cathéter sur des cultures de fibroblastes a également été mise en évidence [23]. Plus récemment, un cathéter urinaire couplant une acylase et une α-amylase a été décrit [24]. Il consiste en un cathéter de silicone recouvert par une alternance de couches d'enzymes et de polyéthylènimine liées par des interactions électrostatiques, cependant aucune mention n'est faite sur le relargage potentiel de l'enzyme. L'efficacité in vivo de ce dispositif a été démontrée ainsi que sa capacité à retarder la formation du biofilm par *Escherichia coli*, *Staphylococcus aureus* et *P. aeruginosa* jusqu'à 7 j.

Application topique et perspective de pansements fonctionnalisés

Un modèle de brûlure chez la souris infectée par *P. aeruginosa* PAO1 a été mis en place pour évaluer l'efficacité de la lactonase de *Bacillus* sp. ZA12 [29]. Les animaux ont été brûlés au troisième degré avant d'être infectés en voie sous-cutanée par une dose létale correspondant à 10^6 bactéries. L'application topique d'un gel contenant la lactonase a permis de prévenir une infection systémique par la bactérie réduisant ainsi la mortalité. La combinaison de la lactonase avec un antibiotique, la ciprofloxacine, a permis de guérir les souris soulignant l'action synergique des deux traitements. Pour la première fois, l'efficacité d'une enzyme à activité QQ, administrée par voie cutanée pour limiter l'infection d'une plaie, a été démontrée ouvrant de nombreuses perspectives pour le développement de pansement anti-infectieux.

Traitement des infections pulmonaires

L'effet in vivo d'un variant de l'enzyme *Sso*Pox issue d'expériences d'évolution dirigée administrée par voie

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intratrachéale a été étudié [30]. Un modèle d'infection pulmonaire à *P. aeruginosa* PAO1 chez le rat a permis d'évaluer l'effet de l'administration concomitante ou retardée de l'enzyme. La mortalité a ainsi été réduite de 75 % jusqu'à 20 % dans ce modèle. La capacité de l'enzyme à inhiber la formation du biofilm de la bactérie in vitro, à hauteur de 65 %, a été mise en évidence. La lactonase administrée s'est avérée particulièrement efficace pour bloquer la virulence de *P. aeruginosa*, notamment rencontrée chez les patients atteints de la mucoviscidose.

L'utilisation du QQ constitue une alternative prometteuse aux agents antibactériens classiquement utilisés comme les antibiotiques et antiseptiques. Les enzymes dégradant les AHL ont été largement décrites comme limitant la virulence et la formation du biofilm. Bien que des études toxicologiques ont pu démontrer que l'utilisation d'une lactonase n'induit pas d'inflammation ni de réponse cytokinique en application topique ou en injection intrapéritonéale [29], la plupart des enzymes utilisées dans des stratégies de QQ ne sont pas humaines ni humanisées, il se peut qu'elles entraînent des réactions allergiques ou immunitaires. Cependant, de nombreuses techniques sont connues pour diminuer l'allergénicité des protéines. Classiquement, la PEGylation est une technique simple qui consiste à masquer des molécules bioactives en leur attachant des chaînes de polyéthylène glycol (PEG). Cette technique peu agressive permet de diminuer la toxicité et l'immunogénicité des protéines tout en améliorant de nombreux paramètres pharmacocinétiques de ces dernières [31,32]. Une autre approche consiste à piéger les enzymes dans des liposomes ce qui permet de cibler les zones à traiter et de réduire l'immunogénicité des enzymes [33]. Cette technique a notamment été utilisée avec des oxydases dans des tests d'élimination de biofilm in vitro. Enfin, une technique plus récente consiste à utiliser les globules rouges du patient comme transporteur d'enzyme réduisant presque entièrement les risques de rejet de la part de ce dernier [34]. Ces techniques, appliquées aux enzymes à QQ, permettront de développer de nouveaux dispositifs médicaux et ouvriront la voie vers des traitements prophylactiques et thérapeutiques contre les infections bactériennes.

Néanmoins, de nombreux verrous technologiques doivent être levés afin de valider le potentiel des enzymes. La production à grande échelle, ainsi que la stabilité et compatibilité avec les contraintes industrielles doivent faire l'objet d'une attention particulière. Dans cette optique, des enzymes robustes issues d'environnements extrêmes ont été plus particulièrement étudiées. Parmi celles-ci, les PLL s'avèrent être des candidats prometteurs.

Phosphotriesterase-Like Lactonases

Des enzymes à large promiscuité

Les PLL sont des lactonases naturelles (EC 3.1.1.25) montrant une activité de promiscuité sur les molécules organophosphorées. Ces enzymes sont très proches des phosphotriestérases (PTE) dont elles constituent le plus probable ancêtre [35—37]. Elles appartiennent à la superfamille des amidohydrolases et leur structure tridimensionnelle est formée par un tonneau $(\beta/\alpha)_8$. Elles comportent un site actif contenant deux cations métalliques divalents coordonnés par quatre histidines, un acide aspartique et une lysine carboxylée [38]. Le centre bi-métallique participe à la catalyse en tant qu'acide de Lewis impliqué dans l'activation d'une molécule d'eau en ion hydroxyde pour l'attaque nucléophile du substrat. Deux sous-familles, les PLL-A et PLL-B, ont été identifiées sur la base de leur similarité de séquences et de la longueur de deux boucles caractéristiques, les boucles 7 et 8. Les PLL-A sont capables de dégrader les AHL et les oxo-lactones contrairement aux PLL-B spécifiques des oxo-lactones [39]. La capacité des PLL-A à dégrader les AHL est particulièrement intéressante pour le développement de stratégies basées sur le QQ. Cette sous-famille compte parmi ses représentants de nombreuses enzymes issues d'organismes extrêmophiles qui présentent un fort potentiel applicatif. Les lactonases *Vmo*Lac (issue de la crénarchée extrêmophile *Vulcaniseta moutnovskia*) [39,40], *Gk*L (issue de la bactérie thermophile *Geobacillus kaustophilus*) [41], *Sac*Pox (issue de l'archée acidophile *Sulfolobus acidocaldarius*) [42], *Sis*Lac (issue de l'archée hyperthermophile *Sulfolobus islandicus*) [43] ou encore *Sso*Pox (issue de l'archée hyperthermophile *S. solfataricus*) ont été décrites [38,44,45]. Leur stabilité intrinsèque (T_m de 106 °C et 128 °C pour *Sso*Pox et *Vmo*Lac respectivement) constitue un atout majeur pour le développement de dispositifs médicaux fonctionnalisés par des enzymes. D'un point de vue évolutif, ces enzymes sont éloignées d'autres lactonases appartenant à la superfamille des métallo-β-lactamases qui comprend notamment l'enzyme AiiA de *Bacillus thuringiensis* qui a fait l'objet de nombreux travaux (Fig. 1) [17,46]. Bien que ces enzymes ne présentent pas de similarité structurale, il est intéressant de noter que leurs sites actifs montrent des ressemblances frappantes [37].

Intérêt des enzymes hyperthermostables : le cas de *Sso*Pox

*Sso*Pox est la PLL la mieux caractérisée à ce jour. L'enzyme, isolée d'une archée hyperthermophile des sources d'eau chaude du Vésuve, a été initialement étudiée pour sa capacité à hydrolyser les pesticides et agents neurotoxiques de guerre organophosphorés [45,47,48]. Cette enzyme est extrêmement stable et active sur de larges gammes de températures (10—100 °C) et de pH (5,0—9,0) ce qui lui confère un fort potentiel biotechnologique [44]. Outre son activité phosphotriestérase, *Sso*Pox possède également une activité lactonase qui lui permet de dégrader efficacement les AHL même à température ambiante. La structure tridimensionnelle de *Sso*Pox a été résolue et montre un repliement en tonneau $(\beta/\alpha)_8$ légèrement tordu [49]. Contrairement aux autres PTE qui disposent d'une structure similaire, *Sso*Pox montre de légers changements au niveau des boucles 7 et 8, respectivement plus courte et plus longue que celles des PTE. Ces modifications structurales engendrent la formation d'un canal hydrophobe qui accommode parfaitement les substrats de type lactone.

La capacité de l'enzyme sauvage à hydrolyser une large gamme de lactones a été démontrée. *Sso*Pox est ainsi capable de dégrader les AHLs, les γ-lactones et les δ-lactones avec des efficacités catalytiques allant jusqu'à $8{,}0 \times 10^4\ M^{-1}.s^{-1}$ [35,38,50]. L'activité lactonase de *Sso*Pox

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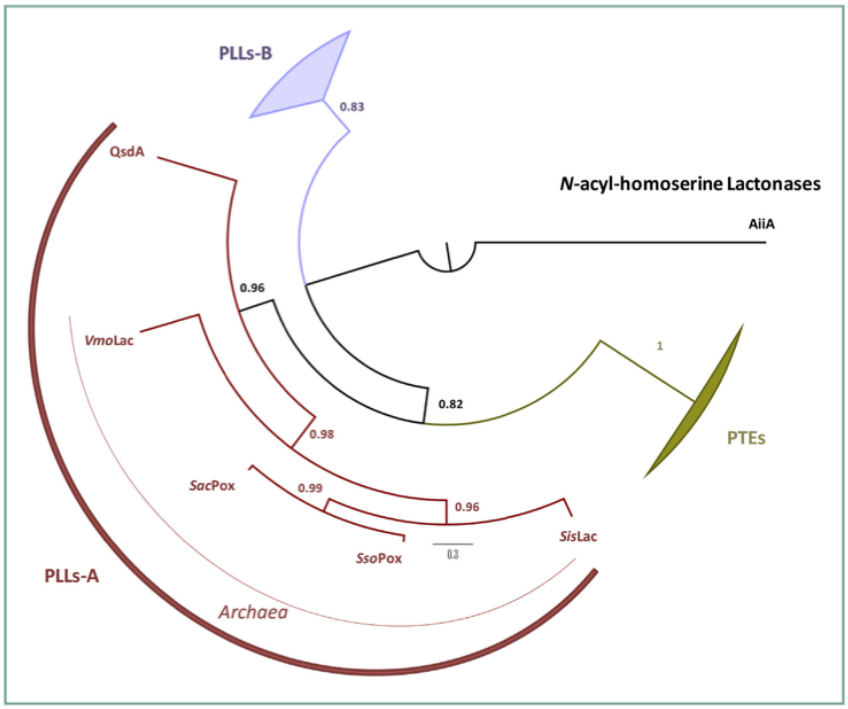

