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Biotechnological exploitation of bacterial communication processes: from quorum sensing inhibition to the generation of synthetic cells interfacing with natural cells

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Abstract

The asocial existence of the bacterial cell has been a major paradigm in microbiology for centuries. In the 300 years since van Leeuwenhoek's descriptions of the microbial world, bacteria have been regarded as deaf mute individual cells designed to proliferate but unable to communicate and interact with each other. The discovery of quorum sensing (QS) communication systems in recent times dramatically changed our way of thinking at bacterial cells. Indeed, it is now acknowledged that *via* QS bacteria can monitor their cell density and consequently coordinate gene expression at the population level, thus displaying collective behaviours that resemble capacities typical of multicellular organisms. Bacterial social phenotypes controlled by QS systems include bioluminescence, motility, biofilm formation, synthesis of secondary metabolites, competence, and production of virulence factors in plant, animal and human pathogens.

In the last decades many scientists aimed at untangling complex QS networks governing bacterial social behaviours, others at exploiting QS circuits as useful tools in synthetic biology applications or as targets for new therapeutic approaches. In particular, bacterial QS systems have been employed in synthetic biology to generate engineered bacterial cells able to synchronize gene expression at the population level. Moreover, since QS controls the expression of virulence traits in different human pathogens, QS systems are considered promising targets for the development of antivirulence drugs that reduce bacterial pathogenic potential with limited selective pressure for the emergence of resistance.

One of the model organisms for QS studies is the Gram-negative bacterium *Pseudomonas aeruginosa*, one of the most dreaded opportunistic human pathogens, in which a complex network of four interconnected QS circuits controls the expression of multiple virulence factors. *P. aeruginosa* infections are difficult to eradicate since this pathogen is particularly resistant to currently available antibiotics. The necessity for new therapeutic options to treat *P. aeruginosa* infections is testified by a recent release from the World Health Organization ranking this bacterium in the Priority 1 group of antibiotic-resistant pathogens for which new drugs are urgently needed. In this context, QS in *P. aeruginosa* represents both a suitable system to untangle the complex regulatory networks leading to the emergence of social traits in unicellular organisms, and a potential target for the development of antivirulence drugs decreasing *P. aeruginosa* pathogenicity.

This PhD thesis builds up on previous knowledge on QS and virulence in *P. aeruginosa*, on regulatory properties emerging from complex regulatory circuits, on synthetic biology applications and on antivirulence strategies, to exploit the QS communication systems of *P. aeruginosa* for future biomedical applications. In details, experimental and methodological works, as well as

review articles, will be presented aimed at *i)* understanding new regulatory properties emerging from the peculiar topological architecture of the *P. aeruginosa las* QS circuit, *ii)* exploiting the *P. aeruginosa rhl* QS system for the generation of synthetic cells able to interface with natural cells, and *iii)* identifying new antivirulence drugs targeting the *P. aeruginosa pqs* QS system.

Overall, the results produced in this PhD thesis increase our knowledge of the *P. aeruginosa* QS circuits and pave the way for future therapeutic applications based on synthetic cells and on repurposed drugs with antivirulence activity.

Riassunto

Per secoli i batteri sono stati considerati organismi unicellulari incapaci di comunicare e privi di comportamenti sociali, il cui unico scopo era riprodursi. Negli ultimi anni, la scoperta del *quorum sensing* (QS) ha drasticamente cambiato questo pensiero. Il QS, infatti, è un sistema di comunicazione intercellulare mediante il quale i batteri possono attivare l'espressione genica in modo coordinato in funzione della loro densità cellulare, comportandosi come membri di una comunità piuttosto che come singoli individui. I fenotipi controllati dai sistemi di QS in vari batteri includono la bioluminescenza, la motilità, la formazione di biofilm, la sintesi di metaboliti secondari, la competenza e la produzione di fattori di virulenza in patogeni vegetali, animali e umani.

Negli ultimi decenni, molti studi hanno permesso di chiarire i complessi circuiti regolativi che governano i sistemi di QS, mentre altri lavori hanno sfruttato circuiti genetici basati sul QS come strumenti per applicazioni biotecnologiche, o come bersagli per nuovi approcci terapeutici. In particolare, i sistemi di QS batterici sono stati impiegati nella biologia sintetica per generare cellule batteriche ingegnerizzate in modo tale da sincronizzare l'espressione genica a livello di popolazione ed espletare specifiche funzioni in modo coordinato. Inoltre, poiché il QS controlla l'espressione di determinanti di virulenza in diversi patogeni umani, i sistemi di QS sono considerati promettenti bersagli per lo sviluppo di farmaci antivirulenza, in grado di ridurre il potenziale patogeno dei batteri inducendo una minor pressione selettiva per l'emergenza di cloni resistenti rispetto agli antibiotici.

Uno degli organismi modello maggiormente utilizzati per lo studio del QS è il batterio Gram-negativo *Pseudomonas aeruginosa*, un patogeno opportunista umano. In tale batterio, quattro sistemi di QS interconnessi tra loro controllano l'espressione di diversi fattori di virulenza. Le infezioni da *P. aeruginosa* sono difficili da eradicare poiché questo batterio patogeno è

particolarmente resistente agli antibiotici attualmente disponibili. Un recente comunicato dell'Organizzazione Mondiale della Sanità ha classificato *P. aeruginosa* nel gruppo 1 di priorità in una lista di batteri patogeni resistenti agli antibiotici per i quali sono urgentemente necessarie nuove strategie terapeutiche. In questo contesto, il QS in *P. aeruginosa* rappresenta sia un affascinante e complesso modello di studio per chiarire i circuiti di comunicazione e regolazione genica che portano all'insorgenza di tratti sociali negli organismi unicellulari, sia un potenziale bersaglio per lo sviluppo di farmaci antivirulenza in grado di ridurre il potenziale patogeno di tale batterio.

Questa tesi di dottorato si basa sulle precedenti conoscenze acquisite circa il funzionamento dei sistemi di QS di *P. aeruginosa* e del loro impatto sui processi di virulenza di tale batterio, su studi finalizzati alla comprensione di proprietà emergenti dei circuiti regolativi complessi, e su approcci biotecnologici nell'ambito della biologia sintetica e delle strategie antivirulenza, al fine di sfruttare i sistemi di QS di *P. aeruginosa* per future applicazioni biomediche. In particolare, in questa tesi di dottorato saranno presentati lavori sperimentali e metodologici volti a: *i)* comprendere nuove proprietà regolative che emergono dalla particolare architettura del sistema di QS *las* di *P. aeruginosa*, *ii)* sfruttare il sistema di QS *rhl* di *P. aeruginosa* per la generazione di cellule sintetiche in grado di comunicare con le cellule naturali, e *iii)* identificare nuovi farmaci antivirulenza in grado di inibire il sistema di QS *pqs* di *P. aeruginosa*.

Nel complesso, i risultati ottenuti in questa tesi di dottorato hanno consentito di migliorare la nostra conoscenza dei sistemi di QS di *P. aeruginosa* e di gettare le basi per future applicazioni biomediche basate su cellule sintetiche in grado di interagire con cellule naturali e sull'utilizzo in terapia di farmaci con attività antivirulenza.

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Chapter 1

Introduction and aims

1.1 Bacterial chemical communication *via* quorum sensing

The asocial existence of the bacterial cells has been a major paradigm in microbiology for a long time. Since van Leeuwenhoek's descriptions of the microbial world up to few decades ago, bacteria were primarily considered as autonomous unicellular organisms with no capacity for collective behaviours. In 1973, François Jacob stated: "It is perfectly possible to imagine a rather boring Universe without sex, without hormones and without nervous systems peopled only by individual cells reproducing *ad infinitum*. This Universe in fact exists. It is the one formed by a culture of bacteria" (Dunny and Winans, 1999). However, the first evidence of bacterial social behaviours can be traced back to 1965, when Alexander Tomasz reported that the ability of a *Streptococcus pneumoniae* population to enter the competence state is governed by a self-produced extracellular factor. With a remarkable and inspired intuition, Tomasz stated: "Since the activator - a cell-produced chemical - seems to impose a high degree of physiological homogeneity in a pneumococcal population with respect to competence, one is forced to conclude that in this case a bacterial population can behave as a biological unit with considerable coordination among its members. One wonders whether this kind of control may not be operative in some other microbial phenomena also" (Tomasz, 1965). Few years later, Kenneth H. Nealson, Terry Platt and J. Woodland Hastings unravelled the population density dependency of light emission in the bioluminescent marine bacterium *Vibrio fischeri* (Nealson *et al*., 1970), thus paving the way for the identification of the first signal molecule used by bacteria to coordinate gene expression at the population level (Eberhard *et al*., 1981).

A huge amount of experimental evidences collected in the last decades has revealed that bacteria are highly social organisms that preferentially live in communities, in which the expression of certain phenotypes in individual cells is coordinated by cell-to-cell communication at the population level (Williams and Càmara, 2009). Bacterial social life relies on their ability to exchange information *via* chemical communication systems.

One of the most studied bacterial cell-to-cell communication systems is quorum sensing (QS), by which a bacterial population co-ordinately reprograms gene expression in response to cell density *via* self-produced signal molecules (Joint *et al*., 2007; von Bodman *et al*., 2008). Any QS system consists of the genes coding for the QS signal synthase (or the QS signal pro-peptide) and for the QS signal reception apparatus (Williams, 2007). Signal molecules are small diffusible compounds or secreted modified peptides, which activate or repress QS target gene(s) once a crucial threshold concentration of the signal has been reached. Some QS signal molecules interact with receptors at the cell surface, while others act following internalization, depending on their ability to freely

diffuse across the cell envelope (Williams and Càmara, 2009). As the bacterial population density increases, so does the concentration of the QS signal molecules in the external environment (Fig. 1). Once a crucial threshold concentration has been attained, activation of a signal receptor or of a signal transduction system leads to the induction or repression of QS target gene(s).

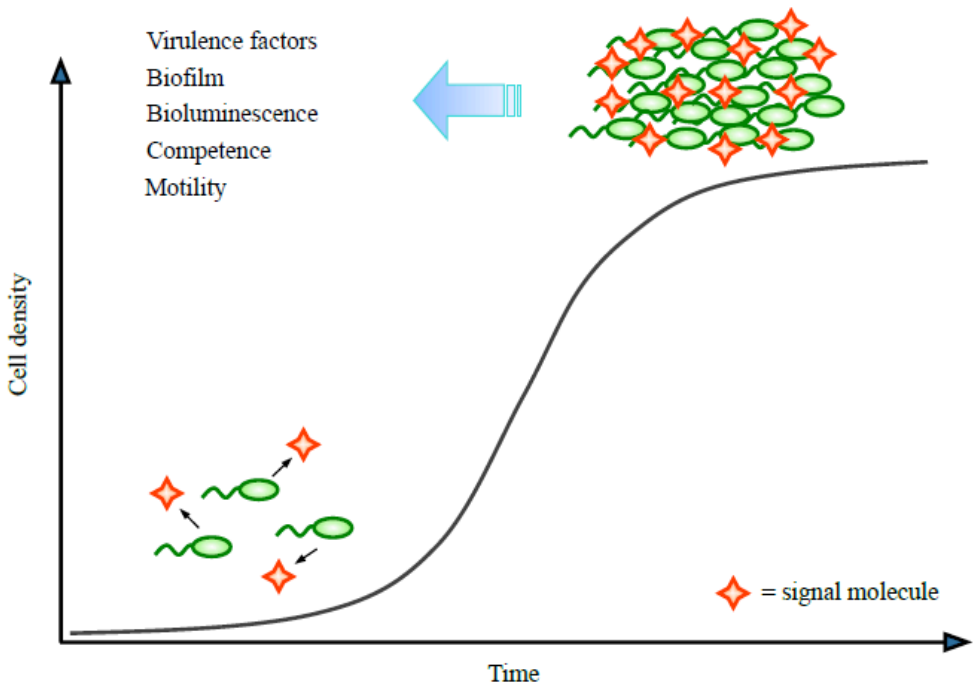

Figure 1. Schematic representation of the QS process. The signal molecule is constitutively produced at basal levels by bacterial cells. At low cell density, the signal molecule concentration is not sufficient to trigger a physiological response in the bacterial population. With increasing cell density, the signal molecule accumulates until a threshold level is reached, and then it activates a specific receptor functioning as a transcriptional regulator, which alters the expression of target genes in all the members of the bacterial population. Some of the phenotypes controlled by QS are indicated in the picture. Image modified from Dunn and Stabb, 2007.

Since the genes coding for the signal synthase (or for the signal pro-peptide) are positively controlled by the activated signal reception system, QS circuits are subject to an auto-regulatory positive feedback (autoinduction) that amplifies signal molecule production; for this reason, signal molecules are also referred to as autoinducers. Consequently, the size of the "*quorum*" depends on the relative rates of production and loss of the signal molecule, which in turn vary depending on local environmental conditions and metabolic status of the cell population (Williams, 2007; Williams and Càmara, 2009).

QS systems allow bacteria to trigger complex activities such as control of secondary metabolism, root nodulation, bioluminescence, protein secretion, motility, virulence factor production, plasmid transfer, and biofilm formation. Moreover, QS plays a critical role in both pathogenic and symbiotic

bacteria-host interactions. In general, QS facilitates the coordination of population behaviours to enhance access to nutrients or specific environmental niches, collective defence against other competitors or community escape where survival of the population is threatened (Williams, 2007; Garg *et al*., 2013).

The first QS system was characterized at the molecular level in the early '70s in *V. fischeri* (Nealson *et al.,* 1970), a bioluminescent marine bacterium that establishes a symbiotic relation with the Hawaiian squid *Euprymna scolopes*. At sunset, *V. fischeri* colonizes the light organ of the squid wherein it grows until it reaches a high cell density that induces the expression of genes required for bioluminescence. Light emission by *V. fischeri* camouflages the squid at night by eliminating its shadow within the water column, thus reducing predation by benthonic fishes. The *V. fischeri* QS system is based on the autoinducer synthase LuxI, which produces the signal molecule 3-oxo-hexanoyl-homoserine lactone ($3OC_6$-HSL), a member of the *N*-acyl-homoserine lactones (AHLs) family, and on the cytoplasmic receptor of the signal molecule, the LuxR protein (Fig. 2).

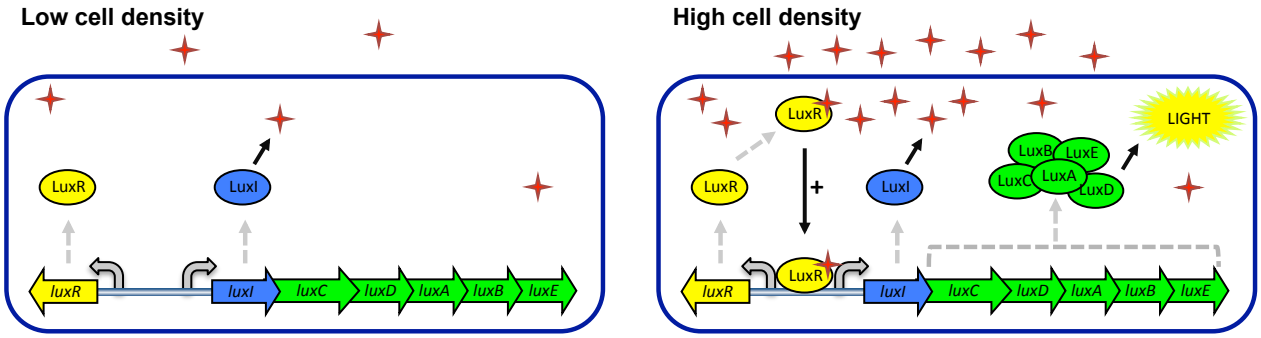

Figure 2. Schematic representation of the *lux* QS system in *V. fischeri*. At low cell density, LuxI produces the signal molecule $3OC_6$-HSL at a basal level. The concentration of $3OC_6$-HSL increases during bacterial growth. At high cell density, the concentration of $3OC_6$-HSL reaches a threshold level at which it binds to and activates LuxR. Activated LuxR increases the expression of LuxI and of the LuxCDABE enzymes, required for light emission. Modified from Waters and Bassler, 2005.

As the cell density of the bacterial population increases, so does the concentration of $3OC_6$-HSL, that freely diffuses among the bacterial cells and accumulates until its level reaches a critical threshold. Once reached this crucial concentration, $3OC_6$-HSL binds to its cognate receptor LuxR. The LuxR/$3OC_6$-HSL complex is now active and works as a transcriptional regulator by triggering the expression of the luciferase operon (*luxICDABE*), required for bioluminescence. Since the first gene of the *luxICDABE* operon codes for the synthase LuxI, the LuxR/$3OC_6$-HSL complex also triggers the production of the $3OC_6$-HSL itself, resulting in a positive feedback loop that enhances

the production of the signal molecule (Nealson *et al*., 1970; Engebrecht *et al*., 1983; Kaplan and Greenberg 1985; Waters and Bassler, 2005).

Bacteria produce chemically different classes of signal molecules (Fig. 3). Indeed, QS systems rely on different mechanisms of signal synthesis and response in Gram-negative and Gram-positive bacteria. The most intensively investigated QS systems in Gram-negative bacteria employ AHLs as signal molecules, and resemble the QS system of *V. fischeri* described above. AHL biosynthesis is typically catalysed by LuxI-family synthases, which transfer an acyl group from an acylated acyl carrier protein (acyl-ACP) to the methionyl amine of *S*-adenosyl-L-methionine (SAM), after which cyclization of the methionyl moiety to homoserine lactone occurs. The length of the acyl side chain (usually from 4 to 18 carbons), saturation and oxidation state at position 3, determine the resulting AHL structure, and thus signal-specificity. Short-chain AHLs generally freely diffuse across membranes, while there is some evidence for active efflux of AHLs with longer acyl side-chains. AHLs generally function by binding to a cognate intracellular receptor protein belonging to the LuxR-family. In most cases, the LuxR-like receptor-AHL complex binds to target promoters, activating gene expression (Miller and Bassler, 2001; Atkinson and Williams, 2009).

In Gram-positive bacteria, QS systems generally rely on genetically encoded peptides as signal molecules, often termed "autoinducing peptides" (AIPs). AIPs are expressed as inactive pro-peptides *via* canonical ribosomal synthesis, and later processed and modified to generate the active QS signal. AIPs are not freely diffusible across membranes, thus their perception by the receiver cell is usually mediated by transmembrane sensor kinases, which transduce the signal from the membrane to cognate response regulators inside the cell *via* a phosphorylation cascade (Miller and Bassler, 2001; Atkinson and Williams, 2009).

Another class of QS signal molecules produced by both Gram-negative and Gram-positive bacteria is composed by furanones, which are synthetized by the enzyme LuxS starting from *S*-ribosyl-L-homocysteine (SRH), which is hydrolyzed to homocysteine and 4,5-hydroxy-2,3-pentanedione (DPD). DPD spontaneously cyclizes generating a series of furanones, collectively known as autoinducer-2 (AI-2) (Miller *et al*., 2004). AI-2 cannot freely diffuse across membranes, and the putative transmembrane proteins involved in its export outside the cell have not been identified yet. The AI-2 transduction mechanism is mediated by a transmembrane sensor histidine kinase, which activates a phosphorylation pathway that involves a cognate response regulator. The activated response regulator ultimately binds to target promoters and controls gene transcription (Waters and Bassler, 2005).

Some bacteria possess several interacting QS circuits, that can employ multiple signal molecules from the same or different chemical classes (Williams, 2007). Moreover, bacteria have evolved

complex social interactions, including inter-species and inter-kingdoms interactions. For example, bacteria belonging to different species can produce and perceive the same signal molecule, while other bacteria may perceive the signal molecules produced by other species, even if they are not able to produce these molecules by themselves. Some bacteria produce enzymes capable of degrading QS signal molecules produced by other species. In other words, different bacterial species can "speak" the same language, "eavesdrop" other bacterial conversations, or interfere with communication systems of other species. The same interactions of the QS communication processes can also occur between bacteria and eukaryotic organisms, including plants and humans (Stacy *et al.,* 2012; Kendall and Sperandio, 2016).

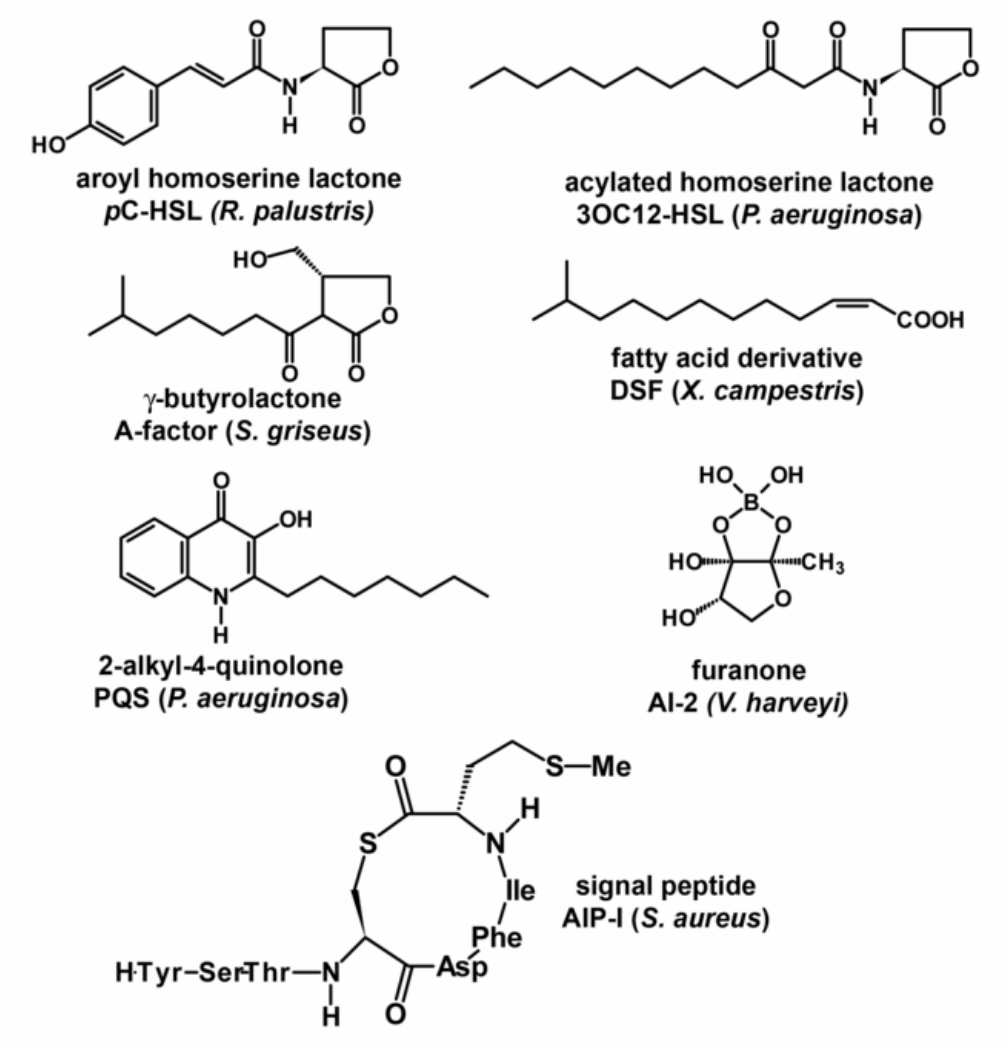

Figure 3. The structures of some common QS signal molecules. Image modified from Atkinson and Williams, 2009.

Besides the interest of QS in fundamental biology to elucidate the mechanism controlling collective behaviours, including coordinate expression of virulence factors in bacterial pathogens,

and to unravel the evolution of social traits in individual cells, in the last decade QS communication systems have been exploited for biotechnological applications. In particular, bacterial QS systems have been employed in synthetic biology to generate engineered bacterial cells able to synchronize gene expression at the population level (Choudhary and Schmidt-Dannert, 2010). As an example, a synthetic genetic circuits in which the *lux* QS system of *V. fischeri* controls the expression of the gyrase inhibiting toxin CcdB was used to limit the growth of engineered *Escherichia coli* cells, with the aim of cell density self-maintenance in a productive scenario (Wang *et al*., 2014).

Moreover, since QS controls the expression of virulence traits in different human pathogens, including *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Vibrio cholerae*, QS systems have been employed as targets for antivirulence drugs (LaSarre and Federle, 2013; Rampioni *et al*., 2014a). Examples of biotechnological applications exploiting QS for biomedical purposes will be discussed in the following sections.

1.2 Exploitation of quorum sensing in synthetic biology

Synthetic biology is an interdisciplinary branch of biology that applies engineering principles to biology in order to build novel organisms or bio-inspired systems that do not exist in nature, for the development of new biotechnological approaches (Arkin, 2009). To this aim, one of the main focuses of synthetic biology is the characterization and definition of standard biological parts that can be easily assembled by a modular architecture design to generate organisms (or parts of organisms), whose behaviour is predictable and orthogonal to other concurrent processes, so displaying new functional synthetic devices (Canton *et al*., 2008; Ausländer *et al*., 2017). The need for standards to be used in synthetic biology led to the recent development of the BioBriks database (Sleight *et al*., 2010), in which well-characterized biological parts (*e.g.*, genes, promoters, ribosome binding sites, transcriptional terminators) and devices (*e.g.*, operons, transcriptional regulatory units) are listed and described for modular assembly of new genetic circuits. The standard parts and devices can be assembled *via* classical molecular biology techniques inside an appropriate natural or artificial host, also known as “chassis”, in order to generate synthetic organisms able to perform specific functions in a controllable and predictable way (Danchin, 2009; De Lorenzo, 2011). In most of the cases, biological chassis are based on bacterial cells, *i.e. E. coli* cells (Brandley *et al*., 2016), despite many synthetic biology studies are conducted on eukaryotic cells (Black *et al*., 2017) and on simplified cells models, such as lipid vesicles (Cashera and Noireaux, 2014).

Synthetic biology approaches often take advantage of mathematical simulations generated from *in silico* modelling in order to foresee and sometimes avoid unpredictable interactions that may

occur between the synthetic genetic/metabolic route and the cellular environment hosting the artificial devises (Endler *et al*., 2009; Manzoni *et al*., 2016). Overall, synthetic biology integrates and exploits most of the impressive recent technological advancements in manipulating molecules (nucleic acid synthesis and sequencing), analysing and categorizing data (high throughput screening, easy access to informatics databases), and creating complex models (bioinformatics, systems biology).

There are countless applications in which the synthetic biology engineering approach could provide a significant advancement, including industrial productions, environmental remediation, computing, and human health (Cheng and Lu, 2012; Goss *et al.,* 2012). Other than practical applications, the synthetic biology "understanding by building" approach also pursues the genuinely scientific goal of using engineering manipulations of biological parts to better understand biological systems. The aim is the construction of artificial models of natural biological processes to experimentally explore aspects of life that are difficult to be studied through the classical analytical approach (*i.e.* taking apart biological systems), or that are not accessible in natural scenarios of research (Salehi-Reyhani *et al*., 2017). The construction of synthetic cells from separate parts, consisting in the assembly of synthetic cells endowed with a minimal degree of complexity, is a paradigmatic expression of a synthetic biology research line joining both biotechnological and theoretical aspects (Luisi *et al.,* 2006; De Lorenzo and Danchin, 2008).

In the last years, synthetic biology approaches have exploited bacterial QS systems for the development of engineered organisms for biomedical applications (Saeidi *et al*., 2011; Din *et al*., 2016). As an example, in 2011 Saeidi and colleagues genetically engineered a non-pathogenic *E. coli* strain to detect and kill the severe human pathogen *P. aeruginosa* (Saeidi *et al*., 2011). Briefly, the researchers functionalised *E. coli* with a synthetic genetic devise including sensing, lysis and killing modules (Fig. 4A). The sensing module was designed based on one of the four QS system of *P. aeruginosa*, the *las* QS system, in which the LasR receptor activates the expression of target genes upon binding to the QS signal molecule *N*-3-oxo-dodecanoyl-homoserine lactone ($3OC_{12}$-HSL) (Fuqua *et al.*, 1996). As shown in Fig 4B, *E. coli* cells containing a synthetic gene circuit for constitutive expression of LasR and for LasR/$3OC_{12}$-HSL-dependent expression of the green florescent protein GFP were able to emit fluorescence in response to exogenous $3OC_{12}$-HSL. At this point, the *gfp* gene was replaced with the gene coding for the lysin E7. In this case, *E. coli* cells underwent lysis in the presence of $3OC_{12}$-HSL (Fig. 4C). Finally, the synthetic circuit was implemented with the gene coding for the pyocin S5, under the control of a promoter activated by the LasR/$3OC_{12}$-HSL complex. The resulting genetic devise allowed to the engineered *E. coli* cells to sense *P. aeruginosa, via* the interaction between the LasR receptor expressed by *E. coli* and the

$3OC_{12}$-HSL QS signal released by *P. aeruginosa*, and to consequently produce the lysine E7 and the pyocin S5. Lysine E7 provoked *E. coli* lysis and hence release of pyocin S5 in the medium, thus killing the pyocin S5-sensitive *P. aeruginosa* strain Ln7 (Fig. 4D; Saeidi *et al*., 2011).

Overall, the work by Saeidi and colleagues describes the generation of an engineered non-pathogenic *E. coli* strain able to detect and kill a bacterial pathogen, by exploiting a QS system.

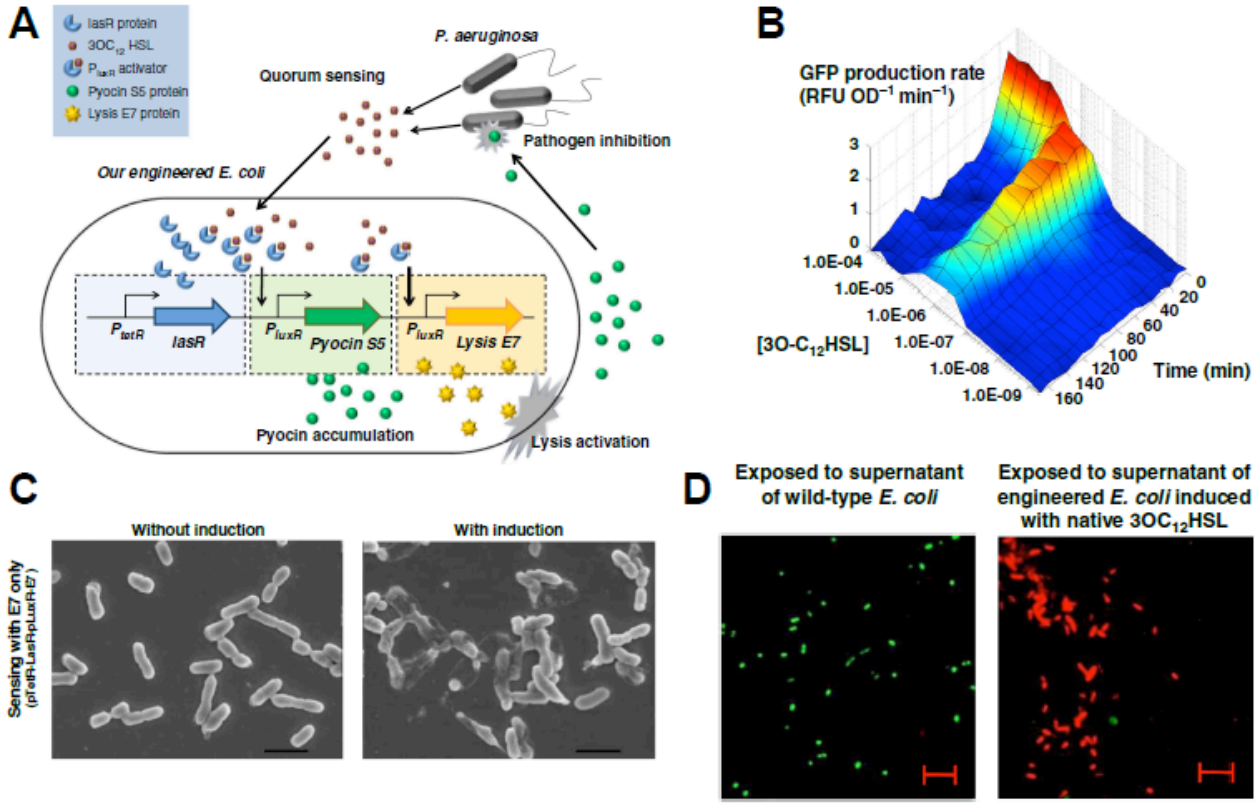

Figure 4. (A) Schematic representation of "Pathogen Sensing and Killing" system. *luxR* promoter is activated by LasR/$3OC_{12}$-HSL complex, leading to the production of lysine E7 and pyocin S5 within the *E. coli* chassis. After lysine E7 attains the threshold concentration that causes the chassis to lyse, the accumulated pyocin S5 is released into the exogenous environment and kills *P. aeruginosa*. **(B)** GFP production rate *per* cell over time at different $3OC_{12}$-HSL concentrations. **(C)** Effect of $3OC_{12}$-HSL-dependent expression of lysine E7 on *E. coli* cell morphology. The results show that the surface of *E. coli* cells carrying the genetic devise in which the LasR/$3OC_{12}$-HSL complex activates the expression of lysine E7 was damaged in the presence of $3OC_{12}$-HSL. **(D)** *P. aeruginosa* Ln7 incubated with supernatants from wild type *E. coli* (left panel) or from the engineered *E. coli* strain carrying the genetic device in which the LasR/$3OC_{12}$-HSL complex activates the expression of lysine E7 and pyocin S5, grown in the presence of $3OC_{12}$-HSL (right panel). *P. aeruginosa* cells were stained using the LIVE/DEAD cell viability assay. Image modified from Saeidi *et al*., 2011.

In a recent paper, Din and collaborators generated an *ad hoc* engineered bacterium in which single cells are able to synchronize their anti-tumour activity *via* a QS circuit (Din *et al*., 2016). The

rationale of this work is based on the ability of some bacteria, including *Salmonella enterica* serovar Thyphimurium, to co-localize with solid tumours once injected in the bloodstream of mice presenting tumour masses (Maeda, 2013). On this basis, Din and collaborators engineered an attenuated *S. enterica* serovar Thyphimurium strain endowed with synchronized autolysis and release of a therapeutic protein. In this case, the *lux* QS system of *V. fisheri* was exploited to generate a synthetic genetic circuit in which the constitutively expressed LuxR receptor, once activated at a specific cell density by the signal molecule $3OC_6$-HSL (produced by the LuxI synthase) was able to bind to target P*luxI* promoters triggering the expression of four different genes: *i*) *luxI*, resulting in the typical auto-regulatory positive feedback of QS mechanisms (activator module); *ii*) *sfGFP*, coding for a brighter variant of the GFP protein (reporter module); *iii*) *φX174 E*, coding for a lytic protein (lysis module); *iv*) *hlyE*, coding for haemolysin E, a toxin that kills tumour cells by creating pores on their membranes (therapeutic module) (Fig. 5A). Therefore, the $3OC_6$-HSL produced by the bacterial cells is expected to activate the QS circuit and to drive the LuxR-dependent expression of: *a)* sfGFP, leading to fluorescent emission; *b)* the anti-tumour effector haemolysin E; *c)* the lytic protein φX174 E, causing bacterial lysis and consequent release of haemolysin E. Firstly, the authors demonstrated that the engineered *S. enterica* strain expressed sfGFP and underwent coordinated lysis when a certain cell density was reached (Fig. 5B). Few bacterial cells surviving the lysis stage were able to grow and to reach the cell density at which a new cycle of sfGFP expression and lysis occurred, thus generating an oscillatory pathway. Moreover, engineered *S. enterica* strain was able to co-localize with the tumour mass *in vivo* (Fig. 5C; in this case the genes for bioluminescence emission in place of the *sfGFP* gene were used), and to provoke lysis in HeLa tumour cells (Fig. 5D). Finally, the *S. enterica* strain carrying the synthetic gene circuit was shown to increase the survival rate of mice with hepatic colorectal metastases when administered in combination with the chemotherapeutic agent 5-fluorouracil (5-FU) (Fig. 5E; green line)

Overall, the work by Din and co-workers represents an outstanding example of possible exploitation of QS systems for the generation of engineered bacterial cells endowed with the ability to accomplish specific tasks in a coordinated and programmable way, and paves the way for the use of these powerful tools in future theranostic (*i.e.* therapeutic and diagnostic) applications. Moreover, this work highlights the importance of basic knowledge on the regulatory properties conferred to the expression of target genes by the peculiar topological organization of their regulatory elements.

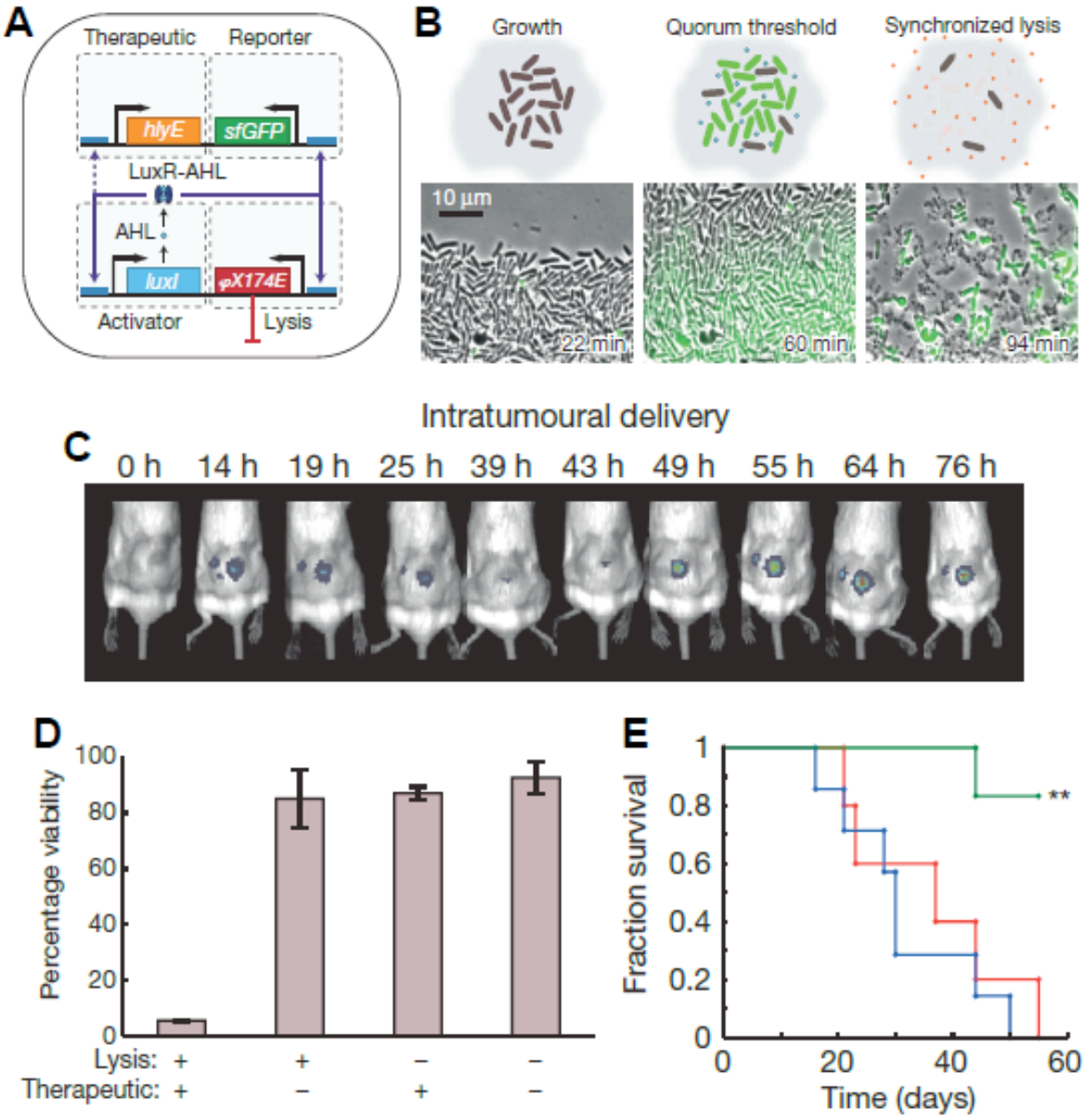

Figure 5. (A) The circuit contains an activator and lysis plasmid. When the population reaches the quorum threshold at a critical AHL concentration, the *luxI* promoter drives the transcription of gene *φX174 E* for lysis, *luxI*, *hlyE* and *sfGFP* or *luxCDABE,* as the reporter module. The *luxI* or the *tac* promoter also drives the transcription of the therapeutic gene for the stabilized circuit used *in vivo*. LuxR expression is driven by the native *luxR* promoter. **(B)** The main stages of each lysis cycle. Shown below the schematic depictions are typical time series images of the circuit harbouring cells undergoing the three main stages of quorum firing in a microfluidic growth chamber. **(C)** *In vivo* imaging over time of a mouse bearing two hind flank tumors injected once with the engineered bacterium strain in which LuxR/3OC$_6$-HSL complex drives the expression of *φX174 E* for lysis and *luxCDABE* operon for the bioluminescence emission. **(D)** Percentage viability of HeLa cells cocultured with supernatant from the engineered bacterium strain expressing both the φX174 E lytic protein (Lysis +) and/or the haemolysin E (Therapeutic +). **(E)** Fraction survival over time for the mice having hepatic colorectal metastases, with the dosing schedule of both engineered bacteria and a common cytotoxic chemotherapeutic, the antimetabolite fluorouracil (5-FU; green line). Image modified from Din *et al*., 2016.

Indeed, the engineered *S. enterica* strain developed by Din and co-workers was endowed with alternate cycles of growth and lysis, thus behaving as a biological oscillator (Din *et al*., 2016). A similar behaviour was exploited by the same group to develop an *E. coli* biosensor strain able to emit pulsed fluorescence with different periods of oscillation depending on the concentration of a pollutant in the growth medium. Also in this case, a QS system was employed to synchronize pulsed fluorescence in *E. coli* engineered cells at the population level (Prindle *et al*., 2012). Oscillatory gene expression, as well as other temporal and spatial dynamic properties of gene regulation, including robustness, fold-change detection, acceleration or deceleration of the response time, delay of the on or off states, all rely on specific network motifs. These are simple recurrent regulatory patterns composed of few interconnected transcriptional regulators, that constitute the "building blocks" of the intricate full regulatory pathways found in living organisms (Alon, 2007). Network motifs are overrepresented in regulatory pathways because they have been evolutionarily selected to perform well-defined regulatory functions (Fig. 6; Milo *et al*., 2002; Riccione *et al*., 2012).

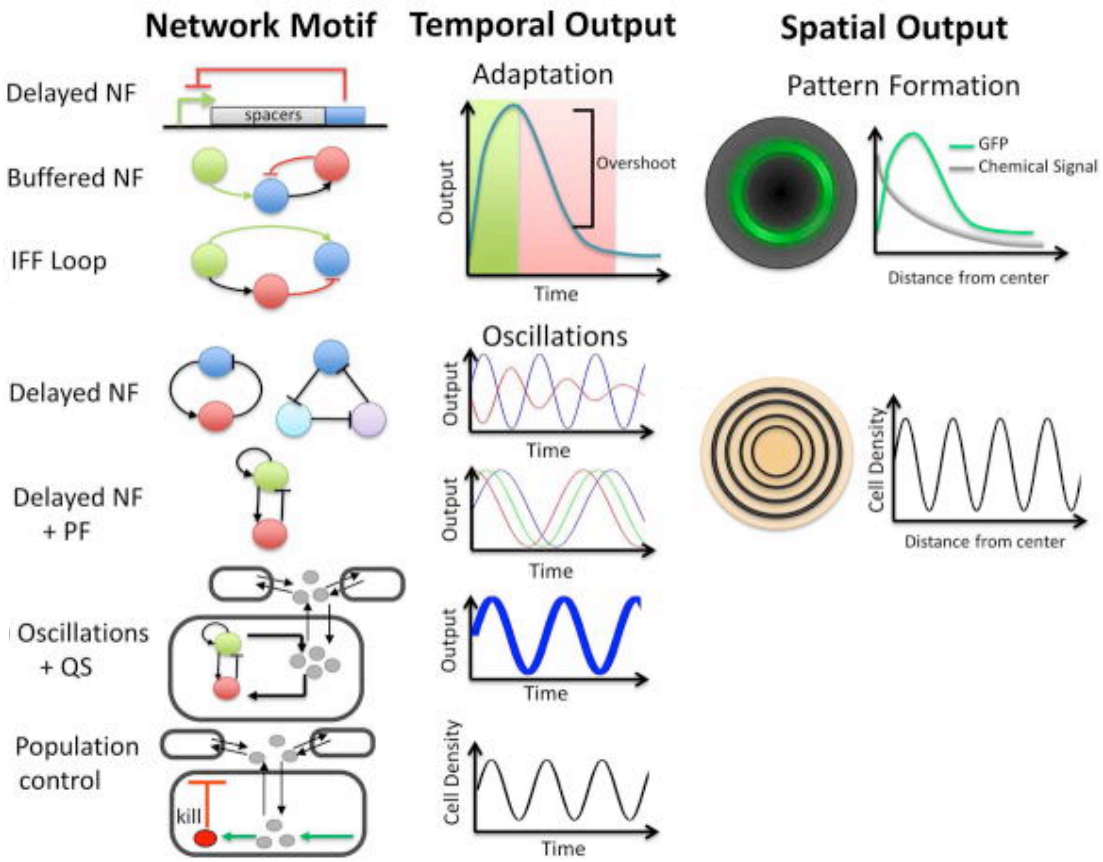

Figure 6. Examples of network motifs that generate autonomously regulated gene regulation dynamics and spatial distribution. NF, network motif; IFF Loop, incoherent feedforward loop; PF, positive feedback; QS, quorum sensing. Image modified from Riccione *et al*., 2012.

However, regulatory properties conferred from network motifs to their target genes are still poorly understood in most cases. A deeper understanding of the regulatory spatial-temporal dynamic patterns of gene expression associated to peculiar network motifs would allow future generation of engineered bacterial strains with implemented and more refined functional capabilities, calling for further studies focused on their investigation (Riccione *et al.,* 2012). An experimental work investigating the regulatory properties of a network motif governing QS in *P. aeruginosa* will be presented in Chapter 2 of this PhD thesis.

Moving back to the exploitation of QS systems in the synthetic biology field, a recently proposed approach envisage the use of synthetic cells endowed with communication capability for biomedical applications (Stano *et al*., 2012). In 2007, Leduc and collaborators proposed the potential application of nanotechnology to human health presenting the concept of injectable "nanofactories" (or "soft-nanorobots"). These are lipid vesicles-based molecular "nanomachine" that could be introduced into the human body to produce therapeutics *in situ*, to convert pre-existing materials into therapeutic compounds, or to transform molecules that a patient is unable to process, owing to some medical condition, into other compounds that the body can process (Leduc *et al.,* 2007). Examples of nanotechnology-enabled materials for drug delivery include nanoparticle therapies using liposomes to specifically encapsulate anti-cancer drugs (Farokhzad *et al.,* 2006). However, instead of delivering the drug itself, Leduc and co-workers envisaged the delivery of a nanofactory that could sense the need and consequently produce the drug on-site. As depicted in Fig. 7, researchers suggested the following features as key elements of future nanofactories: *i)* structural scaffold, like for example, lipid vesicles (liposomes). They have been extensively studied for drug delivery, because their structure permits the encapsulation of both hydrophilic and hydrophobic drugs, and at the same time they are biocompatible, consisting in the same lipids of biological membranes; *ii)* import/export capability to control the entry or exit of molecules *via* transmembrane transporters or α-haemolysin, a bacterial toxin that creates 2 nm channels in the membrane; *iii)* sensorial systems, like sensor proteins able to perceive stimuli; *iv)* internal set of enzymes for biochemical synthesis or conversion of molecules or drugs; *v)* external targeting capability, to localize the nanofactory to a specific tissue/cell type. Mechanisms for tissue-specific delivery could be taken from approaches that have already proved to be effective, such as receptor-ligand binding, or antibody-antigen binding (*i.e*. the so-called immuno-liposomes); *vi)* triggered self-destruction capability, in order to have control over the presence and activity of the nanofactory (Leduc *et al.,* 2007). Therefore, nanofactories are designed as minimal synthetic cells (or synthetic cells) endowed with functional properties typical of natural cells, such as the ability to interrogate their environment and to consequently accomplish programmable tasks, including modification of their

environment itself *via* production of chemical compounds. In this context, the ability to generate synthetic cells able to exchange information with natural cells *via* chemical communication would pave the way toward the realization of nanofactories for future biomedical applications.

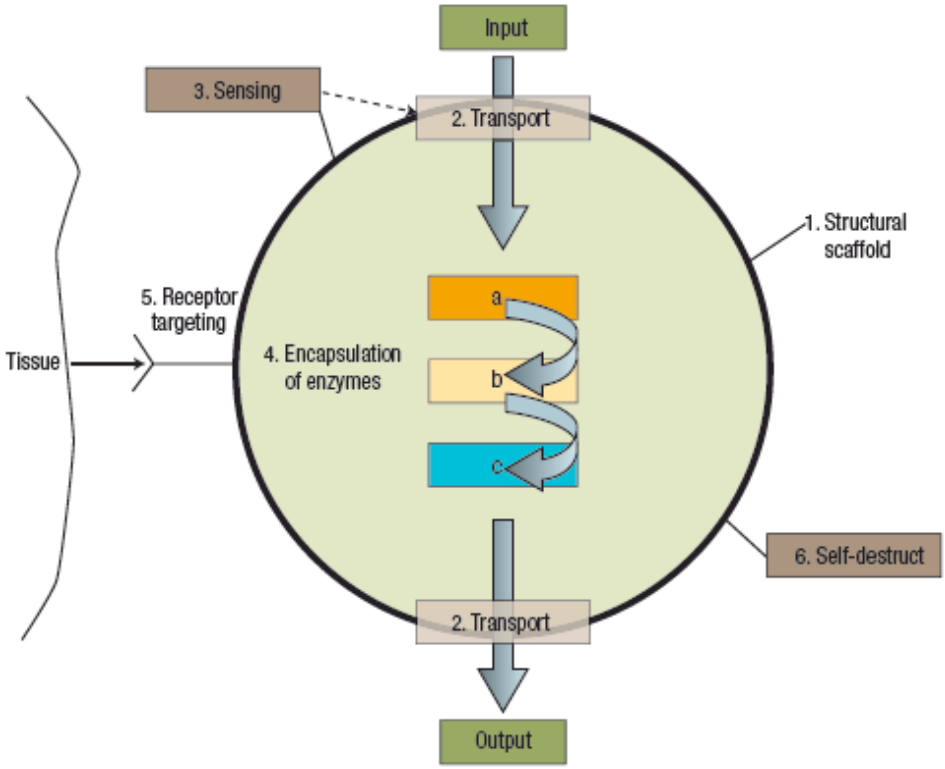

Figure 7. Schematic of six mechanisms in a biologically inspired nanofactory: *i)* a structural shell or scaffold; *ii)* transport to convey biomolecules to and from the environment; *iii)* sensing functionality; *iv)* encapsulation of biochemical machinery; *v)* targeting of the factory within the body; *vi)* externally triggered degradation to terminate a treatment in a controlled fashion. Image modified from Leduc *et al.,* 2007.

The first experimental report toward the generation of nanofactories (or synthetic cells) communicating with natural cells has been published in 2009 by the group of Ben Davis (Gardner *et al*., 2009). The authors encapsulated the precursors of the formose reaction inside lipid vesicles (also known as liposomes), and observed that one class of products of the intra-vesicular reaction escaped the vesicles through a channel formed by α-haemolysin. These products spontaneously reacted with the borate ions present in the external medium to generate furanosyl-boronates structurally similar to the QS signal molecule AI-2, that in *Vibrio harveyi* triggers bioluminescence. Remarkably, these molecules were functional in inducing light emission in a *V. harveyi* mutant strain unable to synthesize AI-2 (Fig. 8). Despite its great interest as a proof of concept study, the synthetic cellular model described by Ben Davis and collaborators has important limitations, since it

cannot be further implemented to produce a wide range of signal molecules, and it cannot be engineered to respond to signals sent by natural cells.