Figure 1. Arbre phylogénétique des PLL et des familles d'enzymes associées. L'outil web www.phylogeny.fr/simple_phylogeny.cgi a été utilisé pour l'alignement de séquences et l'arbre a été construit avec FigTree v1.4.0. Les séquences suivantes ont été utilisées : AiiA (P0CJ63), PTEs (Q93LD7, P0A434), *Sac*Pox (V9S7Z1), *Sso*Pox (Q97VT7), *Sis*Lac (C4KKZ9), *Vmo*Lac (F0QXN6), QsdA (B1N7B5), PLL-B (A4IN23, Q5KZU5, Q9RVU2).
Phylogenetic tree of PLLs and related enzymes. Webtool www.phylogeny.fr/simple_phylogeny.cgi was used for sequence alignment and phylogeny, the tree was obtained with FigTree v1.4.0. Sequence used for the analysis were: AiiA (P0CJ63), PTEs (Q93LD7, P0A434), SacPox (V9S7Z1), SsoPox (Q97VT7), SisLac (C4KKZ9), VmoLac (F0QXN6), QsdA (B1N7B5), PLLs-B (A4IN23, Q5KZU5, Q9RVU2).

a par ailleurs été améliorée par ingénierie enzymatique. Le résidu W263, situé dans le site actif et impliqué dans les mouvements de boucles, a été particulièrement considéré. Une expérience de mutagenèse à saturation a permis d'identifier un variant extrêmement efficace, *Sso*Pox-W263I, montrant des efficacités catalytiques allant jusqu'à $5{,}8 \times 10^6\ M^{-1}.s^{-1}$ [50]. Le variant *Sso*Pox-W263I a été co-cristallisé avec un analogue de substrat, le *N*-décanoyl-L-homocystéinethiolactone (Fig. 2). Ce variant est particulièrement intéressant en vue d'applications biotechnologiques, car si son activité lactonase a été grandement améliorée, il maintient également une forte stabilité ($T_m = 88\ °C$). L'enzyme sauvage et ses variants sont capables d'hydrolyser une vingtaine de lactones soulignant leur grande promiscuité de substrats. Ils résistent de plus à l'action des protéases, détergents et des solvants organiques [47,50]. Ces enzymes sont donc attractives pour le développement de dispositifs médicaux innovants pour lutter contre la virulence bactérienne et la formation du biofilm.

QQ et résistance

Les antibiotiques ont été massivement utilisés durant les dernières décennies pour traiter les infections bactériennes chroniques et aiguës. Ces agents anti-bactériens induisent une très forte pression de sélection sur les bactéries en les tuant ou en empêchant leur développement. Ceci à conduit à l'apparition de nombreux mécanismes de résistance chez les bactéries diminuant considérablement l'efficacité des antibiotiques et entraînant l'augmentation des doses efficaces [51—53]. Le QQ apparaît comme un traitement complémentaire, voire dans certains cas alternatif, aux antibiotiques, dans la mesure où il peut être utilisé pour limiter la virulence et la formation de biofilm sans tuer les bactéries [54—57]. Toutefois, si la pression de sélection appliquée par le QQ est plus faible que celle induite par un agent antimicrobien classique, elle n'en demeure pas nulle pour autant [58—60]. En présence de l'inhibiteur de QS C-30, une furanone de QQ très efficace, *P. aeruginosa* a rétabli par des mutations compensatoires son système de QS, notamment en augmentant l'efflux du C-30 [55,56]. Des bactéries ayant perdu leur capacité à réguler leur métabolisme en fonction de la densité de population bactérienne ont également été découvertes et seraient par conséquent insensibles au QQ [62]. Néanmoins, la diffusion d'une telle résistance est supposée lente puisqu'une étude récente a montré que des mutants bactériens QS-négatifs ont une moins bonne valeur sélective que leurs homologues QS-positifs [63], et les communautés bactériennes adoptent des contre-mesures pour pénaliser les bactéries insensibles au QS qui bénéficient

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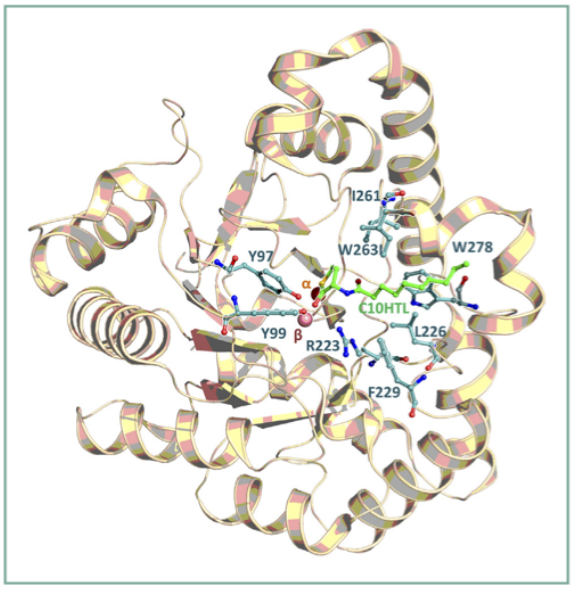

Figure 2. Structure de *Sso*Pox-W263I (PDB ID : 4KF1) en complexe avec l'analogue de substrat C10HTL. Les résidus catalytiques ainsi que les cations métalliques sont également présentés.
Structure of variant SsoPox-W263I (PDB ID: 4KF1) bound with substrate analogue C10HTL. Catalytic residues and metallic cations are emphasized in blue sticks and spheres respectively.

des composés extracellulaires produits par les bactéries QS-positifs [61,62].

L'émergence des phénotypes de résistance sera dépendante de la stratégie de QQ utilisée. La stratégie la moins agressive consiste probablement à utiliser des enzymes. En effet, contrairement aux inhibiteurs de QS, les enzymes peuvent agir depuis l'extérieur de la cellule en dégradant les AI sécrétés dans l'environnement. Outre les phénomènes de résistance précédemment décrits, des mécanismes de résistance putatifs aux enzymes à activité QQ ont toutefois été proposés [58,59]. Une augmentation de la production d'AI pourrait permettre de contrer l'utilisation d'enzymes. Une plus grande affinité ou sensibilité du récepteur cellulaire des AI permettrait également de réduire l'influence des enzymes [64,65]. Toutefois, ces mécanismes pourraient être contournés en augmentant la quantité d'enzyme dans l'environnement, ou en augmentant son activité et son affinité. Une autre stratégie pourrait consister à modifier la structure même de l'AI. Néanmoins les enzymes à activité QQ, et notamment les PLL, sont capables de dégrader une grande variété de lactones et devraient pouvoir supporter des modifications chimiques mineures. Une autre option pourrait consister en la production par les bactéries d'un inhibiteur des enzymes du QQ.

Conclusion et perspectives

Le QQ semble une stratégie prometteuse pour renforcer notre arsenal contre les infections bactériennes. En effet, contrairement aux approches classiques de désinfection et de traitements antibiotiques, le QQ est une approche plus douce, induisant potentiellement une plus faible pression de sélection, ne tuant pas les bactéries mais en les maintenant dans un état non virulent, sans former de biofilm. Par ailleurs, si le QQ est traditionnellement vu comme une approche uniquement préventive, de récents travaux suggèrent qu'il pourrait, grâce à l'ingénierie moléculaire, être utilisé en traitement en combinaison avec des antibiotiques [66]. De nombreuses études ont mis en évidence la corrélation entre formation du biofilm et résistance aux antibiotiques chez de nombreuses bactéries [67—73]. En effet, le biofilm limite la diffusion des agents antimicrobiens et favorise le transfert horizontal de gènes, notamment pour l'acquisition de résistances [66,74]. Dans la mesure où le QQ est généralement associé à une diminution de la quantité de biofilm, il pourrait limiter l'apparition de bactéries résistantes et permettre une meilleure accessibilité des bactéries aux antibiotiques [66].

Le QQ, et plus particulièrement les enzymes capables de dégrader les AI, constituent des alternatives prometteuses aux traitements classiques. Leur incorporation dans des dispositifs médicaux tels que des pansements ou des cathéters constituerait une rupture technologique majeure. Pour ce faire, le développement d'enzymes pouvant résister aux contraintes industrielles (chaleur, pH, stockage, stérilisation) est crucial. Les enzymes issues d'organismes extrêmophiles ont été le sujet de nombreuses études. Les PLL, notamment l'enzyme *Sso*Pox, ont fait l'objet d'une attention particulière car elles sont à la fois très actives pour la dégradation des lactones et extrêmement stables. Les preuves de concept de l'efficacité de ces enzymes ont d'ores et déjà été démontrées, restent maintenant le développement et l'évaluation de dispositifs médicaux de nouvelle génération pour la lutte contre les infections bactériennes.