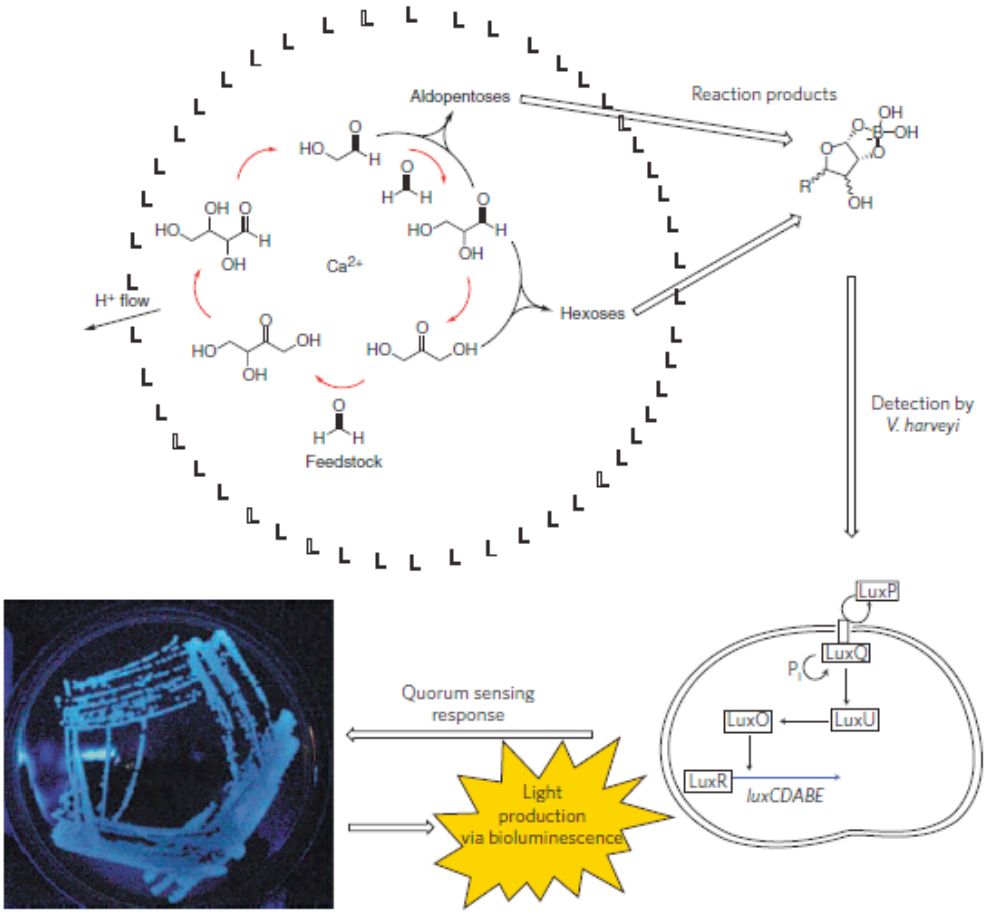

Figure 8. The components of the autocatalytic formose reaction are encapsulated within a lipid (L) vesicle, or liposome (top). The increased pH outside the vesicle initiates the production of carbohydrates. Carbohydrate-borate complexes are formed (top right), and these diffuse through the medium to interact with the bacterium *V. harveyi* (bottom right). Successful binding to the LuxP/LuxQ signal transduction proteins of the *V. harveyi* bacterium results in a protein phosphorylation response, and the subsequent expression of the genes *luxCDABE* (Pi, inorganic phosphate). The proteins produced give rise to a detectable bioluminescent output (bottom left, a photograph of an agar plate of *V harveyi* stimulated with formose). In this way, the products of the protocellular metabolism of a synthetic cell allow signaling to the cells of a natural organism. Image modified from Gardner *et al*., 2009.

In the wake of this research framework, our group proposed the generation of synthetic cells able to communicate with natural cells *via* QS sensing molecules (Stano *et al*., 2012). The synthetic cells envisaged by our group are liposome-based micro-compartments containing a minimal genetic circuit for the expression of a QS signal molecule synthase, hence endowed with the ability to synthesize QS molecules to be perceived by bacterial cells. This goal is achievable, at least in

principle, by encapsulating inside liposomes a plasmid containing a QS synthase gene, a cell-free DNA transcription (TX) and mRNA translation (TL) kit, and the substrates of the synthase. To this aim, an important step forward for the synthesis of proteins inside liposomes was achieved in 2001, when Takuya Ueda and collaborators introduced the PURE system (Protein synthesis Using Recombinant Elements) (Shimizu *et al*., 2001). It consists in a multimolecular TX-TL kit containing the minimum number of molecular components necessary for protein expression from DNA, hence it fits perfectly with the requirement of full-characterized parts/devices/systems in synthetic biology, and it is considered the "gold standard" cell-free TX-TL system for constructing synthetic cells (Stano *et al.*, 2011). On these bases, preliminary experimental work and *in silico* modelling studies investigating the possibility of generating synthetic cells that are able to synthesize QS signal molecules *via* the PURE system have been published by our group in the last years (Stano *et al*., 2012; Rampioni *et al*., 2013; Rampioni *et al*., 2014b; Stano *et al*., 2014).

Recently, the group of Sheref Mansy also exploited the PURE system to generate synthetic cells able to synthesize molecules perceived by bacterial cells. In this case, the expression of LuxI-like synthases inside liposomes, in the presence of acetyl coenzyme A and SAM, resulted in the production of QS-like molecules able to activate *E. coli*-based biosensor strains for AHLs detection (Lentini *et al*., 2017).

Theoretical and experimental works towards the generation of synthetic cells able to communicate with bacterial cells will be presented in Chapters 3 and 4 of this PhD thesis.

1.3 Quorum sensing as a target for antivirulence drugs

In recent times, the massive use of antibiotics as traditional approaches to combat bacterial infections has dramatically accelerated the emergence of antibiotic-resistant strains. Indeed, we are currently witnessing an alarming increase of multi-drug, or even pan-drug resistant pathogens, a problem that especially concerns a group of severe pathogens particularly relevant from a clinical point of view, known with the acronym ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, P. aeruginosa*, and *Enterobacter* species). ESKAPE pathogens currently cause the majority of hospital infections and effectively "escape" the effects of almost all available antibacterial drugs (Rice, 2008; Boucher *et al*., 2009). The concurrent increase of antibiotic resistance and decrease of new availability antibiotics is leading to the so called "post-antibiotic era" (Mohr *et al*., 2016), with limited treatment options for many bacterial pathogens and urgent need for new strategies to combat bacterial infections (Fernebro, 2011; Rampioni *et al.,* 2017). Indeed, many pharmaceutical companies consider the development of new

antibiotic drugs a less attractive investment compared with more lucrative therapeutic areas, since even if a new antibiotic is discovered, its lifetime is expected to be short due to rapid emergence of resistance (Luepke *et al*., 2017). In this context, antivirulence drugs provide a new attractive strategy for antibacterial therapies.

Whereas conventional antibiotics target bacterial viability, antivirulence drugs target bacterial functions required for the infection process, such as toxin function, toxin delivery, virulence gene regulation, or cell adhesion. Thereby, antivirulence drugs "disarm" rather than kill pathogens and, as a consequence, they are thought to alleviate the problem of emerging resistance (Rasko and Sperandio, 2010). Nonetheless, researchers are investigating whether rapid resistance to antivirulence drugs might also evolve (Mellbye and Schuster, 2011; Allen *et al*., 2014). *In vitro* social-evolution experiments suggest that resistant variants will not emerge if the antivirulence therapy specifically targets public goods, like virulence factors secreted by the producer cells and shared among all the members of a bacterial population. In this context, a resistant mutant to an inhibitor of an antivirulence drug should not experience any fitness benefit relative to sensitive clones because it would be the only cell in a sensitive population capable of producing public goods, which would not be sufficient to sustain the growth of the whole population. Additionally, the production of public goods is metabolically expensive for the cell, thus a resistant mutant to a drug that inhibits the production of public goods should be negatively selected with respect to the sensitive strains as a consequence of the metabolic burden associate to public goods production. Overall, mutants resistant to antivirulence agents targeting the production of public goods are not expected to emerge in a population of sensitive bacteria (Mellbye and Schuster, 2011; Allen *et al*., 2014; Rampioni *et al*., 2017). Besides exerting less selective pressure toward resistance, the antivirulence approach potentially has other advantages compared to the use of antibiotics, including preservation of the endogenous microflora and expansion of molecular targets for the development of new therapeutic agents (Rampioni *et al*., 2017). Furthermore, antivirulence drugs could be administered in combination with conventional antibiotics to enhance their efficacy (Rasko and Sperandio, 2010).

QS is considered an ideal target for antivirulence drugs, since in different bacterial pathogens this communication system controls the expression of multiple secreted virulence factors (*i.e.* public goods), and it is also related to antibiotic tolerance, because it is required for biofilm formation. Accordingly, many bacterial pathogens show decreased virulence in infection models when genes coding for to their QS systems are deleted. Strategies focused on inhibiting QS communication systems are generally referred to as quorum quenching (QQ) (LaSarre and Federle, 2013; Rampioni *et al*., 2014a).

The majority of QS systems are organized in a classical scheme, based on: *i*) the biosynthesis of the signal molecule by a sender cell; *ii*) the availability and functionality of the signal molecule; *iii*) the reception of the signal molecule and the consequent physiological response by a receiver cell. This recurrent scheme suggests that interference with QS can be applied at several levels by inhibiting signal molecules production, lowering signal activity/availability, or inhibiting signal reception (Fig. 9).

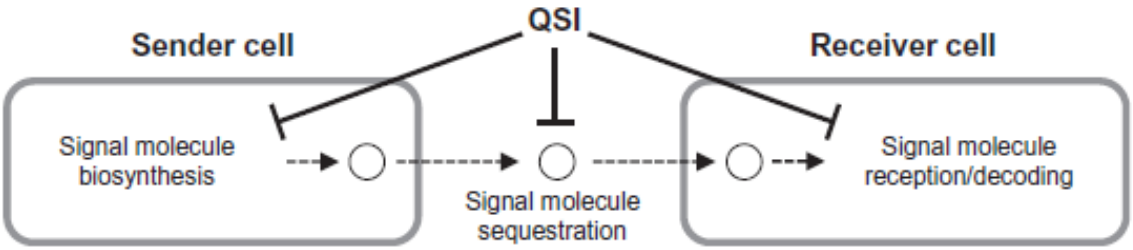

Figure 9. Schematic representation showing potential drug-targets of a QS system. A QS inhibitor (QSI) may target the biosynthesis of the signal molecule by the sender cell, functionality and availability (*i.e.* by sequestration) of the signal molecule, or reception/decoding of the signal molecule by the receiver cell. Image modified from Rampioni *et al*., 2014a.

A first strategy focuses on finding enzymes capable of hindering the signal molecule by modifying or degrading it, thereby altering its functionality. Well-known AHL-inactivating enzymes are lactonases and acylases produced by microorganisms themselves, plants and animals as a mechanism of defence (Chun *et al*., 2004). An example is PvdQ, an acylase produced by *P. aeruginosa*, which degrades the *las* signal molecule $3OC_{12}$-HSL. While PvdQ shows little activity against AHLs with side chains less than 10 carbons in length, a structure-based design approach led to the generation of a PvdQ variant with modified substrate specificity and activity towards shorter chain AHLs, such as *N*-octanoyl-homoserine lactone (C_8-HSL). This PvdQ variant was able to decrease the amount of C_8-HSL present in *Burkholderia cenocepacia* cultures, and was effective in protecting *Galleria mellonella* larvae from *B. cenocepacia* infection (Koch *et al*., 2014). The group of Paul Williams identified enzymes able to degrade the QS signals 2-alkyl-4-quinolones (AQs) produced by *P. aeruginosa*. In a first work, they observed that the structure of the substrate of the Hod dioxygenase from *Arthrobacter nitroguajacolicus* is similar to the *P. aeruginosa* signal molecule PQS, and they found that the Hod dioxygenase is able to bind and inactivate PQS, catalysing its conversion to *N*-octanoyl-anthranilic acid and carbon monoxide (Pustelny *et al*., 2009). In a second work, the same group demonstrated that *Achromobacter xylosoxidans* is endowed with enzymatic activity responsible for the conversion of PQS to the inactive molecule 2-

heptyl-2-hydroxy-1,2-dihydroquinoline-3,4-dione (Soh *et al*., 2015). A different QQ strategy has been developed by Kaufmann and colleagues, who used monoclonal antibodies to sequester AHLs, thereby limiting their bioavailability in the bacterial population (Kaufmann *et al*., 2006).

Even though not many works focused on targeting signal molecules biosynthesis, Christensen and co-workers identified molecules capable of inhibiting LuxI-like biosynthetic enzymes, thus decreasing AHLs production, through a cell-free screening assay (Christensen *et al*., 2013). Moreover, the elucidation of the AQs biosynthetic pathway paved the way for the discovery of novel QS inhibitors targeting the enzymes necessary for the production of these signal molecules (Calfee *et al*., 2001; Weidel *et al*., 2013; Thomann *et al*., 2016).

The majority of QQ strategies are directed to the inhibition of the QS signal molecule receptors. Most of them focus on signal molecule analogues capable of binding to the cognate QS receptor without triggering its activation. For example, Hartmann and colleagues described the synthesis of antagonists of the QS receptor PqsR of *P. aeruginosa*, based on its natural agonist 2-heptyl-4-quinolone (HHQ) (Klein *et al*., 2012), and the group of Paul Williams found a potent PqsR inhibitor by means of a rational design approach, based on the structural information obtained from the crystal structure of PqsR bound to one its natural ligands, 2-nonyl-4-quinolone (NHQ) (Ilangovan *et al*., 2013).

A number of QS receptor inhibitors have been also identified by high throughput screening (HTS) campaigns performed on *ad hoc* engineered biosensor strains (Rampioni *et al*., 2014a). As an example, this method led to the identification of garlic extract and 4-nitro-pyridine-*N*-oxide (4-NPO) as potent inhibitors of the QS receptor LasR of *P. aeruginosa* (Rasmussen *et al*., 2005). In this context, it is interesting to highlight that inhibitors identified *via* screening systems employing heterologous organisms as biosensors (most frequently engineered *E. coli* or *Chromobacterium violaceum*) may lack activity or even function as agonists when tested on the target pathogen (Galloway *et al*., 2011). Novel agonists may however still prove useful in the search for antagonists as exemplified by the HHQ analogue 2-heptyl-6-nitroquinolin-4*(1H)*-one, which acted as an antagonist in an *E. coli*-based biosensor strain but as an agonist in *P. aeruginosa*, as a consequence of a metabolic modification. However, a subsequent synthetic modification of 2-heptyl-6-nitroquinolin-4*(1H)*-one resulted in a strong PqsR antagonist in *P. aeruginosa* (Lu *et al*., 2012 and 2014).

Recent availability of crystal structures of QS receptors, like LasR and PqsR, also allows identifying antivirulence drugs through *in silico* approaches (Rampioni *et al*., 2014a). *In silico* approaches allow to preselect promising candidates *via* virtual computer-based screening, and to subsequently verify by wet-lab experiments the functionality of a reduced number of compounds,

by saving time and costs (Vasker, 2014; Reuter *et al*., 2015). Two studies highlighting the feasibility of *in silico* molecular docking to identify QS inhibitors have been recently published (Tan *et al*., 2013; Kalia *et al*., 2017). In both works, a library of natural compounds was tested through an *in silico* screening for the ability to bind to the *P. aeruginosa* QS receptor LasR, thus potentially inhibiting its functionality.

Overall, many studies succeeded in identifying antivirulence drugs targeting QS systems at different levels. Unfortunately, most of these drugs have toxic effects on eukaryotic cells, thus limiting their therapeutic potential (Rampioni *et al*., 2014a; Maura *et al*., 2016). A possible solution to this problem is searching for QS inhibitors among molecules already approved for their use in humans. This strategy is called drug repurposing, or drug repositioning, and shows remarkable advantages compared with *de novo* drug discovery (Wermuth, 2006). Indeed, drug repurposing approaches have higher probability to identify safe and bioavailable molecules with favourable pharmacological properties, that reducing the time and costs generally associated with standard drug discovery processes (Rampioni *et al*., 2017). Drug repurposing strategies can be based on serendipity, rational repurposing, or random screening. In the first case, the new activity of a drug already approved to treat different pathologies is discovered just as a casualty, while in the rational repurposing the similarity between the primary molecular mechanism targeted by the drug and the new molecular mechanism to be targeted drives the repositioning of the molecule toward new therapeutic approaches. In the random screening approach, libraries of drugs already approved for the treatment of various diseases are screened for a possible secondary activity. The majority of drugs endowed with a secondary antivirulence activity has been identified by this latter approach (Rampioni *et al*., 2017).

The screening is usually performed by using *ad hoc* engineered whole-cell biosensors, as is the case of an interesting study conducted by Francesco Imperi and co-workers, which screened a library of FDA (Food and Drug Administration)-approved compounds for their ability to inhibit the *las* QS system in *P. aeruginosa* (Imperi *et al*., 2013). The screening system was designed to select for molecules able to decrease light emission in a coculture of *P. aeruginosa* PA14 wild type and of the QS biosensor strain PA14-R3, unable to synthesize the signal molecule $3OC_{12}$-HSL (Δ*lasI*) and carrying a chromosomal transcriptional fusion between the $3OC_{12}$-HSL-activated promoter P*rsaL* and the *luxCDABE* operon for light emission (P*rsaL::lux*). Therefore, in this coculture system, *P. aeruginosa* PA14 produces the QS signal molecule $3OC_{12}$-HSL and PA14-R3 responds to this molecule by emitting light, unless a tested drug hampers the synthesis or reception of $3OC_{12}$-HSL (Massai *et al*., 2011). By using this coculture screening approach, the researchers succeed in discovering the new anti-QS property of the "old" anthelmintic drug niclosamide, which strongly

inhibits the *las* QS system in *P. aeruginosa*, leading to reduced expression of *las*-controlled virulence factors and protecting *G. mellonella* larvae from *P. aeruginosa* infection (Fig. 10; Imperi *et al*., 2013). This work can be considered as a proof of concept of the functionality of the drug repurposing approach in finding compounds that hamper QS signalling and reduce virulence in bacterial pathogens.

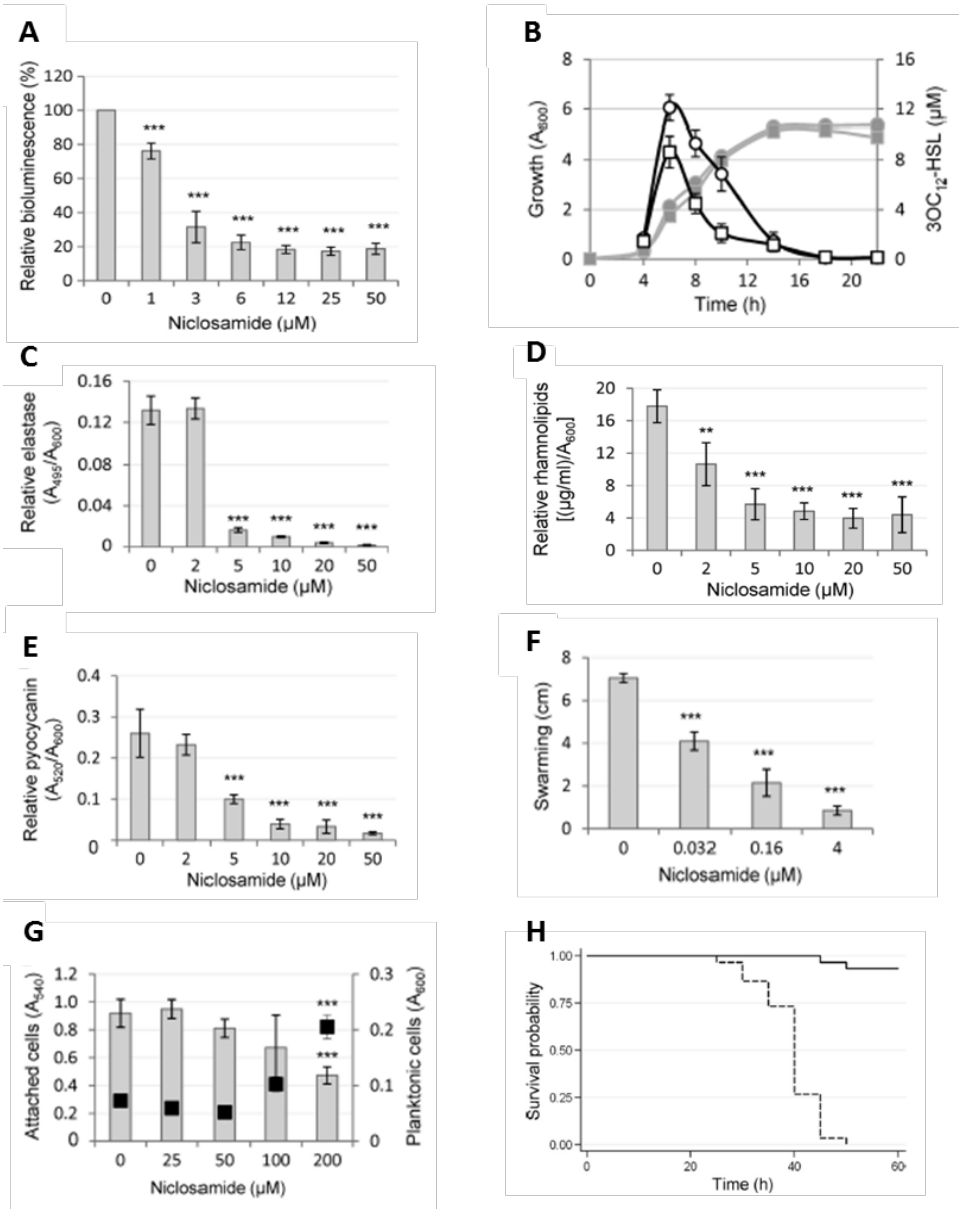

Figure 10. Effect of the anthelmintic drug niclosamide on: **(A)** the PA14/PA14-R3 coculture system; **(B)** *P. aeruginosa* PA14 growth curve (left *y* axis; grey lines and filled symbols) and $3OC_{12}$-HSL production (right *y* axis; black lines and open symbols); **(C)** elastase, **(D)** rhamnolipids and **(E)** pyocyanin production; **(F)** swarming motility; **(G)** biofilm formation, assessed as the amount of attached cells (left *y* axis; grey histogram) versus planktonic cells (right *y* axis; black squares); **(H)** *G. mellonella* larvae survival to *P. aeruginosa* PA14 infection (dashed line, larvae infected with *P. aeruginosa*; solid line, larvae infected with *P. aeruginosa* and treated with niclosamide). Image modified from Imperi *et al*., 2013.

A methodological work describing the coculture-based screening for the identification of anti-QS drugs and an experimental work using this approach to identify new FDA-approved inhibitors of the *pqs* QS system of *P. aeruginosa* will be presented in Chapters 5 and 6 of this PhD thesis, respectively.

1.4 Aims of the thesis

As discussed in the introduction sections 1.1 – 1.3, QS systems can be exploited in the biotechnological field for biomedical purposes. In particular, synthetic biology approaches can make use of synthetic gene circuits based on network motifs and on QS systems to generate engineered bacteria or synthetic cells endowed with the ability to accomplish programmable tasks useful in the therapeutic and diagnostic fields. Moreover, QS systems are suitable targets for the development of antivirulence drugs that reduce bacterial pathogenic potential with limited selective pressure for the emergence of resistant strains.

In this context, the aims of this PhD work have been: *i*) the investigation of new regulatory properties arising from the peculiar topological architecture of the network motif governing the *las* QS system in the human pathogen *P. aeruginosa* (Chapter 2); *ii)* the generation of synthetic cells interfacing with *P. aeruginosa via* QS chemical communication for the development of innovative drug-delivery approaches (Chapters 3 and 4); (*iii*) the identification of FDA-approved compounds targeting the *pqs* QS system of *P. aeruginosa* for the development of new antivirulence therapeutic approaches (Chapters 5 and 6).

Each of the following Chapters will be preceded by a short summary to present the publications (Chapters 2-5) or manuscript in preparation (Chapter 6) collected in this PhD thesis.

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Chapter 2

The multi-output incoherent feedforward loop constituted by the transcriptional regulators LasR and RsaL confers robustness to a subset of quorum sensing genes in *Pseudomonas aeruginosa*

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Preface to Chapter 2

Network motifs are simple regulatory patterns occurring in transcription networks in all living organisms much more often than what would be expected for random networks. It is generally acknowledged that network motifs are overrepresented in regulatory pathways because they have been evolutionarily selected to confer peculiar properties to the expression of their target genes. For example, distinct network motifs have been studied governing oscillatory or bistable phenotypes in bacterial and mammalian cells.

The paper presented in this Chapter aims at unravelling new regulatory properties conferred by the network motif controlling the *las* quorums sensing (QS) system of the human pathogen *Pseudomonas aeruginosa* to the expression of its target genes. Experiments performed in *P. aeruginosa* mutant strains revealed that the peculiar architecture of the *las* QS system splits the regulon controlled by the QS receptor LasR in two distinct sub-regulons, with different responsiveness to variations in LasR level. This emerging regulatory property increases *P. aeruginosa* phenotypic plasticity, thus contributing to its adaptability to fluctuating environments.

Overall, data produced in this work contribute to clarify an important aspect of the QS-mediated regulation of virulence factors production in *P. aeruginosa*, and unravelled new regulatory properties emerging from a peculiar network motif. Notably, synthetic biology approaches make use of network motifs, as well as of QS systems, to functionalize engineered microorganisms for biotechnological and biomedical applications. In this context, basic knowledge produced in this work could be also applied for future development of engineered organisms and synthetic cells with improved functional abilities. The latter issue will be reviewed in Chapter 3.

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The multi-output incoherent feedforward loop constituted by the transcriptional regulators LasR and RsaL confers robustness to a subset of quorum sensing genes in *Pseudomonas aeruginosa*†

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Quorum sensing (QS) is an intercellular communication system which controls virulence-related phenotypes in the human pathogen *Pseudomonas aeruginosa*. LasR is the QS receptor protein which responds to the signal molecule *N*-(3-oxododecanoyl)homoserine lactone ($3OC_{12}$-HSL) and promotes signal production by increasing the transcription of the $3OC_{12}$-HSL synthase gene, *lasI*. LasR also activates the expression of other genes, including *rsaL*, coding for the RsaL protein which acts as a transcriptional repressor of *lasI*. Direct gene activation and RsaL-mediated gene repression, both exerted by LasR on the expression of the output gene *lasI*, generate a regulatory network motif known as the type 1 incoherent feedforward loop (IFFL-1) that governs $3OC_{12}$-HSL production. In addition to *lasI*, RsaL directly represses a set of LasR-activated genes; hence, the IFFL-1 generated by LasR and RsaL is a multi-output IFFL-1. Here we demonstrate that the multi-output IFFL-1 constituted by LasR and RsaL confers robustness with respect to fluctuations in the levels of LasR to the phenotypes controlled by both these transcriptional regulators (*e.g.* $3OC_{12}$-HSL synthesis and pyocyanin production). In contrast, other virulence-related phenotypes controlled by LasR but not by RsaL (*e.g.* elastase and protease production) are sensitive to changes in LasR levels. Overall, the multi-output IFFL-1 generated by LasR and RsaL splits the QS regulon into two distinct sub-regulons with different robustness with respect to LasR fluctuations. This emerging regulatory property enhances the phenotypic plasticity of *P. aeruginosa*, thus contributing to its adaptation to changing environments.

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Introduction

Network motifs are simple regulatory patterns occurring in transcription networks much more often than what would be expected for random networks, and constitute the "building blocks" of intricate regulative pathways. It is generally acknowledged that network motifs are overrepresented in regulatory pathways because they have been evolutionarily selected to perform well-defined regulatory functions.[1,2]

One of the most common network motifs is the type-1 incoherent feedforward loop (IFFL-1), in which an activator X, upon the perception of an input stimulus, directly activates an output gene Z and simultaneously represses it by promoting the expression of a Z-repressor Y, thus exerting an "incoherent" (or "paradoxical") regulation (Fig. 1A).[2–4] The IFFL-1 is among the most frequent network motifs in eukaryotic systems, including yeast and animal cells.[1,3,5] For example, in animal cells the IFFLs-1 are involved in the transcription networks of embryonic and hematopoietic stem cells, in innate immune regulation, in fly eye development, in the control of neuronal regeneration, in long-range cell migration, and in other processes.[6–13] The IFFLs-1 are also well represented in bacteria where, among other functions, they have been demonstrated to control galactose metabolism in *Escherichia coli* and sporulation in *Bacillus subtilis*.[14–16]

The opportunistic pathogen *Pseudomonas aeruginosa* has three main quorum sensing (QS) systems, namely *las*, *rhl* and *pqs*, based on the production, secretion and perception of different signal molecules. QS signals are produced at a basal level at low

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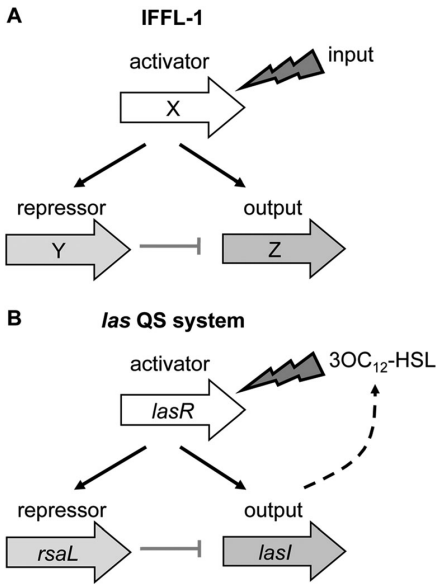

Fig. 1 Schematic representation of an IFFL-1 and the *las* QS system. (A) The activator X, upon the perception of an input signal, activates the transcription of the output gene Z and of the repressor of Z, Y. (B) The activator LasR, upon the perception of the input signal $3OC_{12}$-HSL, activates the transcription of the output gene *lasI* and of the gene coding for the repressor of *lasI, rsaL*. Arrows indicate positive regulations; T-bars indicate negative regulations; dashed arrow represents information flow.

cell density and accumulate in the extracellular milieu during growth. When QS signals reach a threshold concentration, corresponding to a certain cell density (the *quorum*), these molecules bind to and activate cognate transcriptional regulators, thus eliciting coordinated reprogramming of gene expression in the whole bacterial population. In *P. aeruginosa* the *las* QS system is arranged as an IFFL-1, since the intracellular receptor LasR (X) activated by the input signal *N*-(3-oxododecanoyl)homoserine lactone ($3OC_{12}$-HSL) simultaneously triggers the expression of the output gene *lasI* (Z) and the *lasI*-repressor gene *rsaL* (Y) (Fig. 1B).[17–19] Therefore, LasR has dual action on *lasI* expression: (i) positive direct regulation and (ii) negative indirect regulation mediated by RsaL. The *lasI* gene encodes for the $3OC_{12}$-HSL biosynthetic enzyme LasI. LasR is considered a key element of *P. aeruginosa* virulence because it activates genes involved in the production of virulence factors (*e.g.* elastase, proteases, pyocyanin) and it is also required for the full activation of the other two *P. aeruginosa* QS systems, the *rhl* and *pqs* systems, both positively contributing to the expression of *P. aeruginosa* virulence-related traits.[20,21]

The *las* IFFL-1 seems to be multi-output, since RsaL directly binds to and represses the promoters of multiple *P. aeruginosa* genes that are directly activated by LasR, besides *lasI* (*e.g.* genes for pyocyanin biosynthesis).[18,19,22,23]

The *las* IFFL-1 is embedded in the complex regulatory network of *P. aeruginosa*, since the expression of the *las* genes is finely regulated at the transcriptional and post-transcriptional levels in response to various metabolic and environmental stimuli.[21,24] The richness and complexity of regulatory networks and metabolic pathways confer a high phenotypic plasticity to this opportunistic pathogen that accounts for its ecological success in very diverse environments, including the human host.[25,26]

Theoretical and computational analyses suggest that IFFL-1 network motifs confer peculiar properties to the expression of their output gene(s). It has been proposed that the IFFLs-1 can influence the output gene Z by: (i) accelerating the response time, (ii) generating transcriptional pulses, (iii) generating non-monotonic input functions, (iv) providing fold-change detection properties, and (v) conferring robustness with respect to transcriptional noise.[3,14–16,27–34] The first three properties have been validated by using synthetic gene circuits[27,29] and/or by investigating the behaviour of natural gene circuits in whole cells.[14–16] Conversely, the ability of the IFFLs-1 to provide fold-change detection properties to their output genes has only been suggested by theoretical studies and *in silico* modelling.[30] The robustness with respect to transcriptional noise has been experimentally validated only in mammalian cells for regulatory systems in which the repressor element Y is a microRNA (miRNA).[31,32,35,36]

Both theoretical models and synthetic gene circuit experiments deal with the IFFL-1 as a three-gene circuit in isolation. In real cells, this circuit is usually embedded in a network of additional regulators and interactors, which complicate its behaviour with respect to *in silico* or *in vitro* observations. Therefore, it is important to experimentally validate the regulatory properties conferred to gene expression by the IFFL-1 in a natural cell scenario. This led us to investigate the *las* IFFL-1 regulatory circuit with respect to the expression of its output genes in the natural scenario of a *P. aeruginosa* culture.

Here, the effect of altered levels of LasR in *P. aeruginosa* was for the first time investigated in a mutant strain carrying the deletion of the *vfr* gene, which encodes the Vfr transcriptional regulator.[37] Vfr directly binds to the *lasR* promoter (P*lasR*) and activates its transcription; hence, a *P. aeruginosa* mutant deleted in *vfr* shows reduced expression of LasR.[38] Subsequently, recombinant strains expressing LasR under the control of an arabinose-dependent promoter were employed, in order to rule out any unpredictable effect of *vfr* mutation. The results obtained with these two complementary approaches consistently showed that the LasR regulon is divided into two distinct sub-regulons. The first sub-regulon is robust with respect to LasR variations, *i.e.* it tends to maintain its steady state for a certain range of fluctuations in the LasR level, and corresponds to the *las* IFFL-1 output genes, such as *lasI* and the genes involved in pyocyanin production. The second sub-regulon is sensitive to LasR variations, *i.e.* its expression parallels the fluctuations in the LasR level, and includes genes directly activated by LasR and not repressed by RsaL, such as those involved in elastase and protease production.

By characterizing an emergent property of the *las* IFFL-1 and its contribution to *P. aeruginosa* phenotypic plasticity, this study provides additional insights into the subtle mechanisms underlying the ecological fitness of this human pathogen.

Methods

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table S1 (ESI†). *Escherichia coli* S17.1 λ*pir*[39] was used for cloning purposes. All *E. coli* and *P. aeruginosa* strains were routinely grown at 37 °C in Luria–Bertani (LB) broth alone or supplemented with 1.5% (w/v) agar.[40] Unless otherwise stated, antibiotics were added at the following concentrations: *E. coli*, 100 μg mL^{-1} ampicillin (Ap), 25 μg mL^{-1} kanamycin (Km), 30 μg mL^{-1} chloramphenicol (Cm), 10 μg mL^{-1} tetracycline (Tc), and 15 μg mL^{-1} nalidixic acid (Nal); *P. aeruginosa*, 400 μg mL^{-1} Cm, 200 μg mL^{-1} Tc, and 400 μg mL^{-1} carbenicillin (Cb). When required, the medium was supplemented with L-arabinose at the concentration reported in the text.

Recombinant DNA techniques

The plasmids used in this study and details of their construction are reported in Table S2 (ESI†). The preparation of plasmid DNA, purification of DNA fragments, restrictions, ligations, and transformations of *E. coli* were carried out by standard procedures.[40] PCR amplifications were performed using Bio Red-Taq DNA polymerase (Bioline) according to the manufacturer's instructions. The oligonucleotides used in this study are listed in Table S3 (ESI†). Automated sequencing was performed by Genechron sequencing services (Genechron). When required, plasmids were transferred from *E. coli* S17.1 λ*pir* to *P. aeruginosa* PAO1 by conjugation.[41]

Construction of *P. aeruginosa* mutants

Mutant strains of *P. aeruginosa* were generated using the pDM4- or pEX18-derivative plasmids as previously described.[42,43] The pEX18Δ*lasR* plasmid (Table S2, ESI†) for the generation of the Δ*lasR* mutant strain was kindly provided by Prof. Martin Schuster (Department of Microbiology, Oregon Sate University, Corvallis, OR, USA).[44] The mutagenic plasmids pDM4Δ*rsaL* and pDM4Δ*lasR*Δ*rsaL* used to generate the Δ*rsaL* and Δ*lasR*Δ*rsaL* mutant strains, respectively, were generated in this study as described in Table S2 (ESI†). Briefly, the flanking DNA regions of the selected genes were PCR amplified using the primers listed in Table S3 (ESI†) and were sequentially cloned in pDM4. The resulting pDM4-derivative plasmids were independently introduced in *P. aeruginosa* PAO1 by conjugation. Clones with a chromosomal insertion of the pDM4-derivative plasmids were selected on LB agar plates supplemented with 400 μg mL^{-1} Cm and 10 μg mL^{-1} Nal. Plasmid excision from the chromosome was subsequently selected on LB agar plates supplemented with 10% (w/v) sucrose. Deletions were checked by PCR analysis.

P. aeruginosa strains for the arabinose-dependent induction of LasR were obtained by inserting a single copy of the genetic cassette *araC*-P_{BAD}*lasR* into a neutral site of the bacterial chromosome, allowing constitutive expression of AraC and carrying the *lasR* gene under the control of the P_{BAD} promoter. For this aim, the mini-CTX1-*araC*-P_{BAD}*lasR* plasmid has been generated as reported in Table S2 (ESI†).

Promoter activity assay

The activities of the *lasR* promoter (P*lasR*) and of the *lasI* promoter (P*lasI*) were measured in *P. aeruginosa* strains carrying the pMP-P*lasR*::*lacZ* and pMP-P*lasI*::*lacZ* plasmids, respectively, by a standard β-galactosidase assay.[45] The pMP-P*lasI*::*lacZ* plasmid was already available,[19] while the pMP-P*lasR*::*lacZ* plasmid has been generated in this study as described in Table S2 (ESI†). Briefly, overnight cultures of the PAO1 strains carrying the pMP190-derivative plasmids were diluted to an optical density at 600 nm wavelength of 0.01 (A_{600} = 0.01) in 10 mL of fresh LB broth and incubated at 37 °C with shaking at 200 rpm. Promoter activity (Miller units) and cell density (A_{600}) were determined after 9 h of growth at 37 °C.

Western blot analysis

Western immunoblotting was performed using a standard technique[40] with anti-LasR serum (1 : 1000)[22] and horseradish peroxidase-conjugate anti-rabbit IgG as secondary antibody (1 : 5000; Promega). Final development was performed with the Amersham ECL chemiluminescent reagents (Amersham Biosciences), and a ChemiDoc XRS + imaging system (Bio-Rad Laboratories) was used for data acquisition. Exposure parameters were kept constant for multiple Western-blot analyses. Band intensity was quantified by using the open-source image processing software ImageJ.

Protease, elastase and pyocyanin assays

In the phenotypic assays, cultures were diluted to an A_{600} of 0.02 in 50 mL of LB broth, and incubated at 37 °C with shaking at 200 rpm. For the experiments with the strains PAO1 Δ*lasR* *lasR*^{ind} and PAO1 Δ*lasR*Δ*rsaL* *lasR*^{ind}, 10 mL of LB broth was supplemented with different concentrations of L-arabinose ranging from 0.4 to 0.025% (w/v). Cell-free supernatants were collected after 9 hours of incubation. The concentrations of $3OC_{12}$-HSL, proteases, elastase and pyocyanin in the cell-free supernatants were measured as previously described.[46–49] The average measurements and relative standard deviations (SD) were calculated from at least three independent experiments. Statistical analysis was performed using Student's *t*-test. Differences were considered statistically significant when the p value was <0.05.

Results

The LasR–RsaL IFFL-1 confers robustness to the *las* QS system in *P. aeruginosa*

In order to investigate the effect of reduced levels of LasR in *P. aeruginosa* PAO1, we used the Δ*vfr* PAO1 mutant.[37] The deletion of *vfr* does not affect the growth rate of *P. aeruginosa*, since PAO1 Δ*vfr* showed the same growth profile and kinetics as the wild type (data not shown). β-Galactosidase assays and Western-blot analysis with the anti-LasR antibody were performed on PAO1 Δ*vfr* and on the wild type strain, both carrying the pMP-P*lasR*::*lacZ* plasmid for the analysis of the *lasR* promoter (P*lasR*) activity. In agreement with a previous report,[38]

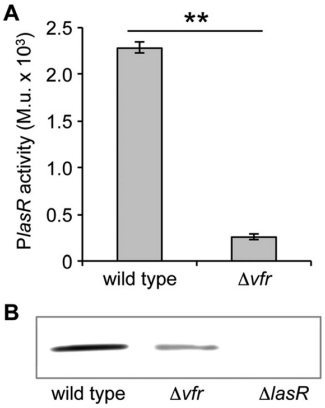

Fig. 2 Effect of *vfr* mutation on P*lasR* activity and LasR levels. (A) P*lasR* activity measured using a β-galactosidase assay in the indicated PAO1 strains carrying the transcriptional fusion P*lasR*::*lacZ*. M.u., Miller units. The average values from three independent experiments are reported with SD. **, $p < 0.01$. (B) Western-blot analysis performed with anti-LasR polyclonal serum on protein crude extracts from the PAO1 indicated strain; a *P. aeruginosa* Δ*lasR* strain was used as a negative control. The Western-blot result here reported is representative of three independent experiments.

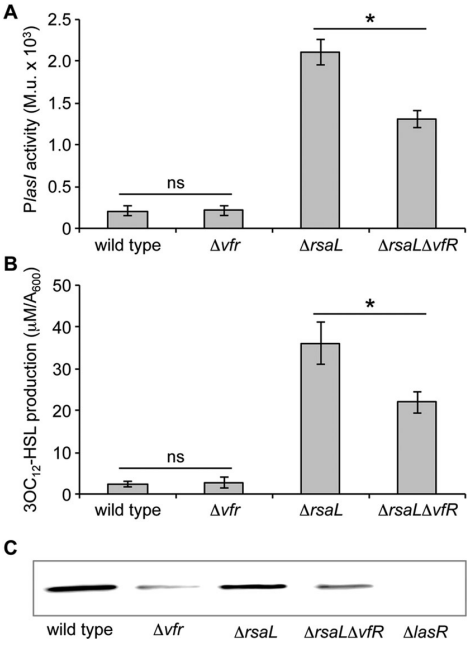

Fig. 3 Effect of *vfr* and *rsaL* mutations on P*lasI* activity, $3OC_{12}$-HSL production and LasR levels. (A) P*lasI* activity measured using a β-galactosidase assay in the indicated PAO1 strains carrying the transcriptional fusion P*lasI*::*lacZ*. M.u., Miller units. (B) $3OC_{12}$-HSL concentration measured in the cell-free supernatant of the indicated PAO1 strains. (C) Western-blot analysis performed with anti-LasR polyclonal serum on protein crude extracts from the indicated PAO1 strains; a *P. aeruginosa* Δ*lasR* strain was used as a negative control. For both (A and B), the average values from three independent experiments are reported with SD. *, $p < 0.05$; ns, not statistically significant. The Western-blot result here reported is representative of three independent experiments.

both P*lasR* activity and the LasR protein level were strongly reduced, though not abrogated, in the Δ*vfr* mutant relative to the wild type (14.8% residual P*lasR* activity and 31.5% LasR protein level in the Δ*vfr* mutant relative to the wild type strain; Fig. 2), confirming the activating role of Vfr on LasR expression. Hence, the activity of the *lasI* promoter (P*lasI*) and the level of the $3OC_{12}$-HSL signal molecule synthesized by LasI were measured in both the wild type and Δ*vfr* strains carrying the pMP-P*lasI*::*lacZ* plasmid, for the determination of P*lasI* promoter activity.[19] While causing a strong reduction of the LasR level (~29.9% relative to the wild type), *vfr* mutation did not affect P*lasI* activity and $3OC_{12}$-HSL production (Fig. 3). These results indicate that both the transcription of *lasI* and the LasI-mediated synthesis of the QS signal molecule $3OC_{12}$-HSL are robust with respect to variations in the concentration of the regulator LasR.

In order to demonstrate that the robustness of the output gene *lasI* is due to the IFFL-1 generated by LasR and RsaL, we disrupted this network motif by deleting the *rsaL* gene in the PAO1 Δ*vfr* mutant, and measured *lasI* transcription, $3OC_{12}$-HSL production, and LasR expression in the PAO1 Δ*rsaL* and PAO1 Δ*rsaL*Δ*vfr* strains, both carrying the pMP-P*lasI*::*lacZ* plasmid.

P*lasI* activity and $3OC_{12}$-HSL production were higher in the Δ*rsaL* mutant relative to the PAO1 wild type (Fig. 3) due to the loss of repression directly exerted by RsaL on *lasI* transcription.[18,19] Notably, P*lasI* activity and $3OC_{12}$-HSL synthesis were significantly reduced in the PAO1 Δ*rsaL*Δ*vfr* strain with respect to PAO1 Δ*rsaL* (60.8% residual P*lasI* activity and 71.9% $3OC_{12}$-HSL level in PAO1 Δ*rsaL*Δ*vfr* relative to PAO1 Δ*rsaL*; Fig. 3). This indicates that *lasI* expression was affected by *vfr* mutation only in the Δ*rsaL* genetic background, while no difference was observed when comparing the PAO1 wild type and Δ*vfr* strains. These results demonstrate that the IFFL-1 generated by LasR and RsaL confers robustness with respect to fluctuations in LasR levels to the expression of the output gene *lasI*, and consequently to the concentration of the QS signal molecule $3OC_{12}$-HSL.

The LasR-regulon is divided into two sub-regulons with different robustness with respect to LasR variations

In many IFFLs, the same X and Y regulators can control multiple output genes, resulting in multi-output IFFLs.[50] The *las* system has the features of a multi-output IFFL-1 because other genes besides *lasI* are directly activated by LasR and repressed by RsaL, such as the $phzA_1$-G_1 and *phzM* genes involved in pyocyanin production, and the *hcnABC* operon accounting for hydrogen cyanide biosynthesis.[19,22,51] In order to assess whether the robustness disclosed by *lasI* is also conserved in other output genes controlled by the multi-output *las* IFFL-1, we investigated the effect of reduced LasR levels caused by *vfr* mutation on pyocyanin production in the PAO1 wild type and PAO1 Δ*rsaL* genetic backgrounds. Pyocyanin production was chosen as the marker phenotype due to easy quantitative determination. Our results show that pyocyanin production follows the same rules as $3OC_{12}$-HSL production and P*lasI* transcription, since it

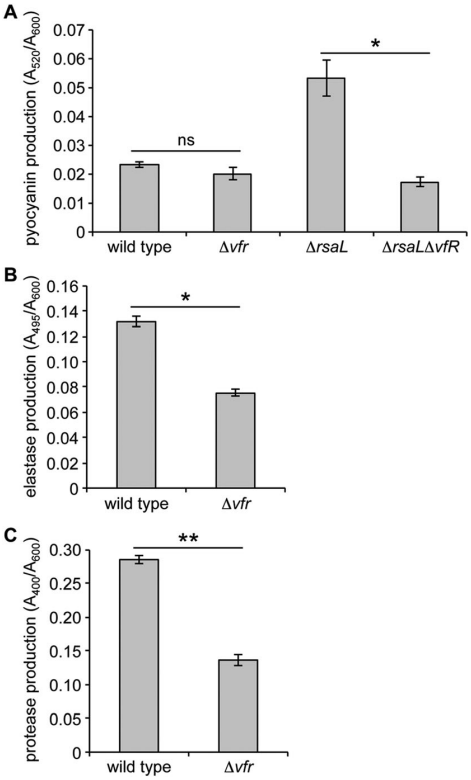

Fig. 4 Effect of *vfr* and *rsaL* mutations on pyocyanin, elastase and protease production. (A) Pyocyanin, (B) elastase and (C) protease production measured in the cell-free supernatant of the indicated PAO1 strains. For the three assays, the average values from three independent experiments are reported with SD. *, $p < 0.05$; **, $p < 0.01$; ns, not statistically significant.

is not significantly affected by the *vfr* mutation in the *rsaL*-proficient strains Δ*vfr* and wild type, but it decreases in PAO1 Δ*rsaL*Δ*vfr* relative to PAO1 Δ*rsaL* (31.7% pyocyanin production in PAO1 Δ*rsaL*Δ*vfr* relative to PAO1 Δ*rsaL*; Fig. 4A). Thus, the *vfr* mutation and hence a reduction in LasR levels affect the expression of the *las* IFFL-1 output genes only in the absence of *rsaL*.

These data indicate that irrespective of the possible influence of different ancillary regulators specifically acting on $3OC_{12}$-HSL or pyocyanin biosynthesis, the regulatory properties generated by the *las* IFFL-1 are conserved on different output genes (*e.g. lasI* and *phz*), as expected for an IFFL-1 multi-output network motif.

A sub-set of LasR-activated genes is not directly repressed by RsaL, *e.g.* the genes involved in elastase (*lasB*) and protease (*aprA*) production.[19,22] Such genes are not outputs of the *las* IFFL-1 and their expression behaviour is predicted not to be robust with respect to LasR variations. In agreement with this hypothesis, elastase and protease production in the Δ*vfr* mutant strain is reduced to 57.3% and 47.6% of the PAO1 wild-type level (Fig. 4B and C), as a consequence of the lower expression of LasR caused by *vfr* mutation.

In the experiments described above, it is not possible to exclude additional effects of *vfr* mutation on the tested phenotypes in addition to the reduced expression of LasR. To address this issue, a single copy of a genetic cassette for L-arabinose-dependent induction of *lasR* was introduced in a neutral site of *P. aeruginosa* PAO1 Δ*lasR* and of *P. aeruginosa* PAO1 Δ*lasR*Δ*rsaL* chromosomes, thus generating the PAO1 $lasR^{IND}$ and PAO1 Δ*rsaL* $lasR^{IND}$ strains, respectively. In these strains, LasR levels can be modulated by the exogenous provision of L-arabinose. In particular, 0.4% (w/v) L-arabinose caused the wild type expression of LasR, whereas no LasR was detectable at L-arabinose concentrations $\leq$0.025% (w/v). At intermediate L-arabinose concentrations, a gradual decrease in LasR expression was observed, *i.e.* ~80%, ~51%, and ~19% relative to the wild-type level for L-arabinose concentrations of 0.2%, 0.1% and 0.05% (w/v), respectively (Fig. 5A and B).

As shown in Fig. 5C, the levels of $3OC_{12}$-HSL produced by PAO1 $lasR^{IND}$ are maintained almost to their maximum value and do not show significant differences for L-arabinose concentrations ranging from 0.4% to 0.1% (w/v), whereas $3OC_{12}$-HSL production is reduced only for L-arabinose concentrations $\leq$0.05% (w/v). In contrast, in the PAO1 Δ*rsaL* $lasR^{IND}$ strain, in which no repressive effect is exerted by RsaL on *lasI* expression, $3OC_{12}$-HSL production gradually decreases as a function of reduced L-arabinose concentration. Indeed, $3OC_{12}$-HSL levels were significantly reduced to about 80% and 60% of the wild-type level for L-arabinose concentrations of 0.2% and 0.1% (w/v), respectively. Based on the Western-blot analysis shown in Fig. 5A and B, ~20% reduction in the LasR level is sufficient to significantly decrease $3OC_{12}$-HSL production in the *rsaL*-deficient genetic background. Conversely, $3OC_{12}$-HSL production is stable up to LasR levels $\geq$51% of the wild-type level in the presence of *rsaL*. A decrease in the $3OC_{12}$-HSL level is apparent in the *rsaL*-proficient genetic background only when LasR drops to ~19% of the wild-type level. If considering that $3OC_{12}$-HSL concentration was not affected by *vfr* mutation (Fig. 2A), leading to a 68.5% reduction of LasR levels relative to the wild strain (Fig. 2B), the range of LasR variations for which the expression of target genes of the *las* IFFL lost the robustness property is likely between ~20% and ~30% of the wild-type level.

Notably, L-arabinose reduction led to a gradual decrease in the levels of elastase and proteases in both the $lasR^{IND}$ and Δ*rsaL* $lasR^{IND}$ strains, in agreement with the fact that the genes involved in the synthesis of these factors are controlled by LasR levels and not by the *las* IFFL-1 (Fig. 5D and E). The decrease in L-arabinose concentration had a stronger impact on elastase and protease levels with respect to $3OC_{12}$-HSL synthesis in both the $lasR^{IND}$ and Δ*rsaL* $lasR^{IND}$ strains, similar to the consequence of lower binding affinity of LasR to the promoter region of the *lasB* and *aprA* genes with respect to the promoter region of *lasI*. Indeed, a lower affinity of purified LasR to the *lasB* promoter region relative to *lasI* has previously been demonstrated.[17]

Unfortunately, it was not possible to quantify pyocyanin levels in these strains, since pyocyanin production in *P. aeruginosa* PAO1 is negatively affected by L-arabinose concentration in the medium (data not shown).

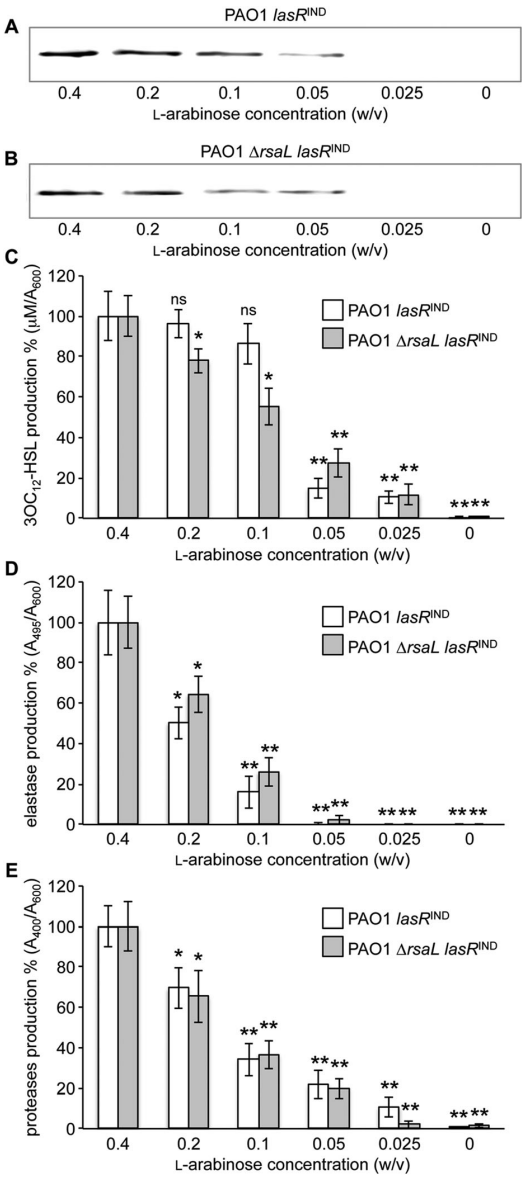

Fig. 5 Effect of LasR variations on $3OC_{12}$-HSL, elastase and protease production. (A) PAO1 *lasR*^{IND} and (B) PAO1 Δ*rsaL lasR*^{IND} protein crude extracts from cultures grown in LB broth supplemented with the indicated L-arabinose concentrations and analysed by Western-blot with anti-LasR polyclonal serum. The Western-blot results here reported are representative of three independent experiments. (C) $3OC_{12}$-HSL, (D) elastase and (E) protease production measured in the PAO1 *lasR*^{IND} strain (white bars) and in the PAO1 Δ*rsaL lasR*^{IND} strain (grey bars) grown in LB broth supplemented with different L-arabinose concentrations, as indicated below the histograms. For both strains, 100% $3OC_{12}$-HSL, elastase and protease production was considered as the maximum value measured with 0.4% (w/v) L-arabinose. For the three assays, the average values from five independent experiments are reported with SD. Significance with respect to 0.4% (w/v) L-arabinose in the same strain is indicated: *, $p < 0.05$; **, $p < 0.01$; ns, not statistically significant.