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2.2 Revue française n°1 : Empêcher les bactéries de communiquer : diviser pour mieux soigner

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REVUE GÉNÉRALE

Empêcher les bactéries de communiquer : diviser pour mieux soigner

Prevent bacteria from communicating: Divide and cure

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MOTS CLÉS
Quorum Sensing ;
Virulence bactérienne ;
Quorum Quenching ;
Biofilm ;
Antibiotiques ;
Phagothérapie

Résumé Le *quorum sensing* (QS) est un système de communication utilisé par de nombreuses bactéries pour synchroniser leur comportement à la densité de population. Pour cela, elles sécrètent et détectent des molécules médiatrices, appelées auto-inducteurs (AI), dont la concentration dans l'environnement augmente proportionnellement au nombre de bactéries. Le QS induit des changements physiologiques et phénotypiques majeurs tels que l'induction de la virulence et la formation de biofilm. Le biofilm constitue un environnement défavorable à l'action des antibiotiques et aux traitements anti-microbiens et favorise l'apparition de résistance. La perturbation du QS, appelée *Quorum Quenching* (QQ), est une stratégie employée par les microorganismes eux-mêmes pour empêcher la mise en place de certains comportements de groupe. Deux stratégies ont été principalement décrites : l'utilisation d'inhibiteurs du *Quorum Sensing* (QSI) et d'enzyme à activité *Quorum Quenching* (QQE) dégradant les AI. De nombreuses études ont été consacrées à identifier des QSI (naturels ou synthétiques) ainsi que des QQE et à démontrer leurs effets anti-virulence et anti-biofilm sur de nombreuses espèces bactériennes. La synergie du QQ avec des traitements traditionnels tels que l'antibiothérapie ou avec des thérapies réémergentes comme la phagothérapie a été mise en évidence. L'efficacité de nombreux QSI et QQE a ainsi pu être démontrée in vitro et in vivo sur des modèles animaux aboutissant à la mise au point de dispositifs médicaux contenant des QSI et QQE dans le but d'améliorer les traitements déjà existants.

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KEYWORDS
Quorum sensing;
Bacterial virulence;
Quorum quenching;
Biofilm;
Antibiotics;
Phagotherapy

Summary Quorum Sensing (QS) is a communication system used by numerous bacteria to synchronize their behavior according to the cell density. In this way, bacteria secrete and sense small mediating molecules, called autoinducers (AI), which concentration increases in the environment proportionally to bacterial cell number. QS induces major physiological and phenotypic changes such as virulence induction and biofilm formation. Biofilm represents a physical barrier which shelters bacteria poorly sensitive to antimicrobial treatments and favors the apparition of resistance mechanisms. Disturbing QS is referred to as quorum quenching (QQ). This strategy is used by microorganisms themselves to prevent the development of specific group behaviors. Two strategies are mainly employed: the use of quorum sensing inhibitors (QSI) and of quorum quenching enzymes (QQE) that degrades AI. Many studies have been dedicated to identifying QSI (natural or synthetic) as well as QQE and demonstrating their anti-virulence and anti-biofilm effects on numerous bacterial species. Synergistic effects between QQ and traditional treatments such as antibiotherapy or with reemerging phage therapy have been put forward. The efficiency of numerous QSI and QQE was thereby demonstrated either with in vitro or in vivo animal models leading to the development of medical devices containing QSI and QQE to improve already existing treatments.

Introduction

Le *Quorum Sensing* (QS) est un système moléculaire par lequel les bactéries communiquent afin d'adapter collectivement leur comportement à la densité cellulaire et à l'environnement dans lequel elles se trouvent (Fig. 1). Ce système de communication permet aux bactéries de mettre en œuvre des processus qui sont coûteux en énergie et inefficaces à faible densité cellulaire mais qui deviennent utiles pour l'ensemble de la communauté à forte densité cellulaire comme la synthèse de facteurs de virulence, la formation de biofilm, et la production de protéases et sidérophores [1]. Le QS consiste en la production et la détection de petites molécules extracellulaires, appelées auto-inducteurs (AI), qui sont libérées et dont la concentration augmente proportionnellement à la densité cellulaire [2]. Chez les bactéries à Gram positif, les peptides auto-inducteurs (AIP) ont été largement étudiés et sont responsables de l'induction du QS. Les AIP sont spécifiques aux espèces et souches et ont été décrits entre autres chez *Staphylococcus* spp., *Clostridium* spp., ou encore *Enterococcus* spp. (Fig. 2) [3]. De nombreuses bactéries à Gram négatif, dont *Pseudomonas* spp., *Acinetobacter* spp. et Burkholderia spp., utilisent une classe différente d'AI : les acylhomosérine lactones (AHL) [4]. Les AHL sont composées d'un cycle lactone et d'une chaîne acyle aliphatique variant en taille et fonction [4]. De nombreuses autres molécules médiatrices ont également été identifiées [5], incluant des acides gras utilisés par *Xanthomonas* spp., *Burkholderia* spp., *Xylella* spp. [6], des cétones (*Vibrio* spp. et *Legionella* spp. [7]), l'adrénaline, la noradrénaline et AI de type 3 (bactéries entérohémorragiques [8]) ou des quinolones (*Pseudomonas aeruginosa* [9]). Enfin, AI-2, le diester de furanosyl borate, est à la fois utilisé par des bactéries à Gram négatif et à Gram positif [10] (Fig. 2). La plupart des bactéries à Gram négatif combinent plusieurs systèmes de QS afin d'intégrer différents signaux, organisés soit de manière hiérarchique, comme pour *P. aeruginosa* chez qui quatre systèmes de QS (las, rhl, iqs et pqs) interagissent en formant un réseau sous le contrôle de las, chaque système induisant l'activation du suivant [11], ou en parallèle, comme pour *Vibrio harveyi* chez qui trois systèmes sont intégrés de manière égale pour activer ensuite une même cascade de régulation [12].

L'ensemble des mécanismes permettant d'interférer avec le QS sont désignés par le terme *Quorum Quenching* (QQ) (Fig. 1). Le QQ est un phénomène naturel, décrit pour la première fois en 2000 avec l'identification d'une enzyme capable de dégrader les signaux AHL d'Erwinia carotovora perturbant ainsi le QS de la bactérie [13]. Depuis, plusieurs stratégies de QQ ont été identifiées et peuvent consister en :

- l'utilisation de molécules inhibitrices pouvant interférer avec la production ou la perception des AI, il s'agit alors d'inhibiteurs du QS (QSI) [14] ;
- la séquestration de molécules du QS par des anticorps [15] et par des macromolécules comme les cyclodextrines [16—18] ou ;
- par l'hydrolyse extracellulaire des AI en utilisant des enzymes à activité QQ (QQE) [19] (Fig. 2).

Plusieurs peptides antagonistes ont été identifiés naturellement ou synthétisés dans le but de bloquer le QS de bactéries à Gram positif. De nombreux QSI, ciblant principalement le QS des bactéries à Gram négatif et le QS dépendant des AI-2, ont également été décrits [14,20]. De tels composés sont soit des produits naturels, comme les polyphénols isolés de thé ou de miel, l'ajoène de l'ail, l'eugénol du clou de girofle et d'autres molécules produites par des organismes marins et des champignons [14], soit des molécules synthétiques, tel le 5-fluorouracile (5-FU) ou l'azithromycine [21,22]. À ce jour, de nombreuses QQE et macromolécules [23,24] ainsi que des QSI, naturels ou synthétiques [25—29] ont été décrits et ont fait l'objet de revues détaillées. Des brevets associés à ces composés [30—32] ainsi que les moyens d'obtenir de nouvelles molécules [33] ont également été recensés. Les mécanismes utilisés par les différents QSI ne sont pas toujours connus et diffèrent très probablement d'une molécule à une autre [34]. Parmi les molécules inhibant le QS, certaines comme

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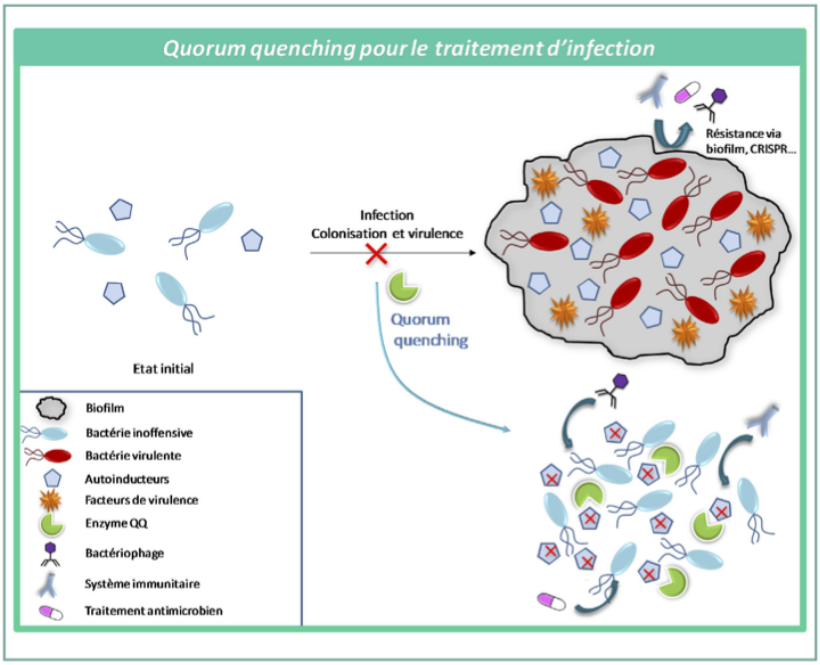