Discussion

The main result of this work is the demonstration that only a subset of LasR-controlled genes is robust with respect to variations in the levels of this regulator, and that this property is dependent on the integrity of the IFFL-1 network motif constituted by LasR and RsaL (Fig. 6).

Previous studies investigated the role of the IFFL-1 based on miRNAs as a repressor (Y) in conferring robustness to the expression of output genes with respect to transcriptional noise, temperature fluctuations, or alteration of the dose of transfected vectors in mammalian cells.[36] However, these properties have been mainly investigated in theoretical studies and have only been confirmed in a few cases with synthetic gene circuits and in mammalian cells.[4,31,32,35] To the best of our knowledge, this is the first case in which the robustness with respect to variations in the levels of the activator protein X has been demonstrated in bacterial cells for a natural IFFL-1 in which the repressor element Y is a protein.

The use of a *vfr* mutant strain allowed the behaviour of the IFFL-1 to be tested, since Vfr is a well-known activator of *lasR* transcription. Previous studies showed that a mutant defective in *vfr* was impaired in elastase and protease production without measuring P*lasI* activity and $3OC_{12}$-HSL production.[38] This led to the wrong conclusion that Vfr could control all the LasR-activated downstream phenotypes. Conversely, here we show that Vfr action is rather restricted to phenotypes not controlled by the *las* IFFL-1 (*i.e.* elastase and proteases).

The results obtained by using LasR-inducible strains show that the robustness of the *las* IFFL-1 output genes with respect

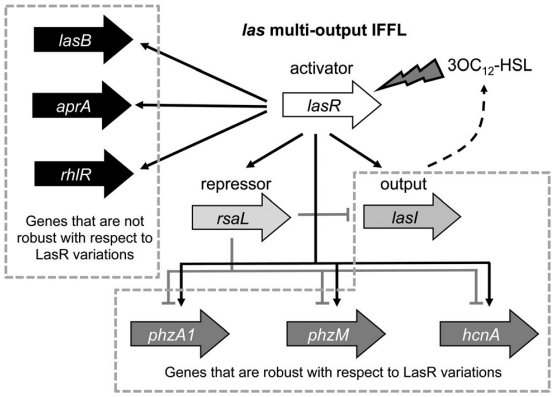

Fig. 6 Schematic representation of the *las* multi-output IFFL. The LasR/$3OC_{12}$-HSL complex promotes the transcription of multiple output genes, including the virulence genes *lasI*, *phzA1*, *phzM*. *hcnA*, *lasB*, *aprA*, and *rhlR*; RsaL represses only a subset of the LasR-activated genes, including *lasI*, *phzA1*, *phzM* and *hcnA*. The expression of genes under dual control of LasR and RsaL is robust towards LasR fluctuations, while the expression of genes only controlled by LasR is sensitive to variations in the LasR level. Hence, the LasR regulon is divided into two distinct sub-regulons differentially affected by LasR intracellular concentration. Note that only selected virulence genes reported to be positively controlled by LasR and negatively regulated by RsaL are depicted in this figure. Arrows indicate positive regulations; T-bars indicate negative regulation; dashed arrow represents information flow.

to LasR variations is conserved in a *vfr* proficient background, excluding possible side effects caused by Vfr and other ancillary *lasR* transcriptional regulators, *e.g.* PA3699[37] or AlgR2 (AlgQ),[52] on the tested phenotypes. This experiment also highlights that the buffering capacity exerted by RsaL on the expression of genes controlled by both LasR and RsaL (*i.e. lasI*) is restricted within a defined range of LasR levels, likely higher than 20–30% relative to the wild-type level under the conditions tested in this study. Indeed, for L-arabinose concentrations $\leq 0.05\%$ (w/v), in which the LasR level drops to $\sim 19\%$ of the wild-type level, the reduction of the RsaL-mediated repression is no longer sufficient to counterbalance the reduction of the LasR-dependent activation on the target genes.

To the best of our knowledge, only the *phzM* gene, involved in pyocyanin biosynthesis, and the *hcnABC* operon, required for the production of the virulence factor hydrogen cyanide, have previously been demonstrated to be directly activated by LasR and repressed by RsaL, besides *lasI* and *phzA1* (Fig. 6).[19,22] However, since both LasR and RsaL are global regulators in *P. aeruginosa*,[19,53] the *las* IFFL could confer robustness with respect to LasR variation and also to the expression of virulence traits other than $3OC_{12}$-HSL, pyocyanin and hydrogen cyanide biosynthesis. Notably, it has been recently reported that both LasR and RsaL also bind to the *pqsH* gene, required for the production of the QS signal molecule PQS, but in this case both LasR and RsaL promote *pqsH* expression.[22,23] In the future, it would be interesting to clarify the contribution of the *las* IFFL to the *pqs* QS system, also considering that LasR promotes the expression of the *pqsABCDE* operon, which is involved in the biosynthesis of the PQS precursor HHQ.[22] Conversely, the *rhl* QS system is under positive control by LasR and is not directly controlled by RsaL,[19,22,23,51] hence this QS system is likely sensitive to perturbations in the LasR level (Fig. 6).

Multiple transcriptomic analyses have also been performed investigating the LasR and RsaL regulons.[19,48,53–57] At present, the dependence of RsaL expression on LasR activity does not allow the identification of a reliable set of genes activated by LasR and repressed by RsaL. In the future, a transcriptomic approach based on *ad hoc* engineered *P. aeruginosa* mutant strains in which the expression/activity of LasR and RsaL is uncoupled could allow the ultimate identification of the genes whose transcription is robust or sensitive with respect to LasR variations. Moreover, a detailed analysis of the binding affinity of LasR and RsaL to the promoter region of target genes would provide valuable information to gain quantitative insights on the extent of the buffering effect exerted by RsaL on LasR-controlled genes.

The data produced in this study may contribute to clarifying some aspects underlying *P. aeruginosa* adaptation to the host environment. *P. aeruginosa* can establish both acute and chronic infections, characterized by different behaviours and distinctive phenotypes. An acute infection like sepsis is rapid, systemic and carried out by a planktonic bacterial community expressing high levels of virulence factors. During chronic infection, as in the lung of cystic fibrosis patients, bacterial proliferation is limited to the infected organ, where bacteria can persist for extended periods of time, forming a biofilm and adopting a slow-growing sessile lifestyle characterized by a reduced expression of some virulence factors, such as elastase and proteases.[58] Conversely, high levels of pyocyanin have been recovered in the sputum of cystic fibrosis patients with *P. aeruginosa* chronic lung infection.[59] Since the majority of virulence phenotypes expressed by *P. aeruginosa* during both acute and chronic infections are positively controlled by QS, it is unclear how *P. aeruginosa* can modulate the differential expression of QS-dependent virulence genes under these two conditions. Our results gain insight into this issue, suggesting that the different response of the two LasR sub-regulons to cues affecting LasR levels could result in different levels of expression of virulence genes required in acute and chronic infections. In this scenario it is tempting to speculate that the virulence phenotypes involved in acute infection (*e.g.* elastase and protease production) require rapid regulation to allow the bacterial population to face environmental changes that occur during the planktonic lifestyle, and therefore their expression levels are promptly adjusted in response to variations in LasR levels. Conversely, other virulence phenotypes (*e.g.* pyocyanin production) likely need to be steadily expressed despite spurious environmental and metabolic fluctuations, and this could be achieved thanks to the properties of the *las* IFFL-1.

This hypothesis is supported by recent reports demonstrating that Vfr expression is decreased upon mutation of the *mucA* gene, leading to the expression of the mucoid phenotype, a hallmark of *P. aeruginosa* chronic lung infection in cystic fibrosis patients.[60,61] Moreover, high levels of c-di-GMP result in decreased levels of the Vfr co-activator c-AMP in the *P. aeruginosa* rugose small colony variants (RSCVs), frequently isolated from cystic fibrosis patients with chronic lung infection.[62–64] Our results suggest that *P. aeruginosa* might respond to decreased expression of Vfr or to reduced levels of cAMP by limiting the expression of the virulence factors elastase and proteases, while maintaining steady expression levels of the genes controlled by both LasR and RsaL, such as those required for pyocyanin biosynthesis.

The importance of the *las* IFFL-1 in *P. aeruginosa* pathogenicity is emphasised by previous work demonstrating that *rsaL* mutation, *i.e.* the disruption of the *las* IFFL-1, reduces the establishment of *P. aeruginosa* chronic lung infection in mice.[26] Moreover, *P. aeruginosa* mutant strains impaired in *rsaL* are defective in biofilm formation.[48] Since biofilm bacteria live in close proximity and differentiate in distinct cell types, biofilm formation is often compared to the differentiation process that occurs in multicellular eukaryotic organisms. In this context, it is interesting to mention that in eukaryotic cells different IFFLs-1 have been described in which microRNAs confer robustness to the regulatory networks controlling cell differentiation. In these cases, the IFFLs-1 allow mammalian cells to maintain their differentiation programme despite possible fluctuations in the environmental cues triggering these developmental processes.[36] Therefore, it is plausible that, similar to the developmental processes in eukaryotic cells, biofilm formation and development in *P. aeruginosa* also requires robust regulatory properties conferred by an IFFL-1 network motif.

Notably, negative built-in regulators similar to RsaL have been described in QS systems in a number of bacterial species,

suggesting that the robustness conferred by IFFL-1 network motifs could be a common feature of many bacterial communication systems.[65,66] A "paradoxical" component structured as an IFFL-1 has also been described in another QS system of *P. aeruginosa*, the *pqs* circuit. Indeed, in the *pqs* QS circuit, the PqsR QS receptor (X) activates the transcription of the output operon *pqsABCDE-phnAB* (Z), thus increasing the expression of PqsE (Y), which in turn down-regulates the transcription of the same output operon (Z).[67–69]

In conclusion, thanks to the properties of the *las* IFFL-1, *P. aeruginosa* can modulate the expression of a sub-set of genes by altering the levels of LasR, while the expression of the *las* IFFL-1 output genes remains robust. This regulatory property could enhance *P. aeruginosa* phenotypic plasticity and could be particularly relevant during infection.

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Electronic Supplementary Material (ESI) for Molecular BioSystems.

The multi-output incoherent feedforward loop constituted by the transcriptional regulators LasR and RsaL confers robustness to a subset of quorum sensing genes in *Pseudomonas aeruginosa*

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Running Head: Regulatory properties of the *las* QS system

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SUPPLEMENTAL MATERIAL

Table S1. Bacterial strains used in this study

Table S2. Plasmids used in this study

Table S3. Oligonucleotides used in this study

Table S1. Bacterial strains used in this study

Strains	Relevant characteristics	Reference/Source
E. coli		
S17.1 λ*pir*	Conjugative strain for suicide plasmids.	*39*
SM10 (pFLP2)	Strain carrying the pFLP2 plasmid (FLP; Ap^R/Cb^R) for Flp-mediated recombination.	*43*
P. aeruginosa		
PAO1	wild type strain ATCC15692.	ATCC
PA14-R3	Biosensor strain for $3OC_{12}$-HSL quantification.	*49*
Δ*vfr*	PAO1 mutant strain with in frame clear deletion of the *vfr* gene.	*37*
Δ*rsaL*	PAO1 mutant strain with in frame clear deletion of the *rsaL* gene, obtained by using the pDM4Δ*rsaL* plasmid (Table S2).	This study
Δ*rsaL*Δ*vfr*	PAO1 double mutant strain with in frame clear deletion of both the *rsaL* and *vfr* genes, obtained by using the pDM4Δ*rsaL* plasmid (Table S2) in PAO1 Δ*vfr*.	This study
Δ*lasR*	A 344 bp region encompassing the *lasR* gene, corresponding to nucleotides 1558474-1558819 of *P. aeruginosa* PAO1 genome, has been deleted from PAO1 chromosome by using the pEX18Δ*lasR* plasmid (Table S2).	This study
Δ*lasR*Δ*rsaL*	A 685 bp region encompassing the *lasR* and *rsaL* gene, corresponding to nucleotides 1558473-1559112 of *P. aeruginosa* PAO1 genome, has been deleted from PAO1 chromosome by using the pDM4Δ*lasR*Δ*rsaL* plasmid (Table S2).	This study
$lasR^{ind}$	PAO1 Δ*lasR* mutant strain carrying the *araC*-P_{BAD}*lasR* transcriptional fusion for L-arabinose-dependent induction of *lasR*, integrated into the chromosome. Generated by using the mini-CTX1-*araC*-P_{BAD}*lasR* (Table S2).	This study
Δ*rsaL* $lasR^{ind}$	PAO1 Δ*lasR*Δ*rsaL* mutant strain carrying the *araC*-P_{BAD}*lasR* transcriptional fusion for L-arabinose-dependent induction of *lasR*, integrated into the chromosome. Generated by using the mini-CTX1-*araC*-P_{BAD}*lasR* (Table S2).	This study

Table S2. Plasmids used in this study

Plasmid	Relevant characteristics and plasmid construction	Reference/Source
pDM4	Suicide vector for construction of deletion mutants; *sacBR*; *oriR6K*; Cm^R.	*42*
pDM4Δ*rsaL*	pDM4-derivative plasmid for in frame clear deletion of the *rsaL* gene. The upstream (UP) and the downstream (DW) DNA regions of *rsaL* were PCR amplified from *P. aeruginosa* PAO1 genome using primers FW61G/RV62G and FW63G/RV64G, respectively (Table S3). The UP and DW PCR fragments were subsequently cloned in pDM4 by XhoI-BamHI and BamHI-XbaI restriction, respectively.	This study
pEX18Δ*lasR*	pEX18Tc-derivative plasmid for deletion of the *lasR* gene from nucleotides 1558474-1558819 of *P. aeruginosa* PAO1 genome.	*44*
pDM4Δ*lasR*Δ*rsaL*	pDM4-derivative plasmid for deletion of the *lasR* and *rsaL* genes from nucleotides 1558473-1559112 of *P. aeruginosa* PAO1 genome. The upstream (UP) and downstream (DW) regions of the genetic *locus* containing the *lasR* and *rsaL* genes was PCR amplified from *P. aeruginosa* PAO1 genome using primers FW675/RV765 and RV62G/FW61G, respectively (Table S3). The UP and DW PCR fragments were subsequently cloned in pDM4 by SpeI-BamHI and BamHI-XhoI restriction, respectively.	This study
mini-CTX1-*araC*-P_{BAD}*tolB*	mini-CTX1-derivative plasmid carrying the *araC*-P_{BAD}*tolB* cassette from pBEM9, cloned by XhoI/EcoRI restriction.	*70*
mini-CTX1-*araC*-P_{BAD}*lasR*	mini-CTX1-*araC*-P_{BAD}-derivative plasmid containing a 720 bp DNA fragment encompassing the *lasR* gene. *lasR* was PCR amplified from *P. aeruginosa* PAO1 genome using primers FW763 and RV764 (Table S3) and cloned in the mini-CTX1-*araC*-P_{BAD}*tolB* plasmid by HindIII-EcoRI restriction.	This study
pMP190	Promoter probe vector; IncQ; 15 kb; Sm^R Cm^R.	*71*

Plasmid	Relevant characteristics and plasmid construction	Reference/Source
pP*lasI*190	A DNA fragment encompassing the *lasI* promoter region (P*lasI*) was PCR amplified and cloned in the pMP190 plasmid upstream of the promoterless *lacZ* gene.	*19*
pP*lasR*190	A DNA fragment encompassing the *lasR* promoter region (P*lasR*) was PCR amplified from *P. aeruginosa* PAO1 genome with primers FW324 and RV325 (Table S3) and cloned by SalI-BglII restriction in the pMP190 plasmid upstream of the promoterless *lacZ* gene.	This study

Table S3. Oligonucleotides used in this study

Name	Sequence (5'-3') [a]	Position [b]	Restriction site
FW324	NNNACGCGTCGACGGTCGCACGCGTGGCG	1557812	SalI
RV325	NNNNGGAAGATCTCAACCAAGGCCATAGCGC	1558166	BglII
FW675	GGACTAGTACCTATGCGCCGCCGTTG	1557572	SpeI
RV676	CGCGGATCCAGCGCTACGTTCTTCTTAAACT	1558149	BamHI
RV765	GCGGGATCCACTCGTGCTGCTTTCGCGT	1558454	BamHI
FW763	TATAAGCTTATGGCCTTGGTTGACGGTTT	1558171	HindIII
RV764	TATGAATTCTCAGAGAGTAATAAGACCCAAA	1558869	EcoRI
FW61G	CCGCTCGAGCGCATCGCCTCCAGCGT	1559614	XhoI
RV62G	TATGGATCCGTGTGAAGCCATTGCTCTG	1559111	BamHI
FW63G	TATGGATCCTTGCATTTCTATATAGAAGG	1558932	BamHI
RV64G	TGCTCTAGACTGGGAACCGTCCATCTAC	1558441	XbaI

[a] Engineered restriction sites are underlined.

[b] Position with respect to *P. aeruginosa* PAO1 chromosome (www.pseudomonas.com). [72]

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[a] Please note that other references cited in this Supplemental Material file are listed in the Reference Section of the manuscript main body.

Chapter 3

Current directions in synthetic cell research

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Preface to Chapter 3

Synthetic biology aims at the generation of novel organisms or bio-inspired systems for new biotechnological approaches. This is mainly achieved by assembling standard biological parts inside different hosts, or "chassis", including natural or synthetic cells.

In the present review the state-of-the-art of different branches of synthetic cells research is shortly illustrated, starting from the pioneer phase in the '90s, to the most recent developments. Briefly, synthetic cells are cell-like molecular systems generated in the laboratory by encapsulating inside lipid vesicles called liposomes, biological or synthetic molecules. Synthetic cells are relevant, for example, in the origin of life studies, or used to reconstruct, in a realist but simplified scenario, a biological process, or besides used as tools for biotechnological applications.

Recent important topics in synthetic cells research fields are reported in the review, including: the reconstitution of advanced architecture and functions, which concerns the study of cellular organelles and membrane proteins; the generation of 2D and 3D arrays to study synthetic cells in a population/community perspective; the manipulation of biochemical signals. In particular, the latter aspect is one of the main focuses of this PhD thesis, concerning the possibility of generating synthetic cells able to interface with natural cells *via* chemical communication, as will be experimentally explored in Chapter 4.

Current Directions in Synthetic Cell Research

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Abstract The construction of synthetic cells of minimal complexity is today one of the most attractive and challenging goals in synthetic biology. Synthetic cells are assembled by combining the methods of liposome technology and microfluidics, and components of cell-free systems. In this contribution, we will shortly illustrate the state-of-the-art of synthetic cell research; next we will present some current trends and scenarios, that could lead to a qualitative jump in the next years. In particular, we will focus on the construction of novel multi-compartment vesicles, the achieving of new functions via the reconstitution membrane-bound proteins, the shift from the isolated cell—to the cell population/community perspective, the exploitation of chemical signalling, and the integration of stochastic mathematical models. The ambitious goal of approaching embodied and minimal cognition from this experimental perspective is also shortly mentioned.

keywords Synthetic cell · Synthetic biology · Lipid vesicles · Origins of life

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1 What Are Synthetic Cells

Synthetic Cells (SCs) can be defined as those cell-like molecular systems constructed in the laboratory by inserting biological or synthetic molecules inside and on the surface of lipid vesicles (liposomes, see Fig. 1). Ideally, SCs mimic biological cells with respect to structure, functions, interactions, and any other possible property (including the ultimate and long-term goal of being alive) with *minimal complexity*.

Historically, SCs have been first explored to shed light on some origin of life questions, but it became clear that they can be created and adapted for investigating biological mechanism or developing biotechnological tools (Fig. 1). SCs are relevant in origin of life studies [26] because they are useful models of primitive cells (protocells), especially when they are built with allegedly primitive membrane forming compounds (e.g., fatty acids), and host compounds and reactive systems of primitive importance (ribozymes, simple peptides, self-replicating molecules, etc.) [52]. One of the goal is to show that simple living cells, maybe imperfect and "limping" ones, can however emerge from the self-organization of non-living molecules.

On the other hand, synthetic cells can be used to reconstruct, in realistic cell-like architecture, those biological processes that can be advantageously studied in a

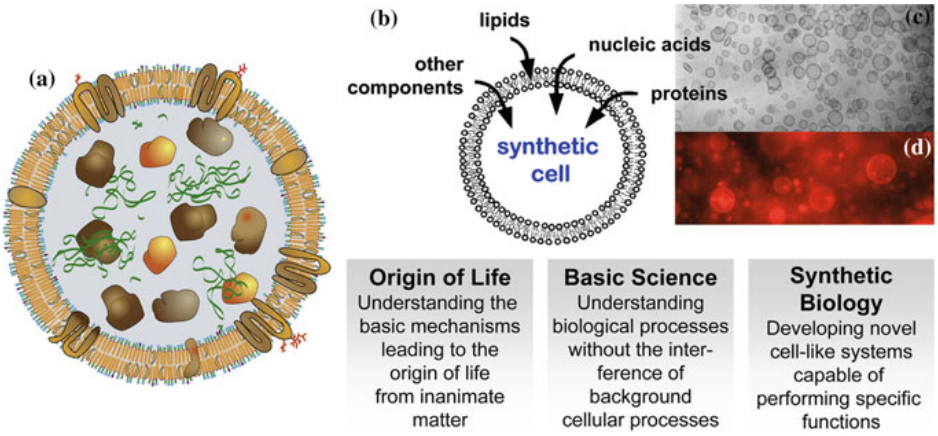

Fig. 1 Schematic drawing of a liposome-based synthetic cell. **a** Synthetic cells are built by incorporating in the aqueous core and in the membrane of lipid vesicles (diameter 0.1–10 μm) the minimal number of molecular components in order to obtain a cell-like structure that performs specific function (ultimately: being alive). The image is taken from [54] with permission from Elsevier [the image has been adapted to represent a synthetic cell, but it actually sketches a biological exosome]. **b** Synthetic cells (or, more correctly, semi-synthetic, *cf.* [26]) can be built by incorporating nucleic acids, proteins, ribosomes and other compounds inside and on the surface of a lipid vesicle. Synthetic cells of conventional size (i.e., diameter below 0.5 μm) can be conveniently imaged by electron microscopy (**c**), whereas *giant* ones (i.e., diameter: 1–50 μm) can be seen by optical microscopy. **d** Synthetic cells can be adapted to perform research in origin of life and basic science, and also be used as biotechnological tools according to synthetic biology

simplified platform. In fact, by reconstructing a certain process in a synthetic cell, its understanding results easier because the interfering "noise" of background cellular processes, as unwanted interactions with other components are purposely removed. The process of interest can be finely tuned owing to the control of synthetic cell composition whereas such control is impossible in living cells.

Finally, SCs can be used as tools for biotechnological applications. Their design and construction is developed under the synthetic biology paradigm, which includes the use of standard parts (http://parts.igem.org), the concepts of modularity and orthogonality [13], and fully programmability (at least in principle). Such bioengineering perspective has led to sketch the possible use of SCs in nanomedicine [25] or in applications of biotechnological flavours, such as sensoring, analysis, programmable on-chip chemical devices, and so on [32, 41].

Starting from the 1990s, several research groups have been involved in synthetic cell research, contributing to setting up the stage for actual and future developments (for reviews, see [47, 49]). It must be noticed that in addition to liposome-based SCs, other artificial compartments have been also explored (*e.g.*, water-in-oil droplets, polymersomes, colloidosomes, coacervates, micelles, oil-in-water droplets).

1.1 From the Pioneer Phase (up to 2001) to the Most Recent Developments

After the 1989 paper on the use of reverse micelles as minimal autopoietic systems [28], early investigation was primarily reported by ETH-Zürich team (Pier Luigi Luisi, Peter Walde and Thomas Oberholzer), which carried out pioneering research on vesicle self-reproduction [55] and performing important proof-of-principle experiments on micro-compartmentalized reactions, namely RNA replication [37], polymerase chain reaction [35], and polypeptide synthesis [36]. Actually, most of the current experimental strategies stems from these seminal papers, with the exception of the recently introduced droplet transfer method and microfluidics-based procedures. The end of the pioneering phase can be symbolically placed in 2001, when the production of a well-folded and thus functional protein inside liposomes was achieved by the Yomo group [58]. Protein synthesis inside liposomes plays a strategic role in SCs research because the proteins carry functions, e.g., they are enzymes, transcriptional factors, membrane channels, and so on.

In the following ten years or so, several fundamental aspects of compartmentalized protein synthesis were explored, and important milestones were reached: (a) the prolongation of intraliposome transcription-translation (TX-TL) reactions for about four days owing to the generation of a α-hemolysin based membrane pore (from inside) [34]; (b) the realization of two-steps genetic cascade reactions inside liposomes [19]; (c) the first report on the synthesis of hydrophobic enzymes (membrane embedded) [22]; (d) the RNA replication via a self-encoded mechanism [21]; (e) the spontaneous formation of super-filled vesicles [27, 48] (Fig. 2); (f) the

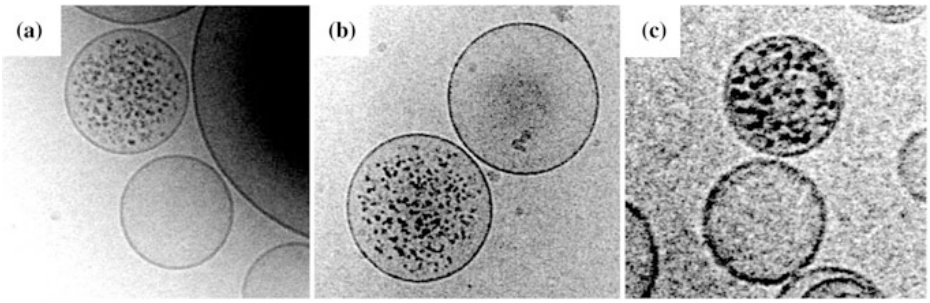

Fig. 2 Cryo-transmission electron microscopy images of ferritin-containing lipid vesicles. Super-filled vesicles coexist with empty vesicles. The internal ferritin concentration reaches high values (in these vesicles about 4–12 times the expected concentration). The diameter of ferritin-filled vesicles are 195, 219, 118 nm, respectively, for picture (**a**), (**b**), and (**c**). A similar behavior is observed with ribosomes [46] and with the full TX-TL machinery (PURE system), see [48]. The fact that solute-filled vesicles form spontaneously might help to explain the origin of solute-rich primitive cellular structures. Reproduced from [27] with permission from Wiley

droplet transfer method [34, 39, 57], which can be used to produce solute-filled giant vesicles with good yields and high encapsulation efficiency; and (g) the ongoing development of microfluidic devices for high-throughput production, in highly reproducible manner, of solute-filled giant vesicles [12]. Many of these results have been obtained by employing the PURE system [45], a standardized and minimal set of macromolecules for synthesizing proteins inside liposomes.

All this—and many other advancements which are omitted here due to space limitation—has brought to a kind of maturation of the field, which now attracts more and more researchers. In other words, times are probably ripe for a sort of qualitative jump.

2 Current Directions in SC Research

What are the current directions in SC research? Here we report a number of topics that we consider important, and that mirror our attitudes, without the presumption of being exhaustive.

2.1 Reconstitution of Advanced Architecture and Functions in SCs

Vesosomes. Whereas current SCs are generally based on a single lipid compartment, generally containing just one lipid membrane (unilamellar lipid vesicle), novel architectures could derive from including small vesicles inside a larger one

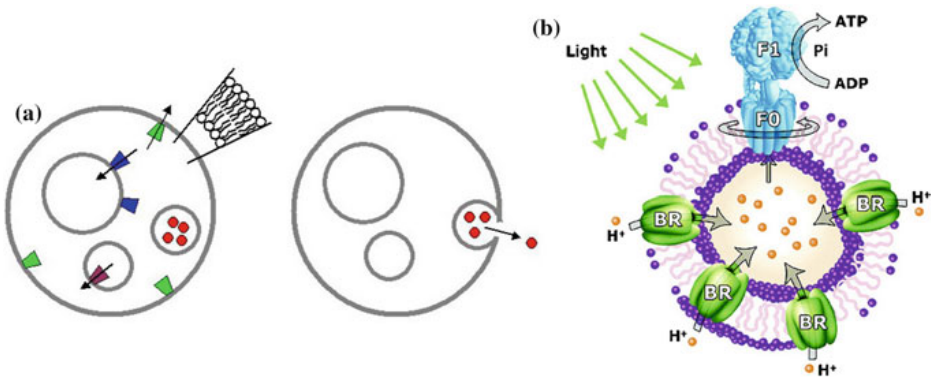

Fig. 3 **a** Vesosomes are multi-vesicular vesicles composed by smaller vesicles inside a larger vesicle. By inserting compartments inside compartments, interesting vectorial properties might arise from oriented membrane proteins. Moreover, the content of internal vesicles can be released in the external medium after membrane fusion. **b** ATP has been produced by coupled reactions between bacteriorhodopsin, a light-driven transmembrane proton pump, and F_0F_1-ATP synthase motor protein, reconstituted in polymersomes. Reprinted with permission from [6]. Copyright 2005 American Chemical Society

(Fig. 3a). Technically, these are called multivesicular vesicles or vesosomes, and it is not rare to find them in vesicle samples formed by classical methods. However, when vesosomes are specifically needed in order to assembly synthetic cells with hierarchical topology (vesicles inside vesicles), their preparation should follow dedicated methods. The construction of vesosomes follows specific protocols [5, 38] but their employment as synthetic cell has not been explored in detail (however, see [16]). The interest in this architecture stems from the cytomimetic role of internalized vesicles: they could play the role of intracellular organelles, allowing novel SC designs in terms of sub-compartmentalization, in particular for exploiting the concentration gradients across their membranes, and expanding the intracellular total membrane area (which will possibly include membrane-bound machineries). Moreover, small vesicles could fuse with the outermost membrane to release their content (a signal molecule for instance) outside the SC in programmable way.

Membrane proteins. Until now, the fundamental relevance of membrane embedded devices (proteins) has been only partially addressed. This is due to the difficulty of reconstituting functional membrane proteins or of synthesizing them directly by SCs. These difficulties are both intrinsic (poor understanding of mechanism of membrane protein folding, refolding, membrane insertion, lipid-membrane interactions) and practical (conflict between required experimental conditions). An early attempt of direct synthesis inside SCs has been reported [22, 44]. Two acyltransferase proteins were successfully produced via transcription and translation reactions, but the main message of the work was more general. In order to accomplish the important goal of membrane protein synthesis in SCs, the vesicle's lipid simultaneously should: (a) form good vesicles; (b) do not chemically interfere with the complicated molecular mechanism of protein synthesis; (c) allow

the correct insertion and folding of the membrane protein. Therefore, a careful design is needed for functionalizing SCs with membrane proteins. There are really several relevant functions done by membrane proteins. One of the most important is the energy production. Attempts to reconstitute parts of the ATP synthase machinery have been published [23]. By coupling bacteriorhodopsin and ATP synthase [6], it has been reported the reconstitution of cytomimetic polymer vesicles, Fig. 3b, which were able to transduce light into chemical energy, i.e., ATP.

2.2 The Population/Community Perspective

This is probably the most fascinating scenario for future SC research, namely the transition from studying individual, non-interacting SCs to a population of mutually interacting ones. The population can be composed of SCs that are free moving, immobilized, networked, and so on, so that one could imagine SC communities, biofilm-like assemblies, or 2D/3D synthetic tissues (Fig. 4). Interesting work has been reported by [18], who assembled vesicles arrays by means of controlled linking (this was achieved by functionalizing the vesicle surface with nucleic acids). Protein synthesis inside such vesicle network has been also published [17]. In the context of origin of life, a simple way to observe formation of "colonies" has been reported [4]. 3D synthetic cell systems have not been announced yet, to the best of our knowledge. Spontaneous organization or directed construction could lead to 3D SCs arrays with featured properties. Ideally, a guided assembly could be realized via 3D printing, possibly on a microsculptured scaffold, and the whole system stabilized by inter-vesicles links or by a gel. Giant vesicles randomly embedded in a gel matrix are routinely prepared in our lab, displaying excellent stability. Clearly, a population of objects becomes interesting when there is a kind of physical or chemical interaction among the parts. The synthetic biology approach allows us to go one step further, foreseeing interactions that are based on exchange of *information*. This consideration brings us straightly to the next scenario.

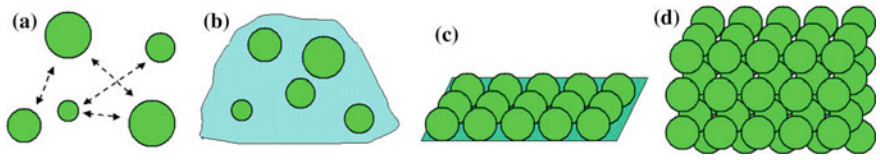

Fig. 4 From one to many. Possible experimental approaches involving synthetic cell arrays. **a** In the population/community perspective, great emphasis is given to vesicle diversity (in size, in content, in reactivity) and to the mutual interaction between them, which can be physical or chemical. **b** One easy way to immobilize synthetic cell is their embedding in a gel matrix. Alternatively, 2D (**c**) or 3D (**d**) arrays can be envisaged. These systems can be models of tissues, can be used as biotechnology tool, or can be studied to simulate primitive cells communities

2.3 Manipulation of (Bio)Chemical Signals: The Bio-Chem-ICTs Perspective

SCs constructed according to the synthetic biology paradigm (standardization of parts, modularity, orthogonality, etc.) not only can serve to first demonstrate that life can emerge from inanimate matter—e.g., consider the first man-made autopoietic cell—but also have the tremendous potential of communicate with each other. This will become possible if SCs will be able to manipulate chemical signals, performing molecular communications [33]. This innovative field can result in novel information and communication technologies based on bio-chemical signals, or bio-chem-ICTs [1]. European networks focus on these issues (cobra-project.eu, fet-circle.eu).

Inspired by previous work [8, 14, 20, 25], we have clearly defined in which way synthetic cells can be used as tools for bio-chem-ICTs [50], and research is in progress in our laboratory to create a communication protocol allowing synthetic and natural cells communicating with each other (Fig. 5a), based on quorum sensing bacterial mechanisms. In particular, our first goal is the creation of synthetic cells capable of communicating with bacteria (Rampioni et al., in preparation), but also with cells of superior organisms. SCs that communicate with biological cells could be used as programmable devices for nanomedicine, as depicted in Fig. 5b.

Another intriguing direction refers to mimicking complex morphogenetic patterns, based on the confinement of chemical oscillators in microcompartments [42]. For instance, the Belousov-Zhabotinsky reaction has been reconstituted inside

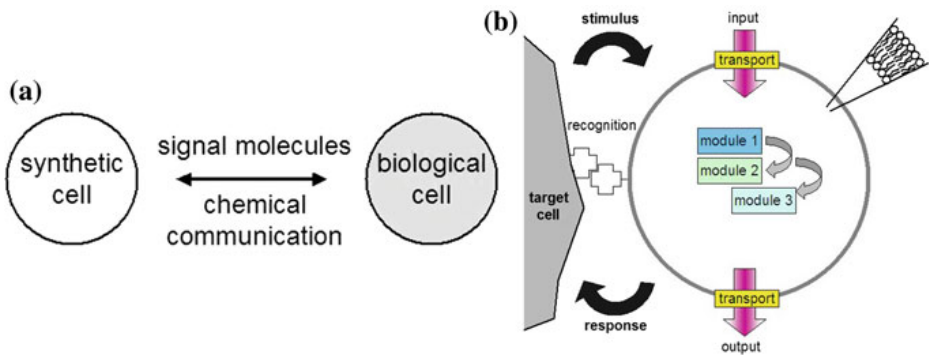

Fig. 5 **a** Is it possible to create a chemical communication protocol for establishing synthetic communication between synthetic and natural cells? Quorum sensing molecules and mechanisms can be exploited to establish a two-way communication channel (synthetic cells/bacteria). Taken from [50]. **b** By advancing the current biotechnology of synthetic cell construction, medical potential applications of more sophisticated, communicating and programmable synthetic cells can be envisaged. A goal would be to construct cell-like systems that, once injected in human body, reach a specic target region and thanks to chemical information processing, is able to act accordingly, for example producing in situ a cytotoxic drug or a stimulus to trigger a cellular response. Very recently, the concept of "pseudo-cell factories" or "nanofactories" (illustrated above) has been proposed. Redrawn, with minor modifications, after [25]

vesicles within liposomes [43, 51, 53]. The overall reaction is driven by the oxidation of an organic substrate, e.g. malonic acid, by bromate in acidic solution in the presence of a catalytic specie in the form of an organo-metal complex such as ferroin (a phenanthroline-iron(II) complex). The oscillatory dynamics is governed by the amount of the inhibitory intermediate bromine and the excitatory intermediate bromous acid present in the vesicles which might diffuse between individual units, thus affecting the overall oscillatory synchrony of multiple drop arrays. These intermediates actually serve as messenger molecules between individual units. In these works it has been proven that networks of oscillating vesicles provide a model to apprehend the propagation and processing of chemical information and the BZ reaction was used as "signal transmitter/receiver" to probe and study complex communication networks, mainly displaying an inhibitory character, i.e., anti-phase oscillations and stationary "Turing" structures. In fact, synchronisation of dynamic elements via chemical communication is a widespread phenomenon in nature and in relevant fields such as biology, physics and chemistry, where coupling and synchronisation are achieved by messenger molecules diffusing from one element to others triggering and spreading a chemical reaction.

2.4 The Integration of Mathematical Models

Engineering inspires synthetic biologists. Such attitude requires the integration of wet-lab approaches with mathematical modelling. Due to the very nature of SCs, which are microscopic compartments often filled with a limited number of molecules, stochastic biochemical modelling plays a major role, but the deterministic approach has been also applied (Fig. 6). We can distinguish models that operate on SCs at different levels. First, models can describe just the mechanisms of internalized reactions, like protein synthesis, transcriptional regulation, enzyme activity, and so on. The diffusion of solutes across the membrane is often coupled to the course of these reactions. The hierarchical vesosome architecture shown in Sect. 2.1 and Fig. 3a also needs a combination of chemical reactions and diffusion/transport modelling. Second, the focus can be on the vesicle transformations such as shrinking, swelling, growth, division, fragmentation, including vesicle formation and the physics of solute capture. These aspects can be complemented by the mechanical analysis of membranes (elasticity, curvature, bending energy,). Third, modelling SCs in a population context (strictly speaking, any systems with more than one SC) considers interaction among vesicles (with exchange of chemical signals), coordinated behaviour, associations in clusters, formation of high-order structures (2D/3D arrays). It is clear that in the more complex cases, a correct model can be obtained only if it spans through different scales, from the molecular nanoscale to the compartment mesoscale and above, blending together chemical and physical processes.

An important SC topic concerns stochastic effects. The SC volume lies in the aL-fL-pL range, implying that solutes are often present in a *small* number. For

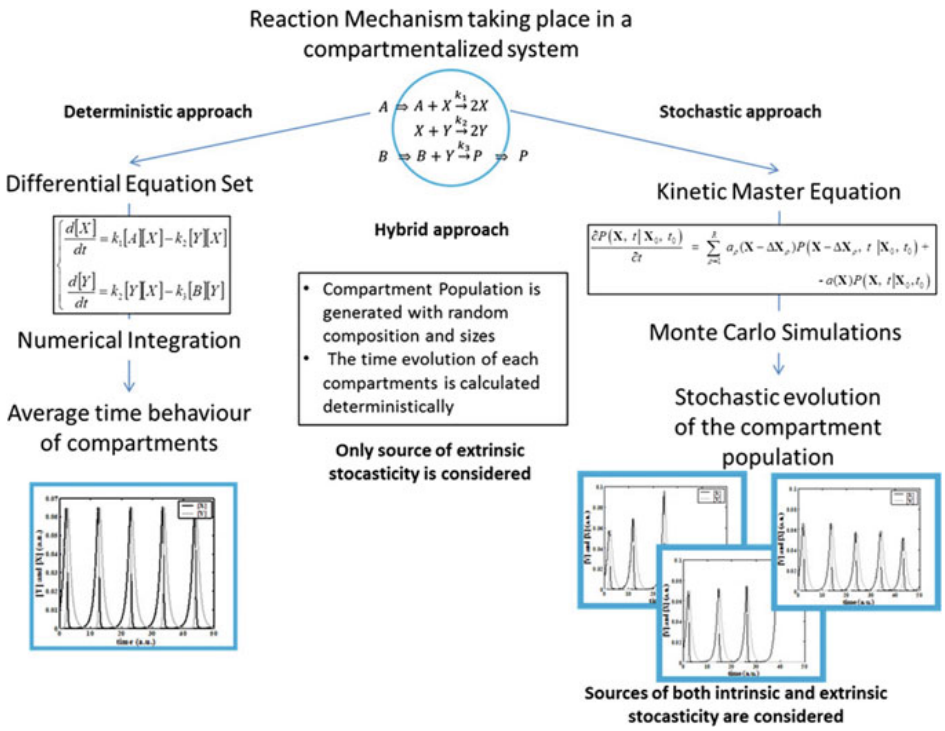

Fig. 6 Typical approaches used in theoretical modelling of compartmentalized reactions (reactions occurring inside vesicles). The specific example is on compartmentalized oscillating chemical systems (i.e., a Lokta-Volterra-like mechanism including five species A, B, X, Y, P, fuelled by external addition of the components A and B, to which the vesicle membrane is supposed to be permeable, as it is for the product P). On the *left*, the 'deterministic approach', generally used in bulk chemical kinetics, makes use of rate laws and ordinary differential equations, which are solved analytically or integrated numerically to find the time-dependent concentrations of all species, known the initial conditions. Such an approach is best suitable for systems with high number of molecules and assumes that all the reacting compartments behave similarly in average. On the *right*, the 'stochastic approach' which expresses the rate law by means of the so-called kinetic master equation, which is solved numerically via Monte Carlo simulations [15, 30]. In contrary to the deterministic approach, the stochastic approach uses the number of molecules as variables, instead of their concentration, and reaction probability instead of reaction rate. Such an approach is generally used for systems with low number of molecules. However, it should be remarked that in some specific examples (e.g., reactive systems with consumption) samples with low numerosity can be correctly modelled by a deterministic approach; and vice versa, some high numerosity systems need the stochastic approach (e.g., the repressilator [56]). More in general, the two approaches especially differ for ill-conditioned systems

instance, in vesicles of 100 nm (diameter), a concentration of 10 μM corresponds to an average of 3 molecules/vesicle. Stochastic phenomena occur both during the formation of vesicles, generating vesicles with different solute content. In turn, these vesicles will originate a kind of "diversity" in terms of the rates of internalized reactions (extrinsic stochastic effects).

We have recently studied both extrinsic and intrinsic stochastic effect in protein-synthesizing SCs, providing detailed analysis of the PURE system mechanism and its energy requirements [3, 24, 29]. Moreover, with reference to the above-mentioned anomalous encapsulation statistics (Fig. 2), a recent in silico study has demonstrated that the measured distribution of protein production inside SCs can be explained only if solute encapsulation (during SC formation) obeys to a power-law distribution, thus rejecting the null hypothesis of random encapsulation [31].

By combining observation and stochastic models it is therefore possible to get insights into the laws governing SC structure and reactivity. Contributing to basic understanding of compartmentalized reactions, such investigations can provide also useful information for modern cell physiology and biophysics.

2.5 Synthetic Biology and Artificial Intelligence: The Potential of Synthetic Cell Research for the Synthetic Exploration of Embodied Cognition

In the last years synthetic biology research, and in particular SC research, has been starting to cross the limits of its original disciplinary area, extending its investigations into the field of the cognitive sciences. These *avant-garde* developments, by shifting the focus of the inquiry from applicative to genuinely basic research purposes, are specifically related to the recent rise of the "Embodied Cognitive Science" (ECS) [7]. ESC directs the attention of research in cognitive sciences towards the fundamental role(s) the biological body plays in cognition. With this new center of interest in the scientific study of cognition, a strong synergy between ECS and biology is growing: a cooperative exploration of the biological groundings of cognitive processes to which, in the last years, SC research has been planning and inceptively starting to actively contribute.

From the methodological point of view, the role of SC research in the scientific exploration of "embodied cognition" relies on the *understanding-by-building* method [40], originally introduced by cybernetics and today increasingly adopted by scientists to study biological and cognitive processes [10]. Its key methodological concept is the following: to test and to develop scientific theories about the mechanism underlying life and cognition, by (i) incorporating related hypotheses in artificial functioning systems, and (ii) comparing the behaviours that these systems produce with those observable in the target natural systems.

Today this "synthetic" or "constructive" approach [10] methodologically supports the involvement of SCs in the exploration of cognition. This can be done by modelling and investigating natural cognitive processes via artifacts building. These artifacts are not limited to computer programs and robotic platforms, but include chemical artificial systems. In other words, the development of artificial intelligence

(AI) research not only through software and hardware, but also wetware models of cognitive processes.

From the theoretical viewpoint, this project finds its groundings in the idea that we can ascribe to minimal living systems (minimal cells) with elementary "cognitive" capabilities. According to this view, introduced by biology of cognition research in the last century and currently developed by radical approaches in ECS [9], these capabilities rely in the "autonomy" of minimal living systems, that is, the property they share with all biological organisms of producing and conserving themselves (their material identity) by actively maintaining a dynamical coupling with their environment [2]. This coupling is what allows us to think minimal living systems as minimally cognitive systems, as it relies on their capability of regulating their dynamics of self-production in response to environmental changes through processes of *perception* and *active reaction*. To be more explicit, perceiving some external variations as perturbations of the internal process of self-production; reacting to them through changes in the metabolic dynamics that compensate these alterations; stably associating to external variations internal patterns of self-regulation, that is, internally generated "operational meanings" allowing the systems to survive in the given environmental conditions.

This theoretical perspective offers to current SC research a significant role in "Embodied AI" [7]: studying minimal forms of embodied cognition—and possibly their evolution—by synthesizing and empirically exploring chemical models of minimal cells, and their interaction with their environment. The centres of interest of contemporary research are multiple, such as (i) exploring the role of regulation in the origins of biological cognition, (ii) understanding and designing minimal forms of collective intelligence, and (iii) studying specific cognitive functions—such as anticipation—in their minimal forms, among others [11].

3 Concluding Remarks

The combination of vesicle technology and cell-free systems is a very fecund vein in synthetic biology. Different approaches coexist, such as the construction of synthetic cells as primitive cell models, or as a tool for study biological processes thanks to simplification, or for designing and building artificial systems for biotechnology. We have firstly summarized the state-of-the-art of synthetic cell research, in particular of the so-called *semi-synthetic* approach [26, 47].

Starting from the recent achievements, it is possible to foresee directions for future studies on next generation synthetic cells, whose developments seem to be at the same time challenging and exciting.

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Chapter 4

Synthetic cells produce a quorum sensing chemical signal perceived by *Pseudomonas aeruginosa*

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Preface to Chapter 4

Recent developments in liposome technology and cell-free protein expression systems allow the generation of synthetic cells endowed with capacities distinctive of natural cells. Indeed, upon the expression of functional proteins encoded by their minimal genomes, synthetic cells can be programmed to display basic metabolic functions, autonomous movements or cell division ability.

Latest advancements in synthetic cell experimentation and in the bio-inspired information and communication technology (bio-ITC) field provide the technological tools and conceptual framework for the design and engineering of programmable synthetic cells capable of communicating with natural cells, hence endowed with cognitive abilities. The generation of synthetic cells able to interface with natural cells would represent a technological breakthrough with significant fallouts in theoretical science, and would pave the way for the flourishing of innovative biotechnological processes, including the engineering of nanofactories or soft-nanorobots for intelligent drug-delivery approaches exploitable in the biomedical field.

In this experimental paper (accepted for publication in *Chemical Communications* on January 4th 2018), the generation of synthetic cells able to establish with bacterial cells a chemical communication channel based on the synthesis of a quorum sensing signal molecule is described.

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Synthetic cells produce a quorum sensing chemical signal perceived by *Pseudomonas aeruginosa*†

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Recent developments in bottom-up synthetic biology (e.g., lipid vesicles technology integrated with cell-free protein expression systems) allow the generation of semi-synthetic minimal cells (shortly, synthetic cells, SCs) endowed with some distinctive capacities of natural cells. In particular, such approaches provide the technological tools and the conceptual framework for the design and engineering of programmable SCs capable of communicating with natural cells by exchanging chemical signals. Here we describe the generation of giant vesicles-based SCs which, *via* gene expression, synthesize in their aqueous lumen an enzyme that in turn produces a chemical signal. The latter is a small molecule, which is passively released in the medium and then perceived by the bacterium *Pseudomonas aeruginosa*, demonstrating that SCs and bacteria can communicate chemically. The results pave the way to a novel basic and applied research where synthetic cells can communicate with natural cells, for example for exploring minimal cognition, developing chemical information technologies, and for the production of smart and programmable drug-producing/drug-delivery systems.

The laboratory construction of semi-synthetic minimal cells (SCs) is based on the encapsulation of chemicals inside lipid vesicles aiming at reconstructing cell-like systems that mimic biological cells.[1–4] Proteins can be synthesized inside SCs starting from nucleic acids and a transcription-translation (TX-TL) machinery,[5–7] allowing the functionalization of SCs in several ways. Among the various potential applications of SCs, engineering of SCs capable of communicating with biological cells by exchanging chemical signals is a frontier goal with relevance for nanomedicine[8] (bio)chemical information technologies,[9] and for exploring conceptual biological questions related to autonomy and cognition.[10–12] Experimental work on this subject has been recently published,[13–16] but only very few systems have been tested to date.

Here we present a further advancement in this novel field, by assaying the capacity of SCs to produce and release a chemical signal recognized as a "self-signal" (quorum sensing, or QS, signal) by the pathogenic bacterium *Pseudomonas aeruginosa*. We have selected this species because it causes hard-to-heradicate infections, and it is a major target for innovative therapies that could be based on nanomedicine. In particular, we have exploited the *rhl* QS system of *P. aeruginosa*, whose signalling molecule is *N*-butanoyl-homoserine lactone (C4-HSL) (Fig. 1).[17,18]

In a "synthetic-to-natural cells" communication channel, natural cells needs to fulfil specific requirements in order to be "signal negative" and able to perceive and transduce the signal molecule sent by the SCs in an easily detectable and quantifiable phenotype. For this reason an appropriate reporter system has been used: a *P. aeruginosa rhlI* mutant impaired in the synthesis of the signal molecule C4-HSL, containing the pKD-*rhlA* plasmid, here called RepC4*lux* (*i.e.*, PAO1 Δ*rhlI* P*rhlA*::*luxCDABE*)[19] (Fig. 1). The pKD-*rhlA* plasmid contains the genetic cassette P*rhlA*::*luxCDABE*, in which the promoter P*rhlA*, activated by the RhlR/C4-HSL complex, controls the expression of the *luxCDABE* operon. Therefore, the RepC4*lux* reporter is expected to emit light as a function of exogenous C4-HSL concentration, but it is unable to synthesize such signal molecule.

First, growth conditions to optimize the response of RepC4*lux* to exogenous C4-HSL were defined and optimized. As shown in the Supporting Information file the RepC4*lux* response to C4-HSL was maximised by varying the experimental parameters affecting

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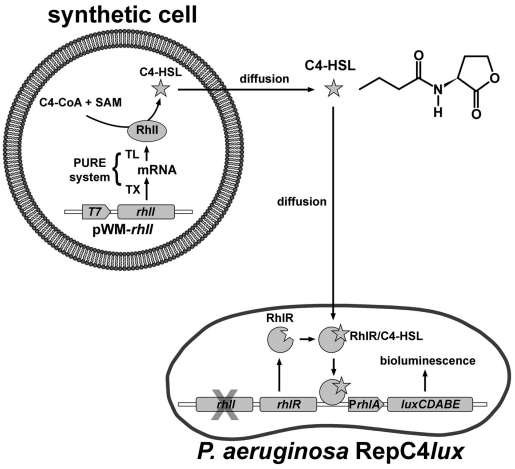

Fig. 1 Design of SCs sending a QS signal molecule to *P. aeruginosa*. The sending system is based on the production of the signal molecule C4-HSL by the synthase RhlI, encoded by *rhlI* gene, and two precursors (C4-CoA and SAM). Inside SCs, the RhlI enzyme is produced by the PURE system by transcription (TX) and translation (TL) steps, starting from a DNA template (plasmid pWM-*rhlI*). C4-HSL spreads through lipid membranes, diffuses in the medium and reaches *P. aeruginosa* cells. The receiving cells (named RepC4*lux*) contain a genetic reporter device for C4-HSL-induced bioluminescence (P*rhlA*::*luxCDABE*) and a mutation inactivating the *rhlI* gene, so that they cannot produce C4-HSL. C4-HSL produced by SCs binds to the receptor RhlR, which in turn triggers *luxCDABE* transcription and bioluminesce emission by RepC4*lux*.

its growth, *i.e.*, the antibiotic concentration, growth temperature and duration, and the presence of a washing step after overnight growth. When grown in optimized conditions RepC4*lux* allows detecting and quantifying C4-HSL as a function of light emission, with a detection limit of ca. 30 nM (Fig. S1), and it has been used in next experiments.