Figure 1. *Quorum Sensing* et *Quorum Quenching* dans les infections bactériennes. Le corps humain abrite naturellement une flore commensale qui n'est pas virulente (A). Quand les conditions de l'environnement bactérien sont perturbées (blessure, lésion, brûlure...), les bactéries trouvent un terrain fertile pour leur développement et colonisent le tissu endommagé. En proliférant, les bactéries produisent des molécules de communication (auto-inducteurs). Si ces molécules ne sont pas dégradées (B), les bactéries peuvent synchroniser leur comportement pour sécréter des facteurs de virulence et produire un biofilm, ce qui peut empêcher l'efficacité d'antibiothérapies et de phagothérapie. L'infection est implantée et peut devenir chronique. Si les auto-inducteurs sont dégradés (C), les bactéries sont incapables de synchroniser leur comportement et restent inoffensives et vulnérables. Les bactéries restent présentes mais l'infection n'a pas lieu.
Quorum sensing and quorum quenching in bacterial infections. Human body naturally hosts a non-virulent commensal flora (A). When the human-bacteria equilibrium is disrupted (wound, burn, lesion...), bacteria find a fertile environment for their development and colonize the damaged tissue. While proliferating, bacteria produce signal molecules (autoinducers). If those molecules are not degraded (B), bacteria can synchronize their behavior to secrete virulence factors and produce a biofilm, which can reduce the efficiency of antibiotic and phage therapies. The infection is established and can become chronic. If autoinducers are degraded (C), bacteria are not able to synchronize their behavior and stay harmless and defenseless. Bacteria remain present without causing infection.

l'azithromycine sont également considérées comme antibiotiques car pouvant inhiber la croissance bactérienne au-delà d'une certaine concentration [35].

Les QQE décrites à ce jour ciblent principalement les systèmes de QS reposant sur l'utilisation d'AHL et d'AI-2. Les *phosphotriesterase-like lactonases* (PLLs), lactonases, acylases et oxydoréductases dégradent les signaux AHL [19] et certaines oxydoréductases ciblent également les AI-2 [13,36]. Le QS est responsable, entre autres, de la formation de biofilm ou de l'induction de la virulence qui favorisent l'implantation des infections et limitent l'efficacité des soins. Le blocage de la communication bactérienne apparaît comme une stratégie prometteuse pour inhiber le comportement virulent des bactéries. Par conséquent, les approches de QQ présentent un intérêt particulièrement important dans le domaine de la santé et pourraient avoir des applications dans de nombreux autres domaines tels que l'agronomie, l'ingénierie hydraulique, et l'industrie marine [37].

L'utilisation massive des antibiotiques dans la médecine moderne a entraîné l'émergence de phénomènes de tolérance et de résistance limitant l'efficacité des soins. De nouvelles approches thérapeutiques sont aujourd'hui nécessaires pour compléter l'arsenal thérapeutique de la lutte contre les bactéries [38]. Le QQ, qui permet d'inhiber la virulence bactérienne sans tuer les bactéries, est particulièrement intéressant [39]. En effet le QQ cible principalement les messages moléculaires de la communication bactérienne et non les messagers (i.e. bactéries) ce qui limite la pression de sélection. Par ailleurs le QQ inhibe la formation du biofilm connu pour contribuer à la résistance aux traitements antibactériens. Le biofilm, dont la formation est induite par le QS, est une structure hétérogène multicellulaire intégrée à une matrice extracellulaire et pouvant adhérer à une surface solide [40]. La matrice, principalement constituée de polysaccharides, protéines et ADN extracellulaire, peut empêcher les antibiotiques de pénétrer efficacement dans les cellules, induisant une tolérance à ces molécules [41]. Les cellules bactériennes intégrées dans la matrice présentent une vitesse de croissance plus lente et un métabolisme différent de celui des bactéries planctoniques qui réduisent l'efficacité du traitement [42]. De plus, les conditions de vie en biofilm allient densité cellulaire élevée et forte pression de sélection, engendrant

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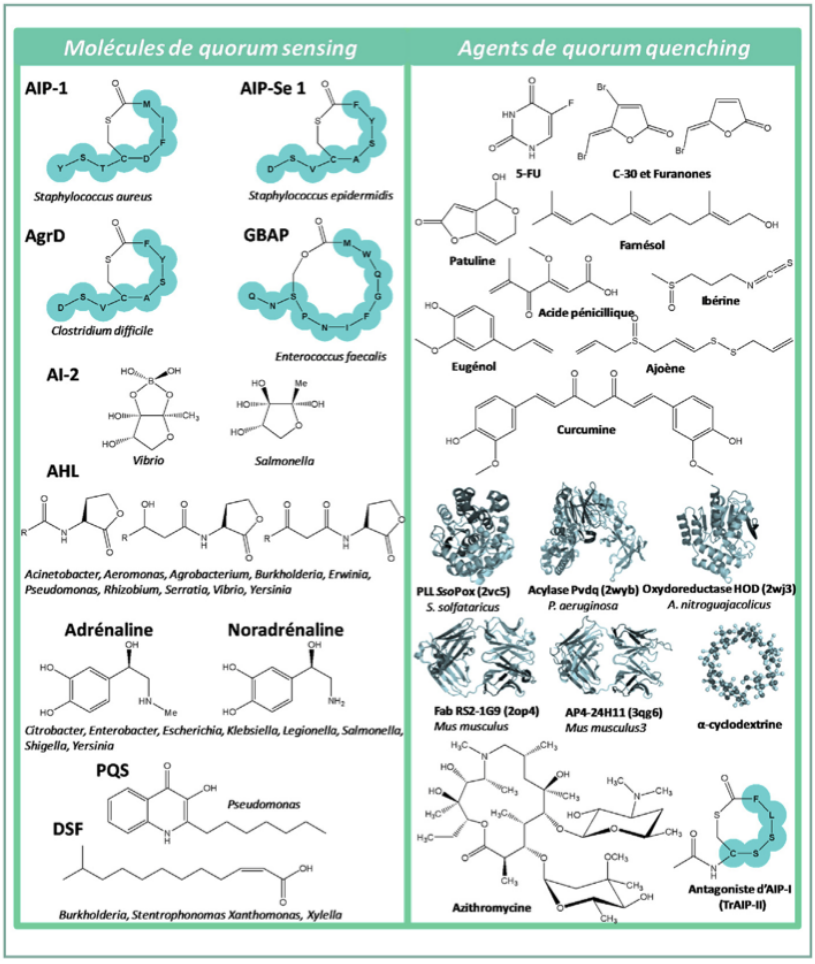

Figure 2. Représentation des auto-inducteurs et des agents de *Quorum Quenching*. À gauche, différents auto-inducteurs sont représentés. De haut en bas : des peptides auto-inducteurs utilisés par des bactéries à Gram positif comme *Staphylococcus* spp., *Clostridium* spp., *Enterococcus faecalis* [3] ; différentes formes d'AI-2, un diester furanosyl, utilisé par les bactéries à Gram positif et à Gram négatif [10] ; un aperçu des différentes molécules utilisées pour le *Quorum Sensing* des bactéries à Gram négatif : les acylhomosérine lactones (AHL) [4], quinolones (PQS) [201], acides gras (DSF) [6], adrénaline et noradrénaline [8]. À droite sont représentés des agents de *Quorum Quenching*. De haut en bas : les inhibiteurs de *Quorum Sensing*, actifs principalement contre le QS dépendant des AHL ou de AI-2 [14] ; les enzymes qui dégradent les AHL (SsoPox, Pvdq) et les quinolones du PQS (HOD) [19] ; les molécules capables de séquestrer les AI, tels les anticorps capables de bloquer les AHL (Fab RS2-1G9) et les AIP (AP4-24H11) [15] et les macromolécules comme les cyclodextrines et leurs dérivés [18] ; un antibiotique, l'azithromycine, ayant des propriétés inhibitrices du QS [21] ; un peptide, TrAIP-II, utilisé pour inhiber le QS de bactéries à Gram positif [20].

Representation of autoinducers and quorum quenching agents. On the left side, autoinducers are depicted. From top to bottom : autoinducers peptides used by Gram positive bacteria such as Staphylococcus spp., Clostridium spp., Enterococcus [3]; various forms of AI-2, a furanosyl diester, used by Gram positive and Gram negative bacteria [10]; an overall view of different molecules used for quorum sensing in Gram negative bacteria : acylhomoserine lactones (AHL) [4], quinolones (PQS) [38], fatty acids (DF) [6], epinephrine and norepinephrine [8]. On the right side, quorum quenching agents are depicted. From top to bottom: quorum sensing inhibitors, principally active against AHL or AI-2-dependant QS [14]; AHL degrading enzymes (SsoPox, Pvdq) and PQS quinolones (HOD) [19], molecules able to scavenge AI, such as antibodies able to block AHL (Fab RS2-1G9) and AIP (AP4-24H11) [15] and macromolecules such as cyclodextrins and derivatives [18]; an antibiotic, azithromycin, possessing inhibitory properties on QS [21]; a peptide, TrAIP-II, used to inhibit Gram positive bacteria QS [20].

une augmentation de la fréquence d'apparition de cellules résistantes générées par mutations ou transferts de gènes [43]. Les biofilms peuvent également abriter des cellules dites persistantes, un caractère non héritable concernant une faible proportion de cellules capables de survivre aux traitements antibiotiques [44]. Le biofilm renforcerait la tolérance des bactéries aux antibiotiques d'un facteur 100 à 1 000 [42]. Les biofilms seraient ainsi impliqués dans 65 à 80 % des infections bactériennes, en ciblant les tissus mous (e.g. les poumons dans le cas de mucoviscidoses), ou en colonisant le matériel médical (e.g. cathéter, endoscopes) [45]. Les Infections associées aux soins (IAS) touchent entre 6 et

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10 % des patients dans les pays développés, les infections les plus fréquentes affectant les voies urinaires [46,47]. L'inhibition de la formation de biofilms dans les dispositifs et environnements médicaux constitue un enjeu majeur pour limiter et traiter les IAS. Il est donc essentiel de développer des traitements alternatifs ou complémentaires aux produits antimicrobiens et antibiotiques conventionnels. En ce sens, le QQ et la phagothérapie sont de plus en plus étudiés [48,49].