A plasmid for the constitutive expression of the RhlI enzyme (pWM-*rhlI*) has been generated. The "empty" vector (pWM-EV), not expressing any protein, has been used in negative control experiments (Fig. S2a). Preliminary assays showed that the pWM-*rhlI* plasmid was functional in driving C4-HSL synthesis (ca. 30 μM) in *Escherichia coli* BL21(DE3), a strain that is unable to synthesize this signal molecule (Fig. S2b). This result demonstrates that an RhlI enzyme that is active in synthesizing the QS signal molecule C4-HSL can be expressed from the T7 RNA polymerase-dependent plasmid pWM-*rhlI* in an *E. coli* BL21(DE3) genetic background.

Next, we tested pWM-*rhlI* in cell-free bulk reactions by using the PURE system,[20] a synthetic biology TX-TL kit of minimal complexity (Fig. 1). Samples including the PURE system, the pWM-*rhlI* plasmid, *S*-adenosyl-methionine (SAM) and butyryl-coenzyme A (C4-CoA) were incubated 3 h at 37 °C, in order to allow the synthesis of the enzyme RhlI, which – if correctly folded – should catalyse the reaction between SAM and C4-CoA, producing C4-HSL. Note that the natural substrate of RhlI is an acyl carrier protein, here mimicked by C4-CoA.[21] To prove the presence of C4-HSL, the cell-free mixtures were afterwards incubated with RepC4*lux*; the latter revealed that the PURE system/pWM-*rhlI* mixture produced ca. 2.4 μM C4-HSL, while the negative control sample (PURE system/pWM-EV) did not (Fig. S3). This key evidence shows that the PURE system is capable of synthesizing catalytically active RhlI, and that can use C4-CoA as a substrate for acyl transfer. The C4-HSL production was completely abrogated, as expected, in the presence of DNase or RNase (Fig. S3).

Solute-filled giant vesicles, here intended as SCs of minimal complexity, represent very reliable cellular models for most synthetic biology investigations.[22] Giant vesicles containing the pWM-*rhlI* or pWM-EV plasmids, the PURE system, SAM and C4-CoA were prepared by the droplet transfer method.[4,7,23,24]

As described in details in the Supporting Information file, the SCs production was optimized, scaled-up, and characterized in size, concentration, droplet transfer efficiency. Analysis of confocal microscopy digital images allowed the determination of SCs diameter (3.5 $\pm$ 1.4 μm, n = 4,000) (Fig. 2a). The Stewart assay[25] was used to quantify POPC and thus calculate the SCs concentration (320 $\pm$ 60 μM POPC, equivalent to ca. 3×10^8 SCs/mL). The droplet-to-vesicle transfer efficiency was estimated by a dye-release assay, resulting to be about 75% (*i.e.*, over 100 water-in-oil droplets, 75 became giant vesicles; the remaining – generally the larger ones – broke during the transfer).

After their preparation, SCs were held 3 h at 37 °C in order to allow the intravesicle synthesis of the enzyme RhlI, and thus of C4-HSL. SCs were then incubated with RepC4*lux* to quantify the C4-HSL produced. As a control, two additional samples were prepared in which DNase or RNase were externally added to SCs just after their preparation. Fig. 2b shows that SCs containing the pWM-*rhlI* plasmid were able to produce ca. 100 nM C4-HSL (expectedly lower than the same reaction performed in bulk, due to the small SCs volume), while no signal molecule was detectable in the sample containing the control vector pWM-EV. Notably, the amount of C4-HSL produced inside SCs is in line with previous *in silico* modelling,[26] and the presence of externally added DNase or RNase did not affect intravesicle C4-HSL synthesis, demonstrating that C4-HSL was indeed produced inside the vesicle lumen. Remarkably, RepC4*lux* successfully perceived the chemical signal sent by SCs and activated its C4-HSL-dependent bioluminescence genetic circuit.

All steps of C4-HSL production were rigorously checked. First, the total RNA was extracted from a mix containing SCs and RepC4*lux*, and submitted to quantitative Real Time PCR with specific primers for the *rhlI* gene to demonstrate the production of *rhlI* mRNA by the PURE system. The *rhlI* mRNA can result only from SCs, since the *rhlI* gene is inactivated in the RepC4*lux* strain. This analysis revealed high levels (150×) of *rhlI* mRNA produced into SCs containing the pWM-*rhlI* plasmid, when compared with the negative control (pWM-EV plasmid) (Fig. S4a). Second, the synthesis of the protein RhlI was verified by a radiolabelling procedure (^{35}S-methionine was included in the PURE system, so that freshly synthesized RhlI is radioactive). RhlI synthesis was followed for the first 3 h. Autoradiography of the resulting PAGE gels revealed the synthesis of ca. 140 nM of RhlI (22.6 kDa) in bulk (Fig. 2cd), and ca. 5 nM when synthesized inside SCs. Third, the

identity of C4-HSL was verified and definitely confirmed by gas chromatography-mass spectroscopy analysis of the ethyl acetate extracts of SCs samples (Fig. S4b). Finally, we confirmed that C4-HSL concentration rapidly equilibrates across the lipid membrane (Figs. S5-S6), as demonstrated by previous studies (∼30 s).[27]

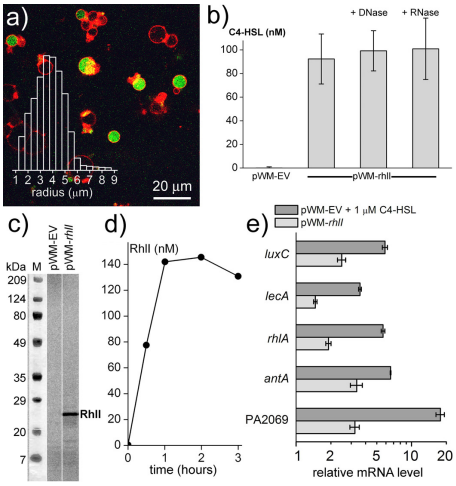

Fig. 2 Production of RhlI and C4-HSL inside SCs. (a) Protein (eGFP) synthesis inside giant vesicles-based SCs prepared by the droplet transfer method, entrapping the PURE system and the plasmid pWM-*egfp*. Membranes are stained with Trypan Blue (size distribution in the inset); (b) Production of C4-HSL inside SCs containing the PURE system and the plasmids pWM-EV or pWM-*rhlI*; (c) gel electrophoresis of ^{35}S-marked protein produced by the PURE system inside SCs containing the plasmids pWM-EV or pWM-*rhlI* (autoradiography). M, molecular weight marker; (d) time course of RhlI production in bulk, estimated by band densitometry; (e) mRNA level of indicated genes in RepC4*lux* after 6 h incubation with C4-HSL-producing SCs (containing the pWM-*rhlI* plasmid), or with SCs containing the pWM-EV plasmid plus 1 μM synthetic C4-HSL, relative to RepC4*lux* incubated for the same time with SCs containing the pWM-EV plasmid. Error bars represent the standard deviations of five experiments, all differences are statistically significant (p <0.001).

In the above experiments we demonstrated that SCs containing the machinery for RhlI synthesis and C4-HSL production are able to activate light emission in the reporter device P*rhlA*::*luxCDABE*, carried by *P. aeruginosa* RepC4*lux*. In other words, SCs send a chemical signal to *P. aeruginosa* which was programmed to emit light as response. However, different genes are known to be activated by the RhlR/C4-HSL complex in *P. aeruginosa* (Fig. 1) in real contexts.[28] Thus we tested whether the SCs-generated C4-HSL signal could activate the transcription of natural (not engineered) *P. aeruginosa* QS-controlled genes. Total RNA was extracted from: (*i*) RepC4*lux* after incubation with SCs containing pWM-EV (negative control sample); (*ii*) SCs containing pWM-EV plus synthetic C4-HSL (positive control sample); (*iii*) C4-HSL-producing SCs. Quantitative Real Time PCR analyses were performed on the resulting cDNAs by using specific primers for 4 genes whose expression is known to be C4-HSL-dependent in *P. aeruginosa*, namely PA2069, *antA*, *rhlA* and *lecA*.[28] As a further control, the RNA levels of the *luxC* gene were monitored in the same strains. The incubation with synthetic C4-HSL or with C4-HSL-producing SCs increased the levels of the tested RNAs in the RepC4*lux* strain with respect to the same strain incubated with SCs containing pWM-EV (negative control sample) (Fig. 2e). Such key observation demonstrates that SCs are able to regulate gene expression in *P. aeruginosa* and therefore altering its behaviour in the same way as it normally happens in a fully biological system. In other words, *P. aeruginosa* responds to a "message" sent by SCs and it is not able to discriminate between SCs and peer cells, at least for what concerns the C4-HSL-based communication system.

It should be noted that the synthetic-to-natural communication system presented above consisted of two sequential steps: first, C4-HSL was produced by SCs in LB medium and the absence of bacteria (Fig. S7); and after, *P. aeruginosa* reporter cells (RepC4*lux*) were incubated with SCs. The next goal was the co-incubation of SCs and *P. aeruginosa* in order to realize a realistic scenario where SCs and natural cells coexist in the same space. The calcein release test,[29] which monitors vesicle stability, revealed that although SCs are stable in the standard bacterial growth medium LB, RepC4*lux* induces SCs lysis when co-incubated with SCs in LB medium (ca. 50% in 3 h; Fig. S8ab). Bacteria crowd around SCs (Fig. S8c; *cf.* with *E. coli*, Fig. S9), causing SCs lysis probably mediated by the secretion of rhamnolipid surfactants[30] (Fig. S10 and discussion in the ESI file).

We reasoned that a gel matrix would contrast direct SCs/*P. aeruginosa* interaction, leading to SCs stabilization (Fig. 3a and Fig. S11). Preliminary experiments showed that giant vesicles are stable when immobilized in agar gel (at least for one week at 4 °C). Therefore we proceeded with co-incubation experiments. C4-HSL-producing SCs and RepC4*lux* were mixed together in warm agar gel and then co-incubated for 3 h at 37 °C in order to assay the bacterial activation in a gel matrix. Such an activation would be observed only if SCs maintain their integrity, produce RhlI and C4-HSL, and the latter molecule diffuses through the gel reaching *P. aeruginosa* cells. A clear increase in bacterial bioluminescence was measured (Fig. 3b), demonstrating the SCs are functional in C4-HSL synthesis in the presence of *P. aeruginosa*, and that the latter perceive and decode the chemical signal. Chemical communication occurred between intimately mixed SCs and natural cells.

A further evidence of *P. aeruginosa* response to SCs synthesizing C4-HSL was obtained also by confocal microscopy. To this aim, a red-fluorescent reporter strain was constructed (RepC4*red*), in which the RhlR/C4-HSL complex drives the expression of the mCherry gene (Fig. S12). Here, a transcriptional fusion between the P*rhlA* promoter region and the mCherry gene was generated, and it was inserted in single copy in a neutral site of the chromosome of the *P. aeruginosa rhlI* mutant strain (details in the Supporting Information file). SCs and RepC4*red* were co-incubated in LB-agar (following the pattern of Fig. 3a, bottom). This was done to further reduce SCs/RepC4*red* contact because of the required longer incubation times (this is due to lower sensitivity of this fluorescence-based assay when compared with bioluminescence). As shown in Fig. 3cd, the RepC4*red* strain showed increased expression of mCherry in response to C4-HSL production

by SCs. In particular, C4-HSL was produced at high concentration in the regions filled with SCs, slowly reaching bacteria by diffusion through the gel.

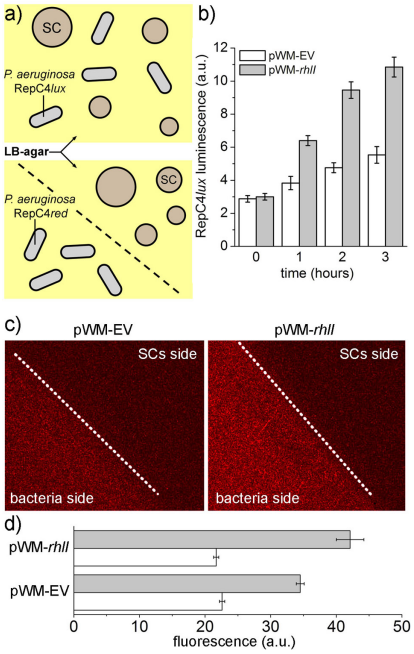

Fig. 3 Co-incubation of SCs and *P. aeruginosa* in LB-agar. (a) Cartoon representing two possible co-incubation desings: SCs and bacteria are mixed together (top), SCs and bacteria are spatially separated (bottom) (see Methods for details); (b) density-normalized luminescence of RepC4*lux* co-incubated as in top panel 'a' with SCs containing pWM-EV or pWM-*rhlI* plasmids. Error bars represent the standard deviations of five experiments, all differences are statistically significant ($p < 0.001$) except for $t = 0$ h; (c) fluorescence confocal micrographs of *P. aeruginosa* RepC4*red* after 6 h co-incubation with spatially separated SCs (as in the bottom panel 'a'); the dashed line represents the boundary between bacteria and SCs, note that individual bacteria cannot be seen at this magnification (the images are 200 μm wide), similarly SCs cannot be seen because have not been marked; (d) quantitation of mCherry produced by RepC4*red* by image analysis (pixel luminosity) (see Fig. S13). The white bars represent the background, the grey bars represents the bacteria-containing regions; error bars represent the standard deviation of six analyzed fields. The difference between the average intensity of bacteria-containing regions is statistical significant ($p < 0.001$).

In conclusions, we have demonstrated that SCs can be built and programmed in order to synthesize the signal molecule C4-HSL from DNA-encoded information. The so-produced C4-HSL can be sensed by the pathogenic bacterium *P. aeruginosa*, which reprograms its gene expression profile as if the signal was sent by other bacteria. Additional genetic devices should be employed for engineering of SCs capable of receiving and decoding a chemical signal, as demonstrated very recently.[16] This will pave the way to envisage the construction of sub-micrometer (not GVs-based) SCs for intelligent drug delivery nanomedicine, *i.e.*, capable of traveling in the body, sensing their environment, send and receive signals, and act in programmable way – for example by producing a drug only when required.[8] The knowledge about the *in vivo* stability of liposomes, generated in the past decades in drug delivery research,[31] will be a starting point for the development of such a scenario (discussed in the ESI file).

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Electronic Supplementary Information (ESI) of the paper:

Synthetic cells produce a quorum sensing chemical signal perceived by *Pseudomonas aeruginosa*

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1. Experimental Procedures

1.1 Bacterial strains, media and recombinant DNA techniques

The bacterial strains used in this study are listed in Table S1. Unless otherwise stated, all *E. coli* and *P. aeruginosa* strains were routinely grown at 37°C in Luria-Bertani broth (LB) with aeration, and antibiotics were added at the following concentration: *E. coli*, 100 µg/mL ampicillin (Ap), 10 µg/mL tetracyclin (Tc); *P. aeruginosa*, 200 µg/mL tetracyclin (Tc), 200 µg/mL kanamycin (Km).

Plasmids used or generated in this study, details on their construction and oligonucleotides used in the work are reported in Table S2 and Table S3, respectively. Preparation of plasmid DNA, purification of DNA fragments, restrictions, ligations and transformations in *E. coli* competent cells were performed with standard procedures [1]. The DNA amplification was performed by Polymerase Chain Reaction (PCR) using the GoTaq® Polymerase (Promega). All plasmids generated in this study were checked by restriction analysis and by sequencing *via* the Genechron Sequence Service (Genechron).

Table S1. Bacterial strains used in this study.

Strain	Relevant characteristic	Reference/Source
E. coli BL21 (DE3)	Strain expressing T7 RNA polymerase	Novagen
P. aeruginosa PAO1	Wild type strain	American Type Colture Collection
P. aeruginosa PAO1 Δ*rhlI*	Strain carrying a mutation in the *rhlI* gene.	University of Washington Genome Centre
P. aeruginosa PAO1 Δ*rhlI* pKD-*rhlA*	Strain carrying a mutation in the *rhlI* gene and containing the pKD-*rhlA* plasmid, in which the expression of the *luxCDABE* operon is under the control of the P*rhlA* promoter; Km^R. Biosensor that emits light in response to C4-HSL, named RepC4-*lux*.	[2]
P. aeruginosa PAO1 Δ*rhlI* P*rhlA*::mCherry	Strain carrying a mutation in the *rhlI* gene and containing the P*rhlA*::mCherry transcriptional fusion integrated into the chromosome; Tc^R. Biosensor that emits fluorescence in response to C4-HSL, named RepC4*red*.	This study

Table S2. Plasmids used in this study.

Plasmid	Relevant characteristics and plasmid construction	Reference/Source
pWM-*egfp*	Plasmid containing the *egfp* gene under the control of the T7 promoter; Ap^R.	[3]
pWM-*rhlI*	The *rhlI* gene was PCR amplified from *P. aeruginosa* PAO1 chromosome using primers FW*rhlI* and RV*rhlI*, and cloned in the pWM-*egfp* plasmid in place of the *egfp* gene by NdeI/BamHI restriction; Ap^R.	This study
pWM-EV	The *egfp* gene was removed from the pWM-*egfp* plasmid by NdeI/BamHI restriction, and the backbone plasmid was ligated after Klenow-mediated fill-in of the protruding ends; Ap^R.	This study
pKD-*rhlA*	pMS402-reived plasmid containing a transcriptional fusion between the RhlR/C4-HSL activated promoter P*rhlA* and the *luxCDABE* operon for light emission; Km^R.	[4]
MINI-CTX-1	Suicide cloning vector allowing single copy integration of genetic cassettes in a neutral site of *P. aeruginosa* chromosome; Tc^R.	[5]

MINI-CTX-P*rhlA*::*mCherry*	The *mCherry* gene was PCR amplified from the pMM45 plasmid (kindly provided by Proff. Miguel Cámara and Paul Williams, University of Nottingham) using primers FW*mCherry* and RV*mCherry*, and cloned in the MINI-CTX-1 plasmid by HindIII/SalI restriction, thus generating the MINI-CTX-*mCherry* plasmid. Then, the P*rhlA* promoter region was PCR amplified from *P. aeruginosa* PAO1 chromosome using primers FWP*rhlA* and RVP*rhlA*, and cloned in the MINI-CTX-*mCherry* plasmid by EcoRI/HindIII restriction; Tc^R.	This study

Table S3. Oligonucleotides used in this study.

Oligonucleotides	Sequence (5' – 3') [a]	Restriction site
FW*rhlI*	TATCATATGATCGAATTGCTCTCTGAAT	NdeI
RV*rhlI*	TATGGATCCTCACACCGCCATCGACAGC	BamHI
FW*mCherry*	TATAAGCTTTGGTGAGCAAGGGCGAGG	HindIII
RV*mCherry*	TATGTCGACTCACTTGTACAGCTCGTCCATG	SalI
FWP*rhlA*	TATGAATTCGCCAGAGCGTTTCGACACC	EcoRI
RVP*rhlA*	TATAAGCTTGCCGCATTTCACACCTCCC	HindIII
FW16SRT	AGTACGGCCGCAAGGTTAAA	-
RV16SRT	CCCAACATCTCACGACACGA	-
FW*antA*RT	GGCTATCACGTCAGCACAGT	-
RV*antA*RT	TTGGGCATCTCGCTGAAGAG	-
FW*PA2069*RT	TACTTCTACGGGCGCATCAC	-
RV*PA2069*RT	GATCGCTGTAGCCGTCGTAG	-
FW*lecA*RT	CAGGGCAGGTAACGTCGATT	-
RV*lecA*RT	CAACCCGGTATTGACCGGAA	-
FW*rhlI*RT	GACGTCTTCGCCTACCTGTG	-
RV*rhlI*RT	CCGTTGCGAACGAAATAGCG	-
FW*rhlA*RT	CATCTGCTCAACGAGACCGT	-
RV*rhlA*RT	TGCCGTTGATGAAATGCACG	-
FW*luxC*RT	TTATTGTCGCTTGGGGAGGG	-
RV*luxC*RT	GGGCAGAAAAACAAGCTCGC	-

[a] Introduced restriction sites are underlined.

1.2 Synthesis of RhlI and production of C4-HSL *in vitro* and inside synthetic cells

The transcription-translation reaction mixture was prepared as specified in Table S4. The PURE system kit has been aliquoted in small fractions and stored at -80° C. The specific components of the PURE system are provided as pre-mixed solutions labelled as "mix A" (enzymes) and "mix B" (buffer and other compounds). The reaction mixture indicated in Table S4 has been used for reactions both *in vitro* and inside synthetic cells. In the latter case, the mixture was used as I-buffer for preparing the w/o droplets, that were transformed in synthetic cells by the droplet transfer method, according to the protocol described in the paragraph 1.3. The O-solution was formed by PURE system buffer (50 mM Hepes-KOH, 100 mM potassium glutamate, 13 mM magnesium acetate, 2 mM spermidine, and 1 mM DTT, pH 7.6) and 200 mM glucose. After centrifugation, the O-solution was removed and the pellet was resuspended in 25 µL of fresh O-solution. When required, DNase or RNase enzymes

(0.4 mg/mL final concentration) were added to inhibit RhlI synthesis. All stock solutions were prepared in RNase-free water and stored at -20°C.

Table S4. Transcription-translation reaction mixture for expression of RhlI and synthesis of C_4-HSL.

Component	Volume (μL)	Final concentration
PURE system, mix A	50	
PURE system, mix B	20	
DNA template	-	8 μg/mL
DTT	-	1 mM
SAM	-	500 μM
Butyryl-CoA	-	2 mM
Sucrose	-	200 mM
RNase-free water	8	
TOTAL	100	

1.3 Preparation of liposomes-based synthetic cells

Liposomes have been prepared by the droplet transfer method [6], see Fig. S0. Since only few practical and mechanistic details on this method were described in previous papers, we optimized a previous procedure [7] in order to obtain a reproducible method for the generation of a large amount of vesicles. In detail, a 3 mM 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) solution in mineral oil (code M5904, Sigma-Aldrich) is prepared by prolonged stirring (2 days). The solution can be stored at 4°C and diluted just before its use to final POPC concentration of 0.5 mM.

A defined small volume of I-solution (V_i), or I-buffer, which will be contained inside liposomes, is added to a defined volume of 0.5 mM POPC in mineral oil (V_{EM}), typically V_i/V_{EM} = 0.033. Typically, 10 μL of I-buffer are emulsified in 300 μL of POPC/mineral oil solution. The w/o emulsion is created by pipetting up and down the V_i + V_{EM} mix, slowly and regularly, until a homogeneous turbid emulsion is obtained. In a 1.5 mL test tube, a volume of POPC-containing interface oil (V_{INT}) is gently stratified over a volume of O-solution (V_o), or O-buffer, in which liposomes will be resuspended, for a defined incubation time before the preparation of the w/o emulsion. Typically, 300 μL of interfacial oil are stratified over 500 μL of O-solution. The w/o emulsion, freshly prepared, is gently poured above the interfacial volume (V_{INT}); within seconds, water droplets quickly sediment filling the whole oil volume (V_{EM} + V_{INT}). The Eppendorf tubes are immediately centrifuged at room temperature or, when needed, at 4 °C, for 10 min at 2,400*g*. After centrifugation, liposomes are generally found on the bottom of the tube. The procedure has been scaled-up in order to be carried out in Falcon tubes (V_i = 50 μL, V_{EM} = 2 mL, V_{INT} = 0.5 mL, V_O = 2 mL) and centrifuged on a swing-arm bench-top centrifuge (10 min at 2,800*g*).

The addition of sucrose and glucose in the I-solution and O-solution, respectively, facilitates the droplet transfer and the sedimentation of vesicles. That happens because the sucrose inside the droplets makes them heavy, and in this way it is easier that they cross the interface during the centrifugation. Importantly, I-buffer and O-buffer must be isotonic, in order to avoid osmotic stress. Preliminary experiments indicated that an improved protocol for liposomes preparation should be based on the use of 200 mM sucrose in the I-buffer and 200 mM glucose in the O-buffer.

Liposomes dimension and size distribution have been established by direct visualization with a TCS SP5 confocal microscope (Leica Mycrosystem, Wetzlar, Germany). Around 4,000 liposomes containing 20 μM of the fluorescent dye calcein, have been photographed and the average of the liposome radius resulted to be 3.5 μm ± 1.4 μm. The amount of liposomes produced with our optimized method has been estimated to be around 320 μM ± 60 μM, in terms of POPC concentration, determined by the Stewart assay [8]. Briefly, 100 μL of a liposomes suspension were mixed with 200 μL of ammonium ferrothiocyanate/KCl and 600 μL of chloroform. After vigorous mixing, followed by an incubation of 30 minutes, the chloroform phase was analysed spectrophotometrically by reading the absorbance at 470 nm, indicative of the POPC/ferrothiocyanate red complex. Absorbance values were converted to concentration by means of a calibration line, using POPC as standard (ε_{470} = 8.5 cm^{-1} mM^{-1}). By hypothesizing a constant radius of 3.5 μm, the number of liposomes in 1 mL of solution resulted to be around 300 million. Finally, we determined the w/o droplets transfer efficiency through the interface, as the number of droplets that successfully become liposomes. This transfer efficiency can be defined as the percentage of solutes found inside liposomes with respect to the total amount of solutes used to prepare the w/o emulsion. To calculate this percentage the water-soluble probe ferroin 2 mM (ε_{508} 11,000 cm^{-1} M^{-1}) has been used. After the formation of liposomes, the absorbance of the supernatants (A_s) was measured and compared with the maximum achievable absorbance (A_{max}), corresponding to 100 % solute release. The transfer efficiency (%), intended as the fraction of V_i found inside liposomes, is calculated as $100 \times (1 - A_s/A_{max})$. In our hands, the transfer efficiency of the w/o droplets resulted to be up to 75% (this value means that 75 droplets, over 100 of them, become liposomes).