Cette revue dresse un bilan des dernières découvertes et des perspectives thérapeutiques du QQ ainsi que son potentiel en tant que traitement complémentaire aux agents antimicrobiens, antibiotiques et bactériophages. Les modèles eucaryotes utilisés pour prouver l'efficacité du QQ et les différents dispositifs médicaux utilisant cette technologie sont aussi décrits.

Le *Quorum Sensing* et la sensibilité aux agents antimicrobiens

Le QS induisant un changement global dans l'expression des gènes et dans la physiologie des bactéries, sa relation avec la tolérance aux antibiotiques est multifactorielle. Il a par exemple été démontré que l'ajout d'AHL à une culture de *P. aeruginosa* augmentait le nombre de cellules persistantes dans la population après traitement à la carbénicilline et à la ciprofloxacine [50]. De plus, l'analyse transcriptomique d'un régulateur transcriptionnel du QS MvfR (PqsR) de *P. aeruginosa* PA14 a révélé que le QS induit l'expression de peroxydases qui fournissent une protection contre les dérivés réactifs de l'oxygène (H_2O_2) et les antibiotiques de la famille des β-lactamines [51]. Chez *P. aeruginosa* PAO1, il a également été montré que VqsM, un régulateur global du QS, est lié à la tolérance aux antibiotiques en induisant l'expression de nfxB, un régulateur de résistance aux antibiotiques, procurant une tolérance accrue aux quinolones, à la tétracycline et à la kanamycine via la régulation de l'expression de l'opéron mexC-mexD-oprJ [52,53].

Bien que les liens unissant QS et résistance aux antibiotiques puissent impliquer de nombreux aspects physiologiques, la plupart des études se sont concentrées sur le rôle du biofilm responsable de nombreuses complications lors des traitements d'infections cliniques [54,55]. La contribution du biofilm à l'antibiorésistance a souvent été observée chez *P. aeruginosa* [56], à la fois sur des souches modèles et cliniques [57], ainsi que chez d'autres espèces bactériennes comme *Klebsiella pneumoniae* [58,59] et *Staphylococcus aureus* [60]. L'environnement particulier offert aux bactéries par la croissance en biofilm favorise le développement de divers mécanismes et phénotypes de défenses : barrière physique limitant la diffusion, modification d'expression de gène, états physiologiques cellulaires différents (e.g. cellules persistantes) [40]. Chez *P. aeruginosa*, les systèmes de QS rhl et las sont essentiels à la formation de biofilm et leur inhibition est corrélée à une sensibilité accrue au système immunitaire de l'hôte et aux composés antimicrobiens [61–63]. En outre, il a été prouvé chez *P. aeruginosa* qu'un autre système du QS, le système pqs, régule l'apoptose induisant une libération d'ADN extracellulaire qui favorise la formation de biofilm et la tolérance aux antibiotiques, bénéficiant ainsi au reste de la population bactérienne [64]. Chez des isolats cliniques d'*Acinetobacter baumannii*, il a été montré que la présence des antibiotiques lévofloxacine ou méropenème induit une surexpression d'une pompe à efflux qui stimule la libération d'AHL, augmentant ainsi la formation de biofilm liée au QS, et donc la tolérance aux antibiotiques [65].

Au vu de l'implication du QS dans la formation de biofilm et la tolérance aux antibiotiques, des études se sont intéressées à la synergie du QQ avec d'autres approches thérapeutiques. Chez *P. aeruginosa*, l'utilisation du benzamide-benzimidazole, un QSI inhibant le régulateur de QS (PqsR) a diminué la formation de biofilm et restauré la sensibilité aux antibiotiques [66,67]. L'administration d'hydrate de baïcaline ciblant les AHL et d'hamamélitanin un QSI à base de peptide, a entraîné une détérioration du biofilm tant chez des bactéries à Gram négatif (*P. aeruginosa* et *Burkholderia cepacia*) que chez des bactéries à Gram positif (*S. aureus*) et a montré des effets synergiques, in vitro et in vivo, en cotraitement avec la tobramycine et la clindamycine ou la vancomycine respectivement [68]. Des aminoglycosides [69,70] aux quinolones [71], en passant par les polypeptides [72,73], les céphalosporines [66] et les glycopeptides [74], l'efficacité d'une large gamme d'antibiotiques s'est vu augmentée par l'ajout de QSI.

Ces résultats suggèrent que les QSI sont particulièrement intéressants pour augmenter la sensibilité des bactéries aux antibiotiques. De plus, des effets synergiques similaires ont également été observés avec une QQE, la lactonase produite par une souche de *Bacillus* sp. ZA12 isolée du sol, en combinaison avec de la ciprofloxacine chez un modèle murin [75].

Relation entre *Quorum Sensing* et sensibilité aux bactériophages

Récemment, l'intérêt pour la phagothérapie a particulièrement grandi, cette stratégie se présentant comme un moyen de traiter les infections causées par des bactéries multirésistantes [76]. Les bactériophages sont les prédateurs de bactéries les plus abondants sur la planète et sont utilisés depuis plus de 80 ans en Europe de l'Est (e.g. Géorgie, Russie) pour traiter les infections bactériennes [77]. Bien que les bactériophages représentent une solution intéressante pour contourner la résistance aux antibiotiques, les bactéries ont également développé des mécanismes de défense pour se prémunir de l'action des phages [78]. Premièrement, l'entrée des phages peut être diminuée par la production de biofilm, ou par la modification de la structure ou de l'expression des récepteurs aux phages [78,79]. Une fois à l'intérieur de la cellule, l'ADN du phage peut être reconnu et dégradé par des enzymes de restriction ou par le système inductible adaptatif CRISPR-Cas (pour *clustered regularly interspaced short palindromic repeat* et CRISPR *associated proteins*) [78,80]. D'autres adaptations métaboliques pourraient aussi procurer une résistance aux bactériophages [81]. Le risque d'infection par les phages augmentant proportionnellement à la densité cellulaire [82], une résistance liée au QS fournirait une protection dans des conditions à hauts risques pour les bactéries se trouvant à forte densité

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tout en limitant le coût énergétique global de ces mécanismes [83].

La relation entre le QS et la sensibilité aux bactériophages a initialement été observée chez *P. aeruginosa* [84], mais l'implication du QS dans la régulation de mécanismes de défenses contre les phages n'a été démontrée que des années plus tard chez *Escherichia coli* [85]. Les auteurs ont montré que les AHL induisaient une réduction du taux d'adsorption des phages lambda et chi en réduisant le nombre de récepteurs à la surface de la cellule d'*E. coli* K-12, qui même si elle n'est pas capable de produire des AHL, est capable de les détecter via SdiA, un régulateur transcriptionnel de type LuxR [86]. Chez *Vibrio cholerae*, des mutations génétiques au niveau des synthases d'AI empêchant la mise en place du QS, a réduit la résistance aux phages, qui a pu être restaurée par ajout d'AI exogènes, AI-2 et CAI-1 [87]. L'augmentation de la résistance aux phages activée par le QS a été expliquée par une sous expression de l'antigène O diminuant l'adsorption de phages et par une augmentation de la production de la protéase dégradant l'hémagglutinine, inactivant ainsi les bactériophages [87]. De manière similaire, l'addition d'AHL synthétiques à une culture d'une souche déficiente en QS de *Vibrio anguillarum* a significativement amélioré sa résistance aux phages [88]. En effet, la production d'AHL est négativement corrélée à l'expression du récepteur aux phages ompK. De plus, il a été récemment démontré chez *Serratia marcescens* que le système CRISPR-Cas se trouvait lui aussi sous la régulation du QS [89]. Cette étude a montré que tant l'acquisition de l'immunité que le mécanisme de dégradation de l'ADN coordonné par le système CRISPR-Cas, étaient négativement impactés par l'absence de signal de QS chez un mutant déficient en synthase d'AI. De plus, l'analyse de données préalablement obtenues [90,91], a suggéré que ce type de régulation puisse également avoir lieu chez *Pectobacterium atrosepticum* et *Burkholderia glumae*. Un contrôle similaire du système CRISPR-Cas par le QS a également été démontré chez *P. aeruginosa* PA14 par l'intermédiaire de délétions des gènes de synthase lasI et rhlI entraînant une sous expression des gènes CRISPR-Cas [92].

Afin d'évaluer la capacité du QQ à augmenter la sensibilité aux phages, les effets de QSI sur les mécanismes de résistance et sur le phénotype de résistance aux phages ont été étudiés. L'utilisation d'acide pénicillique a augmenté la sensibilité de *P. aeruginosa* aux phages en enrichissant la population en cellules viables sensibles [81]. Enfin, il a été observé que la baïcaline inhibait la stimulation du système CRISPR-Cas par le QS chez *P. aeruginosa*, ce qui pourrait empêcher l'utilisation de ce système adaptatif par la bactérie en cas d'infection par des phages [92].

Il a été récemment démontré que perturber le QS d'une espèce induirait une réduction de la biomasse totale de cultures multimicrobiennes lors d'une infection par des phages, laissant penser que le QQ combiné à la phagothérapie pourrait également être efficace pour le traitement d'infections polymicrobiennes [93]. L'inhibition de la communication bactérienne semble être une voie très prometteuse pour développer de nouvelles applications thérapeutiques. En effet, outre sa contribution à la diminution de la virulence et de la formation du biofilm, la combinaison du QQ avec des traitements de phagothérapie pourrait augmenter la sensibilité des bactéries aux phages par effets synergiques.

Effets in vivo des perturbateurs de la communication bactérienne

Afin d'évaluer les bénéfices du QQ, différents modèles animaux ont été testés depuis l'utilisation d'organismes eucaryotes unicellulaires jusqu'aux modèles mammifère en passant par le nématode. Les succès obtenus dans ces différents modèles ont abouti aux premiers essais cliniques chez l'Homme. Les principaux résultats sont présentés ci-après.

Modèles d'infections amibiens

Les amibes sont des organismes eucaryotes unicellulaires qui peuvent adopter une forme de dissémination passive (kyste) ou une forme cellulaire végétative (trophozoïte), elles se nourrissent de bactéries ainsi que d'autres organismes (algues ou champignons). À cette fin, elles utilisent la phagocytose couplée à une digestion lysosomale, semblable au mécanisme d'élimination des bactéries par les macrophages [94—96]. Considérant leurs liens étroits avec les bactéries, les amibes ont été utilisées comme modèle pour tester l'impact des facteurs de virulence [97,98], de la formation de biofilm [99,100] et des systèmes de sécrétion bactériens [101—103]. Classiquement, l'évaluation de la virulence bactérienne chez les amibes repose sur leur capacité à se développer ou non en présence de bactéries pathogènes [98,102]. Le lien entre le QS et les facteurs de virulence a été étudié chez différentes espèces bactériennes en utilisant cette approche, et plus particulièrement pour *P. aeruginosa*. Des mutants de *P. aeruginosa* déficients en QS présentaient une virulence réduite envers l'amibe *Dictyostelium discoideum* [98,102]. La surproduction d'une QQE, l'amidase aliphatique AmiE par *P. aeruginosa* PA14 a entraîné l'inhibition du QS et la réduction de virulence lors d'un test de mortalité sur milieu solide avec D. discoideum [97]. Bien que ce modèle d'étude permette d'obtenir des résultats rapidement et soit approprié à des expériences à grande échelle comme des criblages de principes actifs, son utilisation est limitée tant par les conditions de culture que par les espèces d'amibes utilisées qui peuvent fortement affecter les résultats [104].

Modèles d'infections de *Caenorhabditis elegans*

Le ver rond *C. elegans* est un organisme multicellulaire largement utilisé pour étudier la virulence microbienne [105—108]. À l'instar de l'amibe, *C. elegans* est un modèle approprié à l'évaluation de l'impact du QS sur la virulence bactérienne [107—110]. Cependant, contrairement à l'amibe, *C. elegans* possède un système immunitaire inné, et offre une comparaison plus adaptée à la réponse immunitaire humaine [106] et en fait un modèle particulièrement pertinent pour l'étude de la virulence. Classiquement, *C. elegans* est nourri avec la bactérie d'intérêt puis le taux de survie est évalué. Deux types d'essais peuvent être

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réalisés : un test de mortalité à court terme qui consiste à étudier la survie du ver pendant quelques heures pour mettre en évidence la présence de toxines, et un test à long terme pour observer la mortalité du ver suite à la colonisation bactérienne [111—113].

Dans le but de mettre en évidence les liens unissant communication bactérienne et virulence, de nombreuses expériences se sont concentrées sur l'étude de mutants bactériens déficients en synthèse ou perception d'AI. Plusieurs travaux ont démontré que l'inactivation du QS chez différentes souches de *P. aeruginosa* entraînait une diminution de la mortalité chez les vers [108,109,114,115]. Le modèle *C. elegans* a également permis de mettre en évidence l'implication du QS dans la virulence chez diverses bactéries à Gram négatif dont *Chromobacterium violaceum* [116], *E. coli* [117], *Yersinia pseudotuberculosis* [118], *B. cepacia* [111], *Burkholderia cenocepacia* [119] et *Burkholderia pseudomallei* [120] ainsi que chez des bactéries à Gram positif telles que *Enterococcus faecalis* [107,121] et *S. aureus* [122]. Le QS apparaît donc comme un mécanisme largement impliqué dans la virulence des bactéries.

Outre les mutations génétiques, le modèle *C. elegans* a été utilisée pour prouver l'efficacité QQ de QSI comme le 3,4-dichloro-cinnamaldéhyde, augmentant le taux de survie d'au moins 30 % lors du traitement d'infections à *V. anguillarum*, *V. harveyi* ou *V. vulnificus* [123]. Le taux de survie du ver après infection par *P. aeruginosa* PAO1 s'est vu nettement amélioré par divers traitements à base de QSI tel le 2,5-pipérazinedione et l'acide phénylacétique [124], l'huile de clou de girofle [125], l'extrait de *Terminalia chebula* [126], le menthol [127], l'extrait de *Trigonella faenum-graceum* [128], le coumarate de l'aldéhyde oléanolique [129], l'extrait de feuille de *Mangifera indica* [130], les taux de survie oscillant entre 28 % et 95 % chez les populations traitées contre 0 % à 20 % seulement chez les populations contrôles. L'utilisation de polyphénols de thé a également permis de multiplier par trois le taux de survie après infection par un isolat clinique de *P. aeruginosa* [131]. Des expériences ont aussi été réalisées avec des QQE, comme les lactonases AiiA issues de *Bacillus* sp. testées contre des infections par des souches de *B. cepacia* [132], et des infections par Y. pseudotuberculosis YplII [118]. Plusieurs autres enzymes ont également été testées contre des infections par *P. aeruginosa* PAO1 tels que AiiD une acylase de Ralstonia XJ12B [133], PvdQ une acylase de *P. aeruginosa* PAO1 [134], BpiB09 un réductase déshydrogénase [135], MomL une lactonase de *Muricauda olearia* Th120 [136], toutes ces enzymes entraînant une amélioration du taux de survie du ver. Enfin, l'effet QQ de bactéries a également été mis en évidence dans le modèle *C. elegans*, en effet des bactéries isolées de rhizosphère, eau, mucus ou intestins de flet ont entraîné une augmentation du taux de survie après infection à *B. cenocepacia* et le même effet a été observé avec diverses bactéries de genre *Pseudomonas*, *Pseudoalteromonas*, *Delftia* et *Arthrobacter* lors d'infections du ver avec *P. aeruginosa* PAO1 [137]. Bien que l'impact sur la survie soit variable selon l'essai et les conditions de culture, tous les agents QQ testés ont efficacement diminué la virulence de bactéries à Gram positif [68] et à Gram négatif [138], améliorant ainsi le taux de survie de *C. elegans*. Par exemple en permettant d'atteindre jusqu'à 100 % de protection contre les infections par *P. aeruginosa* PAO1 grâce à l'enzyme BpiB09 ciblant les AHL [135]. Le 4-nitro-pyridine-N-oxide s'est révélé être, quant à lui, le QSI ayant l'effet le plus drastique sur la survie de *C. elegans* après infection par *P. aeruginosa* PAO1, restaurant près de la totalité de la survie des vers [110]. Les QSI naturels les plus efficaces sont des extraits de *Conocarpus* sp., *Callistemon vinimalis* ou *Bucida buceras*, rétablissant le taux de survie jusqu'à 87 % [139]. De plus, un effet synergique avec des antibiotiques a été rapporté pour l'utilisation de la baïcaline et de l'hamamélitanin [68].

C. elegans s'avère être un modèle invertébré extrêmement utile, permettant le criblage à haut débit (pour les mutants bactériens ou les composés QQ) et permettant de mieux comprendre la régulation de la virulence, la modulation par des agents QQ et, de manière générale, par des molécules anti-infections [140]. Cependant, le modèle montre certaines limites, comme les paramètres physiologiques du ver qui diffèrent de ceux des bactéries (e.g. : température de croissance autour de 20°C), et sa physiopathologie qui est très différente de celle des humains. Par ailleurs, comme pour les amibes, une influence des conditions expérimentales sur les résultats a été mise en évidence dans plusieurs études [113,141,142].

Modèles d'infection murins

Les modèles mammifères, comme le rat ou la souris, sont communément utilisés pour étudier le rôle du QS dans les infections bactériennes. En effet, il a été prouvé que les mutations ou délétions de gènes liés au QS réduisaient la mortalité ou la sévérité de pneumopathies [143—146], d'infections de brûlures [108,147], de péritonites [121,148] et d'infections de la prostate [149], à l'exception d'infections causées par *Staphylococcus epidermidis* [150]. Dans la grande majorité des cas, l'approche basée sur le QQ a entrainé une diminution de la mortalité, une accélération de la rémission et une diminution de la colonisation bactérienne.

Dans les modèles d'infections pulmonaires, la colonisation par *P. aeruginosa* et la mortalité associée ont été réduites par traitement aux furanones [151,152], à l'azithromycine pour des concentrations en dessous de sa concentration minimale inhibitrice [153], de l'extrait d'ail [154] et également par l'administration intra-trachéale de la lactonase SsoPox [155]. Dans des modèles de plaie, de nombreux QSI ont permis de réduire la virulence de *S. aureus* [156—159]. Une inhibition similaire de la virulence a été observée avec un anticorps inhibant le QS par la séquestration du peptide autoinducteur (AIP)-4 produit par *S. aureus* RN4850 [15]. Un traitement basé sur un peptide inhibiteur du QS a permis de réduire fortement la colonisation par *S. epidermidis* dans le cas d'une infection contractée après greffe [160], bien qu'il ait été observé que la déficience en système de QS luxS augmente la formation de biofilm in vitro et la virulence dans un modèle animal (Rat) d'infection liée au biofilm chez cette espèce bactérienne [150]. L'efficacité du QQ a aussi été démontrée dans le cas d'un modèle d'infection de brûlure par *P. aeruginosa* traité avec une QQE dégradant les AHL [75] ou avec des inhibiteurs de PqsR (MvfR) [161] et, dans un modèle de blessure par excision en utilisant des polyphénols de thé en tant que QSI [131]. De plus, l'effet combiné de molécules QQ

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et d'antibiotiques a été démontré in vivo sur des bactéries à Gram positif et à Gram négatif. En effet, chez *B. cenocepacia*, la combinaison de baïcaline et de tobramycine a permis de réduire jusqu'à 99 % de la charge bactérienne dans les poumons après trois jours[123]. L'utilisation de ciprofloxacine et de lactonase pour traiter une brûlure infectée par *P. aeruginosa* a permis de supprimer la mortalité et de réduire d'au moins 35 % la dissémination globale des bactéries dans les organes internes des souris après trois jours [75]. Par ailleurs, le cotraitement combinant un QSI et un antibiotique a également réduit drastiquement la colonisation d'un corps étranger artificiel (e.g. cathéter ou implants) par *S. aureus* [156,158], *S. epidermidis* [160] et *P. aeruginosa* [74,162] jusqu'à 5, 4 et 8 ordres de grandeurs de moins par rapport au contrôle non traité respectivement. Ces exemples montrent que le QQ est une alternative de choix pour le traitement des IAS qui permettrait de contourner les résistances liées à l'utilisation des antibiotiques.

Essais cliniques chez les humains avec des inhibiteurs de *Quorum Sensing*

Jusqu'à présent, seuls des QSI préalablement approuvés ou commercialisés ont été utilisés pour des essais cliniques, quoique leur activité initialement démontrée soit liée à leur pouvoir bactéricide, antimicrobiennes (antibiotiques) ou leur cytotoxicité (molécule anticancéreuse) [163,164] plutôt qu'à une action anti QS.

Au début des années 2000, l'azithromycine (Fig. 2) a été utilisée dans des essais cliniques pour traiter des infections chez des patients atteints de la mucoviscidose [165,166] et des patients ayant subi une greffe de poumons [167]. Cet antibiotique, appartenant à la famille des macrolides, a permis d'améliorer la qualité de vie des patients sans diminution de la charge bactérienne [165]. A la même époque, la capacité de l'azithromycine à perturber le QS de *P. aeruginosa*, à des concentrations non bactéricides, a été démontré in vitro [168]. Plus tard, l'effet de l'azithromycine sur le QS de *P. aeruginosa* chez des patients atteints de pneumonie sous ventilation assistée a été évalué [163] permettant de suspecter les effets anti-virulents bénéfiques de l'azithromycine chez un groupe de patients particulièrement vulnérables quoique les résultats ne soient pas assez significatifs.

L'ail est également connu pour ces propriétés QQ [110] et a été utilisé lors d'un essai pour traiter des infections chez des patients atteints de mucoviscidose, aucune preuve manifeste de l'effet de l'extrait d'ail sur la santé des patients ne ressortant toutefois de cette étude [169].

Enfin, il a été démontré que la molécule anticancéreuse [170], 5-FU, un analogue de pyrimidine (Fig. 2), inhibe in vitro la virulence régulée par le QS chez *P. aeruginosa* [22] et ce composé a ensuite été utilisé pour le revêtement de cathéters fonctionnalisés, dont l'efficacité a été prouvée en essais cliniques [164,171].

Utilisation du *Quorum Quenching* dans les dispositifs médicaux

Les dispositifs médicaux sont impliqués dans de nombreuses IAS [172] souvent dues à une colonisation par des bactéries multirésistantes et/ou formant du biofilm. Ces infections causent généralement des complications médicales sévères, forte morbidité et risques de mortalité. Le QQ empêchant la virulence bactérienne [173], le développement de nouveaux dispositifs médicaux utilisant des agents de QQ est particulièrement intéressant. De nouvelles générations de cathéters [174], pansements [37,49], aérosols [155], lentilles de contact [175], dispositifs implantables [176] ou des dispositifs pour l'orthopédie ou le trauma [177] sont actuellement en développement.

Les QSI ont tout d'abord été considérés pour la fonctionnalisation de cathéters. Il a été montré que des furanones fixées de manière covalente permettaient de réduire la formation de biofilm par *S. epidermidis* ATCC 35984 et de contrôler l'infection pendant 65 jours in vivo chez un modèle ovin (Mouton) [178]. Dans une étude clinique sur 960 patients adultes réalisée dans 25 unités de soins intensifs américaines, un cathéter veineux central fonctionnalisé par le 5-FU a donné des résultats comparables aux cathéters recouverts de chlorhéxidine/sulfadiazine, ou d'argent classiquement utilisés, [164,171]. Bien que le lien avec le QS n'ait pas été clairement établi lors de cette étude, les cathéters enduits de 5-FU montraient des niveaux plus faibles de contamination par des bactéries à Gram négatif, comparés aux revêtements traditionnels, ce qui pourrait témoigner de l'interférence avec le QS dépendent des AHL dans cette étude. Il a récemment été prouvé que des revêtements basés sur le poly(éthylène glycol) contenant le QSI DHP (5-methylène-1-(prop-2-enoyl)-4-(2-fluorophenyl)-dihydropyrrol-2-one) réduisaient la colonisation par la souche 38 de *S. aureus* et la souche MH602 de *P. aeruginosa* [179]. Des combinaisons de DHP et de dérivés de furanones ont été fixées de manière covalente à des surfaces en verre et ont permis une réduction significative de l'adhésion de *S. aureus* SA38 et *P. aeruginosa* PAO1 [180]. Le potentiel des dispositifs fonctionnalisés pourraient de plus s'étendre au traitement d'infection fongique puisqu'un vernis libérant de la thiazolidinedione-8 (TZD-8), un QSI utilisé sur des cathéters se révélant actif contre les biofilms de Candida albicans [181].

Outre les cathéters, d'autres systèmes tels que des nanovecteurs de sélénium contenant des polyphénols ont par exemple permis de bloquer le QS de *P. aeruginosa* PAO1 in vitro et in vivo [182]. Des peptides inhibiteurs ciblant le système agr de *S. aureus* ont également été intégrés avec succès dans des biomatériaux. Des peptides macrocycliques chargés dans des nanofibres ont montré une activité biologique contre *S. aureus* [183]. La chimie Clic ou chimie « intelligente » a aussi été utilisée pour revêtir covalement des surfaces avec des peptides pro- et anti-QS, AIP-I et TrAIP-II respectivement. Le revêtement de surfaces en verre a permis de contrôler le QS, en l'activant ou l'inhibant, avec des souches de *S. aureus* [184]. La synergie du peptide FS3, inhibiteur de QS, avec des antibiotiques a également été démontrée : la daptomycine combinée à une prothèse alliée d'un revêtement contenant FS3 a permis de réduire l'infection de greffons par des souches de *S. aureus* d'un facteur 100 par rapport au traitement par l'antibiotique seul [156]. De manière similaire, il a été prouvé que le peptide inhibiteur de RNAIII (RIP) incorporé à un greffon en Dacron était efficace pour réduire l'infection par diverses souches de *S. epidermidis*, réduisant jusqu'à 100 % de

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la population bactérienne quand il est coadministré avec des antibiotiques (Quinupristine-dalfopristine ou Mupirocine) [160].

Bien que les dispositifs inhibant le QS aient été obtenus après immobilisation covalente de QSI ou peptides, les QQE ont aussi été particulièrement étudiées. En effet ces composés agissant sur les AI sécrétés dans l'environnement, ils présentent l'intérêt de ne pas nécessiter de contact direct avec les cellules pour perturber leur communication. Une acylase issue d'*Aspergillus melleus* a été incorporée avec succès dans un revêtement en polyuréthane et dans des cathéters en silicone [185,186]. L'acylase a ainsi permis de réduire la formation de biofilm de *P. aeruginosa* ATCC 10145 sur le revêtement et de *P. aeruginosa* PAO1 dans les cathéters [185,186]. La combinaison de l'acylase avec l'α-amylase de *Bacillus amyloliquefaciens* a retardé le développement de biofilms par *P. aeruginosa* ATCC 10145 et *E. coli* ATCC 25922 jusqu'à sept jours dans un modèle in vivo (Lapin) [187]. Une acylase de rein de porc a également été immobilisée sur des nanofibres de polyanilines carboxylées pour le développement de nanobiocatalyseurs, limitant la formation de biofilm de *P. aeruginosa* PAO1 [188]. Un traitement topique à base de lactonase issue de *Bacillus* sp. ZA12 a aussi été étudié sur un modèle murin de brûlure infectée par *P. aeruginosa* PAO1 [189]. L'application d'un gel contenant la lactonase après une infection de brûlure par 106 bactéries a empêché la dissémination systémique, réduit la mortalité et a montré un effet synergique avec la ciprofloxacine.

La stabilité des enzymes représentant un frein majeur dans le développement de matériaux bio-sourcés, l'utilisation de biocatalyseurs issus d'environnements extrêmophiles a été envisagée. La PLL SsoPox issue de *Sulfolobus solfataricus* a notamment été identifiée, s'avérant particulièrement efficace pour bloquer la virulence bactérienne par QQ [37,49]. Cette enzyme extrêmement robuste [190,191], a dans un premier temps été immobilisée dans des membranes d'alumines nanoporeuses tout en conservant une forte efficacité dans la réduction de sécrétion de facteurs de virulence, pyocyanine et élastase de *P. aeruginosa* PAO1 [192]. Un variant de l'enzyme, SsoPox-W263I, s'est ensuite révélé efficace pour réduire significativement la virulence de 51 isolats cliniques de *P. aeruginosa* issus d'ulcères de pied diabétique et a conservé ses effets envers la souche *P. aeruginosa* PAO1 après immobilisation dans un revêtement de polyuréthane via réticulation au glutaraldéhyde [193]. L'efficacité in vivo de ce variant a également été évaluée par administration intra-trachéale et a entrainé une augmentation significative du taux de survie dans un modèle de pneumonie chez le rat infecté par *P. aeruginosa* PAO1 [155].

En plus de ces études utilisant des agents QQ dégradant les AHL, un rapport récent a décrit l'utilisation de la kinase LsrK ciblant les AI-2. Cette enzyme attachée à une capsule faite de polymères biologiques de chitosane et d'alginate enrichi en ATP, a permis de réduire le QS régulé par les AI-2 [194].

Conclusions et perspectives

Au cours de ces 15 dernières années, de nombreux travaux ont démontré le fort potentiel thérapeutique du blocage des communications bactériennes. Un grand nombre d'études ont mis en évidence les bénéfices de cette approche dans le domaine des dispositifs médicaux fonctionnalisés. Actuellement, les connaissances sur les mécanismes de résistance, que pourraient éventuellement développer les bactéries pour contrer le QQ, sont encore très limitées [195,196]. Tandis que certains QSI ont des effets inhibiteurs de croissance, exerçant ainsi une pression de sélection, les enzymes quant à elles ne nécessitent pas un contact direct avec les bactéries et agissent de manière catalytique depuis l'extérieur de la cellule sans affecter la survie [193]. Par conséquent, les QQE constituent des candidats de choix combinant une pression de sélection minimale, sinon inexistante, et d'importants effets inhibiteurs sur la formation de biofilm et la virulence.

La vaste incidence du QS sur la physiologie des bactéries laisse à penser que le QQ serait une stratégie appropriée non seulement pour réduire la virulence bactérienne mais également pour réduire la tolérance aux antibiotiques et aux bactériophages, ouvrant la voie à de futures polythérapies.

Il est intéressant de relever que la perturbation du signal bactérien, un système de communication rencontré dans la plupart des communautés bactériennes [197], a des répercussions allant au-delà de la physiologie d'une seule population de bactéries. En effet, le microbiote intestinal de poissons nourris avec des bactéries probiotiques, produisant des QQE, a été modifié et la population de la bactérie pathogène *Aeromonas hydrophila* a été réduite [198]. D'une manière similaire, la population et la virulence d'*E. coli* ont été réduites au sein du microbiote intestinal de porcs sevrés nourris avec des bactéries probiotiques inhibant l'activité d'AI-2 [199]. Une étude récente a également montré la capacité d'une souche de *E. coli* surproductrice d'AI-2 à contrer l'impact d'une dysbiose intestinale induite par traitement à la streptomycine, soulignant que le QS joue probablement un rôle au sein des consortia microbiens multiespèces [200]. Des études complémentaires sont toutefois nécessaires pour déterminer les effets induits par les stratégies de QQ tant au niveau d'espèces bactériennes isolées que dans le cas de communautés complexes. Les recherches futures permettront d'affiner les connaissances relatives à l'action des molécules QQ et le potentiel de leur utilisation thérapeutique, seule ou combinée, pour le traitement des infections bactériennes.

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Résumé

De nombreuses bactéries utilisent un système de communication, appelé *quorum sensing* (QS), qui leur permet de synchroniser leur comportement proportionnellement à la densité de population. Parmi elles, certaines bactéries pathogènes de l'homme, des animaux ou des plantes utilisent le QS pour coordonner leur virulence et la formation de biofilm à haute densité cellulaire. Bloquer la communication bactérienne, ou *quorum quenching*, constitue donc une piste prometteuse pour étendre l'arsenal thérapeutique antibactérien en vue de compléter voire remplacer l'action des antibiotiques qui sont associés à de nombreux phénomènes de résistance. L'antibiorésistance serait responsable de 700 000 morts par an ce qui en fait un enjeu majeur de santé publique. Durant cette thèse, un intérêt particulier a été porté à *Sso*Pox, une lactonase hyperstable issue de l'archée extrémophile *Sulfolobus solfataricus*, capable d'hydrolyser les acyl homosérine lactones (AHL) impliquées dans le QS de certaines bactéries à Gram négative pathogènes de l'Homme comme *Acinetobacter baumannii*, *Burkholderia cepacia* ou encore *Pseudomonas aeruginosa*. La grande stabilité de cette enzyme constitue un avantage technologique pour des applications dans le domaine de la santé comme le développement de dispositifs médicaux innovants pour lutter contre les infections associées au soin (IAS) pour lesquelles le taux de multirésistance peut dépasser les 20%. En vue de son incorporation dans des pansements, *Sso*Pox a été étudiée et caractérisée pour son utilisation dans les infections à *P. aeruginosa*. La première partie de ce travail de thèse a consisté à étudier la résistance de l'enzyme aux contraintes industrielles rencontrées lors des procédés de fabrication des pansements. *Sso*Pox s'est montrée tolérante à la chaleur (jusqu'à 150°C), aux solvants organiques, au stockage à température ambiante (plus de 300 jours) ou encore à la stérilisation (éthylène oxyde, autoclave, radiations). Dans une deuxième partie, l'efficacité de l'enzyme pour limiter la production de biofilm et de facteurs de virulence a été démontrée sur 2 souches modèles ainsi que 51 isolats cliniques de *P. aeruginosa*. *Sso*Pox s'est par ailleurs révélée plus efficace que deux inhibiteurs chimiques connus du QS utilisés seuls ou combinés. De plus, l'efficacité de l'enzyme est conservée après immobilisation ce qui est particulièrement prometteur vis-à-vis de son utilisation dans des pansements. Enfin, une dernière partie a été consacrée à l'étude phénotypique et moléculaire du quorum quenching par voie enzymatique de *P. aeruginosa*. *Sso*Pox et une autre lactonase *Gc*L, isolée de *Geobacillus caldoxylosilyticus* et ayant un spectre d'action distinct sur les AHL, ont été comparées. Des expériences *in vitro* ont permis de mettre en évidence des impacts différents entre les deux enzymes sur les facteurs de virulence et la formation du biofilm. Une étude *in vivo* a montré que *Sso*Pox, contrairement à *Gc*L, réduit la virulence de *P. aeruginosa* à l'égard des amibes. L'étude de l'expression des gènes du QS et du protéome ont révélé des impacts distincts entre *Gc*L et *Sso*Pox, cette dernière se montrant globalement plus efficace pour bloquer des protéines intervenant dans le biofilm, la résistance aux antimicrobiens et la virulence. Ainsi *Sso*Pox semble constituer un candidat de choix pour le développement de dispositifs médicaux innovants pour limiter les IAS.

Abstract

Numerous bacteria use a communication system, named *quorum* sensing (QS), to synchronize group behaviors according to population density. Among them, human, animal and plant pathogens use QS to coordinate virulence and biofilm formation at high cell density. Interfering with bacterial communication, or *quorum quenching*, represents a promising target to extent antibacterial drug resources to complement and even replace antibiotics which are associated with drug resistance. Antibiotic resistance would be responsible of 700 000 deaths a year, representing one of the major healthcare problems. Along this PhD project, the focus was brought to *Sso*Pox, a hyperstable lactonase isolated from the thermophilic archaea *Sulfolobus solfataricus,* able to hydrolyze acyl homoserine lactones (AHL) involved in the QS of some human Gram-negative pathogen bacteria like *Acinetobacter baumannii*, *Burkholderia cepacia* or *Pseudomonas aeruginosa*. The high stability of this enzyme is an important advantage for health applications like innovative medical devices to fight healthcare associated infections (HAI) for which the incidence of multidrug resistance can exceed 20%. In view of its incorporation into wound dressings *Sso*Pox was studied and characterized for its use against *P. aeruginosa* infection. The first part of this project was to study the enzyme resistance to industrial constraints encountered during wound dressings manufacturing processes. *Sso*Pox demonstrated a high tolerance to heat (up to 150°C), organic solvents, ambient temperature storage (more than 300 days) and to sterilization processes (ethylene oxide, autoclave, radiations). In a second part, the enzyme efficiency to limit biofilm and virulence factors production was shown on 2 model strains and 51 clinical isolates of *P. aeruginosa. Sso*Pox was also more efficient than two well characterized chemical QS inhibitors, alone or combined. Furthermore, the enzyme kept its efficiency even when immobilized, which is especially promising for its use in wound dressings. Finally, a last part was dedicated to phenotypical and molecular study of enzymatic *quorum* quenching of *P. aeruginosa. Sso*Pox and another lactonase *Gc*L, isolated from *Geobacillus caldoxylosilyticus*, having distinct AHL specificities were compared. *In vitro* experiments highlighted different impacts between enzymes on virulence factors and biofilm production. An *in vivo* study showed that *Sso*Pox, unlike *Gc*L, was able to reduce *P. aeruginosa* virulence toward amoeba. QS gene expression and proteome study revealed distinct impacts between each enzyme treatment, *Sso*Pox showing globally a higher efficiency to block biofilm, antimicrobial resistance and virulence proteins. Thus, *Sso*Pox seems to be a prime candidate for development of innovative medical devices to limit HAI.