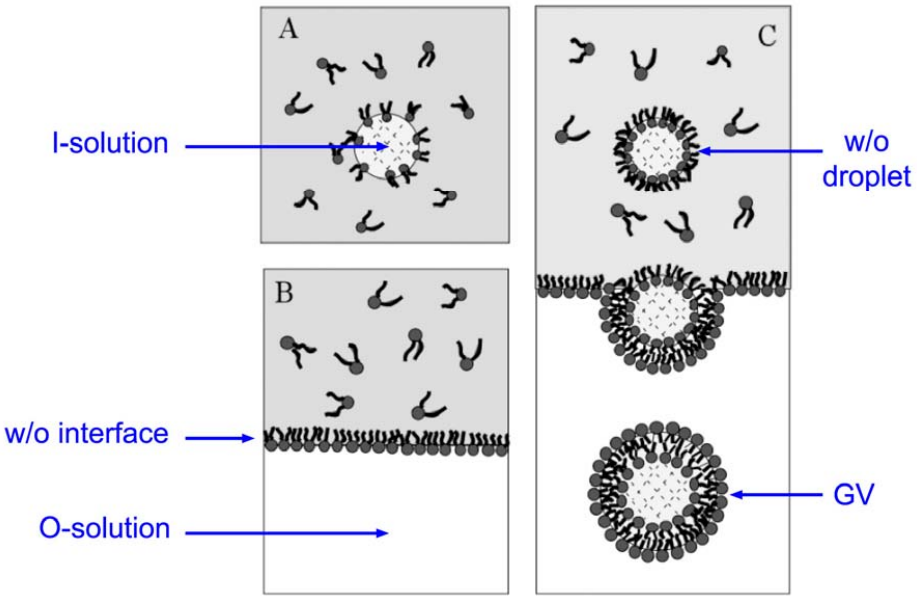

Figure S0. Preparation of GVs by the droplet transfer method (schematic). For details see textual explanation in the experimental part. Adapted with permission from [6]. Copyright 2003 American Chemical Society (in particular, the figure has been modified just by addition of the blue labels). Note that the drawn is not to scale (in particular, the shown curvature is very high; in the case of GVs, actually, the droplets curvature is negligible).

1.4 Encapsulation of C4-HSL inside GVs

C4-HSL-filled GVs were prepared by the droplet transfer method, following the procedure described in Section 1.3, with the difference that I-solution was composed by MC buffer (25 mM Tris-acetate, 100 mM potassium acetate, 1 mM DTT, pH 7.5; MC buffer is generally used in TX-TL reactions) and 200 mM sucrose, whereas the O-solution was composed by MC buffer and 200 mM glucose. Different concentrations of commercial C4-HSL was dissolved in the I-solution before emulsification. A certain amount of C4-HSL ethyl acetate solution was first placed in a vial, and the I-solution (10 μL) added after complete evaporation of ethyl acetate. The C4-HSL-filled w/o droplets were transformed into GVs by centrifugation and collected from the O-solution (500 μL). From the latter, two samples were taken for analysis: 100 μL of supernatant, and the last 100 μL that include the GVs pellet. Analysis consisted in the co-incubation, in liquid LB medium with RepC_4*lux*, as described in Section 1.5. Sodium cholate (2.5 mM) was added to similarly prepared duplicate samples.

Control experiments were run in order to measure the effect of sodium cholate on the RepC_4*lux* response (in particular, the reporter strain was co-incubated with different concentrations of C4-HSL in absence and in presence of 2.5 mM sodium cholate). Depending on incubation time and bacterial density, sodium cholate increases (+12%, 1.5 hours) or decreases (–8-13%, 3-4.5 hours) the bacterial response (data not shown).

1.5 Measurement of bioluminescence

Bioluminescence emitted from the RepC4*lux* strain was determined as a function of population density by using the automated luminometer-spectrometer VICTOR 3V (Perkin-Elmer). An LB grown over-night culture of the RepC4*lux* strain was diluted in fresh LB to an absorbance at 600 nm wavelength (A_{600}) of 2.0. One-hundred eighty μL of this culture was transferred in a microtiter plate (Perkin-Elmer), together with 20 μL of the samples to be tested, or synthetic C4-HSL as a standard. Luminescence and turbidity were determined every hour for 20 hours. Luminescence is given as Relative Light Units (RLU) normalized by cell density (A_{600}). The average data and standard deviations were calculated from three independent experiments.

1.6 Quantification of RhlI expressed inside SCs

The quantification of the expressed RhlI was performed according to the general procedure described by [9]. Briefly, [^{35}S]methionin (>37 TBq/mmol, 10 mCi/ml) was added in the reaction mixture of PURE system and allowed to icorporate into the synthesized RhlI. After the synthesis reaction, the product was subjected by SDS-PAGE and the resulting gel was dried up on a filter paper. The obtained gel image after overnight exposure was analyzed by an imager softwere, such as Image Gauge (Fujifilm). Quantitative determination was performed based on a sandard curve obtained by measuring a series of diluted [^{35}S]methionin solutions.

1.7 Gas cromatography-mass spectroscopy (GC-MS) analysis

The GC-MS analysis was performed with a Shimadzu GC2010 gas chromatograph combined with a mass spectrometer Shimadzu QP2010S. The injector was set at 250°C, the helium flow rate at 1 mL/min. A phenyl-methyl silicone capillary column it has been used, by setting the following temperature program: 150°C for 4 min,

an increase of 10°C/min until 250°C, and finally 10 min at constant 250°C. The mass spectrum was recorded through the ionization due to electrons impact at 70eV, then the mass from 40 to 200 *m*/*z* has been analyzed.

1.8 RNA extraction and Real Time PCR analysis

RNA was extracted from C4-HSL-producing synthetic cells incubated with RepC4*lux*. Samples were treated with RNAprotect Bacteria Reagent (Qiagen), and total RNA extraction was performed with the RNeasy Mini Columns Kit (Qiagen) as the manufacturer's instructions, including the on-column DNase I digestion described by the manufacturer. In addition, RNAs were treated for 1 h at 37°C with DNase TURBO (Ambion). DNase I was removed with the RNeasy Column Purification kit (Quiagen). RNA was quantified with the NanoDrop 2000 spectrophotometer (Thermo-Fisher Scientific). cDNA synthesis was performed with the iScript Reverse Transcription Supermix for RT-qPCR kit (Bio-Rad Laboratories) according to manufacturer's instructions, and quantified with NanoDrop 2000. Real Time PCRs were performed using iTaqTM Universal SYBR® Green Supermix kit (Bio-Rad Laboratories), as the manufacturer's instructions, and Rotor Gene 6000 thermocycler (Corbett Research). Primers employed in this analysis, reported in Table S3, were designed using the Primer-blast software (www.ncbi.nlm.nih.gov/tools/primer-blast). The reaction procedures involved incubation at 95°C for 1 min and 40 cycles of amplification at 95°C for 10 sec and 60°C for 45 sec. Fluorescence was registered in the last 15 sec of the 60°C step. 16S ribosomal RNA was chosen as an internal control (housekeeping gene) to normalize the Real Time PCR data in each single run, and to calculate the relative fold change in gene expression by using the $2^{-\Delta\Delta Ct}$ method. The average data and standard deviations were calculated from three independent experiments.

1.9 Estimation of synthetic cells stability in LB and in a bacterial culture

The stability of synthetic cells in LB and in the presence of bacteria was estimated *via* the calcein dequenching assay [10]. Calcein is a membrane impermeable fluorescent dye with excitation and emission wavelengths of 495 nm and 515 nm, respectively. Above the calcein concentration of about 0.1 mM, fluorescence decreases (by self-quenching) in concentration-dependent manner. In the concentration range corresponding to the self-quenching (0.1-100 mM), calcein dilution brings about a fluorescence increase. Calcein can be entrapped inside synthetic cells at high concentration, so that its fluorescence is low. Stable synthetic cells do not release calcein, and fluorescence remains low. Unstable synthetic cells release calcein in the medium, and fluorescence increases due to the calcein dilution. Quantitative analysis of calcein release can be then correlated with synthetic cells stability. Synthetic cells containing 40 mM calcein were prepared by the droplet transfer method. After the centrifugation, the resulting pellet was washed four times with 300 µL LB, to remove non-entrapped calcein, and used immediately. Bacteria were grown over-night in LB at 37°C, washed by centrifugation (1 min at 12,000 rpm), and resuspended in fresh LB medium to a final A_{600} of 2.0. The calcein fluorescence versus time profile of synthetic cells incubated with bacteria or LB was recorded during time, at 25°C. Sodium cholate 15 mM pH 7.5 was used as detergent to solubilize the synthetic cells and release all calcein, and the corresponding fluorescence value was taken as 100% release.

1.10 Immobilization of synthetic cells and bacteria in a gel matrix

Warm, liquid-like LB-agar 30 g/L, was gently directly mixed (1:1 vol/vol) with synthetic cells/RepC4*lux* or synthetic cells/RepC4*red* mixtures. The so-obtained LB-agar gels, containing synthetic cells and bacteria, were quickly poured in a 96-well microtiter plate (Perkin-Elmer) for light emission analysis. Special microscope slides for fluorescence imaging of RepC4*red* were prepared as it follows. Freshly prepared SCs in LB-agar warm gel was deposited over a microscope slide, in the presence of flat-end cylindrical comb teeths touching the surface microscope slide. The comb teeths function as template. After gelification, the comb teeths were removed, leaving small cylindrical holes in the gel. RepC4*red* dispersed in warm LB-agar, were deposited in the holes, and allowed to gel. The so-obtained system was incubated in a humidified chamber kept at room temperature. A cover glass was applied only before the observation at the confocal microscope in order not to reduce sample aeration (which is an important parameter, as revealed by preliminary experiments).

2. Supplementary Results and Discussion

2.1 Optimization of the RepC4*lux* reporter strain

In a "synthetic-to-natural cells" communication channel, natural cells needs to fulfil specific requirements in order to be "signal negative" and able to perceive and transduce the signal molecule sent by the synthetic cells in an easily detectable and quantifiable phenotype. For this reason an appropriate reporter system has been used: a *P. aeruginosa rhlI* mutant impaired in the synthesis of the QS signal molecule C4-HSL, containing the pKD-*rhlA* plasmid [2], here called RepC4*lux* (main text, Fig. 1). The pKD-*rhlA* plasmid contains the genetic cassette P*rhlA::luxCDABE,* in which the promoter P*rhlA*, activated by the RhlR/C4-HSL complex, controls the expression of the *luxCDABE* operon. Therefore, the RepC4*lux* reporter is expected to emit light as a function of exogenous C4-HSL concentration.

In a preliminary experiment, the RepC4*lux* reporter strain was grown for 14 hours (over-night growth; O/N) in a flask at 37°C, in shacking conditions, in LB supplemented with 200 µg/mL kanamycin (Km) to positively select for plasmid maintenance. This O/N culture was diluted to reach an absorbance at 600 nm wavelength (A_{600}) of 1.0 in fresh LB medium supplemented with 200 µg/mL Km, and grown at 37°C, in shacking conditions, in the absence or in the presence of different concentrations of synthetic C4-HSL in microtiter wells. An A_{600} of 1.0 was chosen because the *rhl* QS system of *P. aeruginosa* starts to be active at this cell density [11]. Cell density and light emission from the resulting sub-cultures were collected after 4 hours of incubation. As shown in Fig. S1 (white columns) the P*rhlA* promoter activity in the RepC4*lux* strain (calculated as total light emission normalized to cell density) was proportional to the amount of synthetic C4-HSL present in the medium, proving the functionality of this reporter system for C4-HSL detection. However, the RepC4*lux* strain could not detect C4-HSL concentrations below 250 nM;. The numerical *in silico* model described in [12], predicted a production of C4-HSL by the synthetic cells of about 100-200 nM. Therefore, different experimental parameters were modified to select for experimental conditions improving the detection limit of the RepC4*lux* reporter system.

RepC4*lux* produces the *N*-3-oxododecanoyl-homoserine lactone (3OC12-HSL) QS signal, and literature data showed that this QS signal molecule may have opposite effects on the *rhl* QS system. In particular, the LasR/3OC12-HSL complex promotes the transcription of the *rhl* genes [13], while the 3OC12-HSL signal molecule decreases the transcriptional activity of the RhlR/C4-HSL complex, likely by interacting with the RhlR

receptor [11]. These literature data do not allow to discriminate between possible beneficial or detrimental effects exerted by 3OC12-HSL on RepC4*lux* sensitivity toward C4-HSL. To address this issue, we tried to add 10 μM synthetic 3OC12-HSL to the culture medium, or to decrease the concentration of this molecule by washing the over-night (O/N) culture before diluting it in the fresh medium. The exogenous provision of 3OC12-HSL did not affect the reporter response to C4-HSL (data not shown), while the washing step slightly increased its performance (Fig. S1, light grey bars). To further improve the sensitivity of the reporter system toward C4-HSL, we increased Km concentration from 200 μg/mL to 500 μg/mL, since this change could possibly select for bacteria with high-copy number of the pKD-*rhlA* plasmid, carrying the Km resistance gene. An increase in pKD-*rhlA* copy number should result in an higher intracellular concentration of the P*rhlA*::*luxCDABE* cassette, possibly decreasing the amount of synthetic C4-HSL required to ensure a measurable response of the RepC4*lux* reporter strain to this molecule. According to our hypothesis, the cultivation of RepC4*lux* at Km concentration of 500 μg/mL resulted in enhanced sensitivity of this reporter strain for C4-HSL, with respect to 200 μg/mL Km (Fig. S1, dark grey bars).

Once our experimental settings have been modified by including the washing step of the O/N culture and the addition of higher Km concentration (500 μM), we tried to further improve the reporter's performance by changing other cultural conditions, such as the medium composition (LB or M9 supplemented with glucose), the temperature (30°C or 37°C), the aeration level (static or shacking incubation), and the starting cellular density of the culture (A_{600} of 1.0, 1.5, or 2.0). This analysis revealed that the sensitivity of RepC4*lux* to C4-HSL was maximal when growing this reporter strain in LB at 30°C in static conditions, starting with an A_{600} of the culture of 2.0 (Fig. S1, black columns).

Despite each single implementation step led to slight sensitivity improvement, the overall optimization process resulted in a 8-fold decrease of the minimal concentration of C4-HSL required to activate a response in RepC4*lux*, from 250 nM to 31.25 nM (compare the white and black bars in Fig. S1). The best experimental setting here described have been applied in all the experiments described in this work, when the RepC4*lux* reporter strain has been used to quantify C4-HSL production.

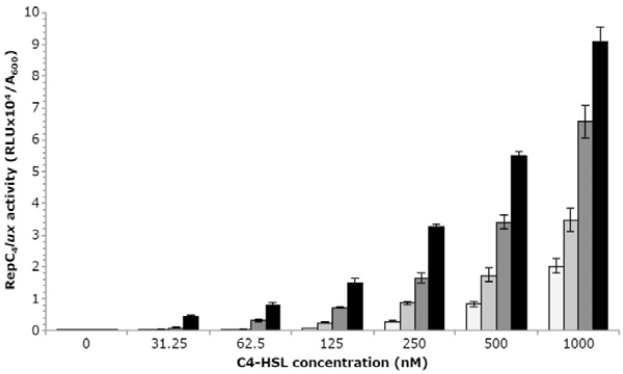

Figure S1. Graph showing the RepC$_4$*lux* reporter activity measured after 4 h of incubation without or with increasing concentrations of synthetic C4-HSL (indicated at the bottom). The activity is reported as relative light units (RLU) normalized to the cell density of the corresponding cultures (A_{600}). Strains were grown in LB supplemented with 200 μg/mL Km, at 37°C, in shacking conditions, starting from an A_{600} of 1.0 (white columns). The same conditions were used together with a washing step of the O/N culture (light grey bars), and with this washing step and an increased Km concentration to 500 μg/mL (dark grey bars). The maximal sensitivity of RepC$_4$*lux* was achieved in LB supplemented with 500 μg/mL Km, at 30°C, in shacking conditions, starting from an A_{600} of 2.0 after a washing step of the O/N culture (black bars). The average of three independent experiments is reported with standard deviations.

2.2 Construction and *E. coli*-based validation of the pWM-*rhlI* plasmid

Gene transcription *via* the PURE system is based on the T7 RNA polymerase, hence a DNA sequence encoding the *rhlI* gene under the control of a T7 RNA polymerase-dependent promoter (*i.e.* the T7 promoter) should be used as template for the PURE translation/transcription machinery. To this aim, a plasmid previously used to express enhanced Green Fluorescence Protein (eGFP) with the PURE system *in vitro* (named pWM-*egfp*) has been conveniently modified [3]. Plasmid pWM-*egfp* contains the *bla* gene for ampicillin resistance, and the *egfp* gene under the control of the T7 promoter [3]. Briefly, the the *egfp* gene contained in pWM-*egfp* was replaced by the *rhlI* gene from *P. aeruginosa* PAO1 (as described in Experimental procedures; Table S2, Fig. S2a). The resulting plasmid pWM-*rhlI* should allow the T7 RNA polymerase-dependent expression of RhlI. As a control, we also generated an empty vector, pWM-EV, by removing the *egfp* gene from the pWM-*egfp* plasmid, and by ligating the plasmid backbone upon Klenow enzyme-mediated fill-in of the restriction sites' protruding ends (as described in Experimental procedures; Table S2, Fig. S2a). To validate the functionality of the pWM-*rhlI* plasmid, both pWM-*rhlI* and pWM-EV were transformed into *E. coli* BL21(DE3) cells (Novagen), containing in the chromosome a genetic cassette for the constitutive expression of the T7 RNA polymerase. *E. coli* BL21(DE3) is not able to synthesize C4-HSL or any other QS signal produced by *P. aeruginosa*. Since the natural substrates for C4-HSL synthesis (SAM and butyryl-ACP) are common metabolites in *E. coli*, the pWM-*rhlI* plasmid is expected to confer to *E. coli* BL21(DE3) the ability to synthesize this QS signal molecule.

To verify pWM-*rhlI* functionality, the *E. coli* BL21(DE3) strains carrying the pWM-*rhlI* or pWM-EV plasmids were grow for 8 h at 37°C and the presence of C4-HSL in the corresponding cell-free spent media was evaluated by using the RepC4*lux* strain. As a positive control, a cell-free spent medium of the C4-HSL producing bacterium *P. aeruginosa* PAO1 was used. A standard curve for C4-HSL quantification was generated by simultaneously incubating the RepC4*lux* reporter strain with increasing concentrations of synthetic C4-HSL. The C4-HSL produced by the *E. coli* strain carrying the pWM-*rhlI* plasmid was about 30 μM, higher than the concentration of C4-HSL produced by *P. aeruginosa* PAO1 (about 14 μM). As expected, no C4-HSL was detectable in the spent medium of the *E. coli* strain carrying the pWM-EV plasmid (Fig. S2b). Overall, these data demonstrate that an RhlI enzyme that is active in synthesizing the QS signal molecule C4-HSL can be expressed from the T7 RNA polymerase-dependent plasmid pWM-*rhlI* in an *E. coli* BL21(DE3) genetic background.

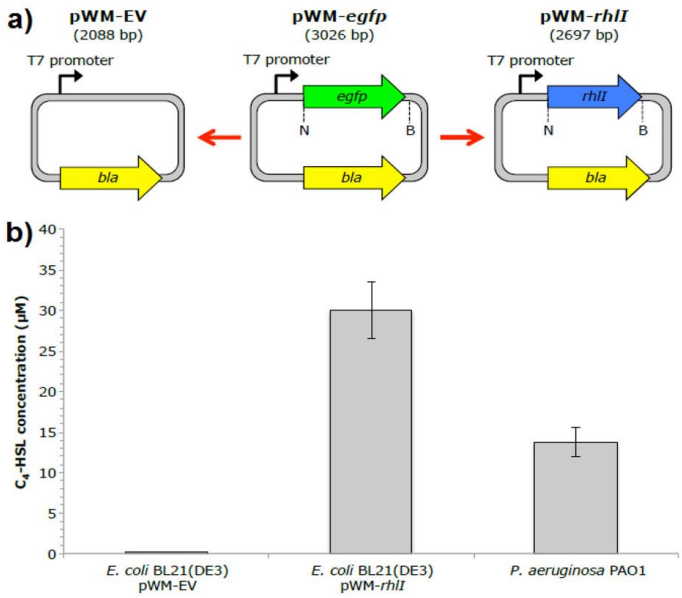

Figure S2. a) Schematic representation of the pWM-EV and pWM-*rhlI* plasmids used in this study, and of the pWM-*egfp* plasmid. *Bla*, ampicillin resistance gene; N and B, NdeI and BamHI restriction sites, respectively; bp, base pair. b) Histogram reporting the concentration of C4-HSL measured *via* the RepC4*lux* reporter strain in cell-free spent media from cultures of the indicated strains. The average of three independent experiments is reported with standard deviations.

2.3 *In vitro* synthesis of C4-HSL *via* the PURE system from a DNA template

The next step of our experimental approach was to test the possibility to synthesize C4-HSL *in vitro* from a DNA template by using the PURE system. To this aim, three independent reaction mixtures (20 µL) were prepared including the PURE system, the pWM-*rhlI* plasmid, 0.5 mM SAM and 0.5 mM butyryl-CoA (herein referred to as C4-CoA) as C4-HSL precursors. The three reaction mixtures were incubated at 37°C for 3, 5 or 7 h. In parallel, control reactions were prepared containing the pWM-EV plasmid instead of pWM-*rhlI*. After the indicated incubation times, C4-HSL concentration in the different samples was evaluated by using the RepC4*lux* reporter strain. As reported in Fig. S3, white bars, our experimental settings allowed producing ca. 1 µM C_4-HSL *in vitro* from the pWM-*rhlI* plasmid after 3 h incubation, while no C4-HSL was detectable when using the control vector pWM-EV. This shows that well-folded and therefore catalytically active RhlI can be synthesized by the PURE system. Note that the presence of the two C_4-HSL precursors (SAM and C4-CoA) does not impair the PURE system functions. Since no increase in C4-HSL production was observed for prolonged incubation times (5 and 7 h; data not shown), the following experiments were performed by incubating the C4-HSL producing SCs for 3 h.

To improve C4-HSL synthesis *in vitro* the concentration of C4-CoA was increased from 0.5 mM to 2 mM. In fact, the Michaelis-Menten constants (K_M) for the two RhlI substrates, SAM and C4-CoA, are 14 and 230 µM, respectively [14]. This means that whereas the SAM concentration (0.5 mM) is well above K_M, ensuring maximal enzyme rate, C4-CoA concentration (500 µM) was just two times the K_M. It is then expected that an increase of C4-CoA concentration brings about an increase of the reaction rate. Indeed, the use of a higher C4-CoA concentration (from 0.5 to 2 mM) led to an increase in C4-HSL synthesis to ca. 2.5 µM (Fig. S3, grey bars). This

final experiment was repeated to include a control sample with the pWM-EV plasmid. Moreover, samples containing the pWM-*rhlI* plasmid were also supplemented with DNase or RNase as negative controls, in order to degrade the pWM-*rhlI* plasmid DNA or the *rhlI* mRNA, respectively. As expected, ca. 2.5 μM C4-HSL was produced only in the sample containing the pWM-*rhlI* plasmid in the absence of DNase and RNase (Fig. S3, grey bars). According to these results, the C4-HSL biosynthetic experiments described in the following experiments were performed with 2 mM C4-CoA.

Previous studies showed that purified RhlI was able to catalyse C4-HSL synthesis from the natural precursors SAM and C4-ACP, and that synthetic C4-CoA could be used by this enzyme as an acyl donor instead of C4-ACP, even if with low efficency with respect to C4-ACP. In fact, a kinetic analysis showed that the K_M for C4-CoA was 40-fold greater than the K_M for C4-ACP [14]. Actually, the use of the unconventional precursor C4-CoA in our approach could partially account for the poor conversion of the substrates into the final product C4-HSL.

Overall, the data here described demonstrate that the synthesis of a signal molecule like C4-HSL is achievable *in vitro* with a well-defined TX-TL system of minimal complexity (*i.e.*, the PURE system) directly from adequate substrates and DNA template.

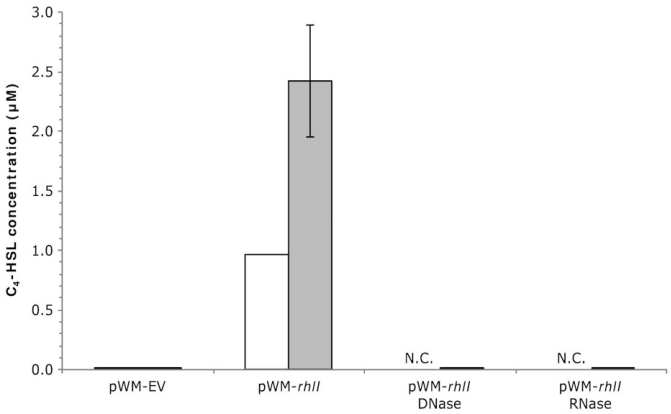

Figure S3. a) Histogram reporting the concentration of C4-HSL measured *via* the RepC4*lux* reporter strain in samples containing the PURE system, 0.5 mM SAM and the plasmid/enzyme indicated below the corresponding bar. Samples also contained 0.5 mM C4-CoA (white bars) or 2 mM C4-CoA (grey bars). N.C., data not collected. For grey bars, the average of three independent experiments is reported with standard deviations.

2.4 Characterization and validation of the communication system between synthetic and natural cells

Each step of the C4-HSL synthesis inside synthetic cells was investigated, in order to verify the transcription of *rhlI* gene into mRNA, the translation of *rhlI* mRNA into RhlI protein, *via* PURE system, and finally the identity of the signal molecule as C4-HSL.

To demonstrate the production of the *rhlI* mRNA inside SCs, total RNA from a mix containing synthetic cells and RepC4*lux* was extracted, retro-transcribed to cDNA and used in Real Time PCR analysis performed with *rhlI* gene specific primers. Please consider that *rhlI* mRNA synthesis in this system can result only from SCs, since the *rhlI* gene has been inactivated in the RepC4*lux* strain. As internal control, the housekeeping gene 16S was used. As shown in Fig. S4a, the amount of *rhlI* mRNA was about 150-fold higher in SCs containing pWM-*rhlI*

plasmid with the respect of those containing the control plasmid pWM-EV, proving the synthesis of the *rhlI* mRNA *via* the PURE system inside SCs.

To verify RhlI synthesis, protein extracts from synthetic cells containing radiolabeled ^{35}S-methionine were analyzed by means of SDS-PAGE. Autoradiography of the resulting gels revealed *de novo* synthesis of a protein with molecular weight corresponding to RhlI (main text, Figs. 2c and 2d).

Finally, to verify the identity of the signal molecule produced inside SCs, a gas chromatography-mass spectroscopy analysis was performed on ethyl acetate extracts of SCs-producing C4-HSL. The resulting *m/z* spectrum (Fig. S4b3) matches exactly with previously published spectrum of synthetic C4-HSL [15](Fig. S4b2), proving that SCs are effectively producing this signal molecule.

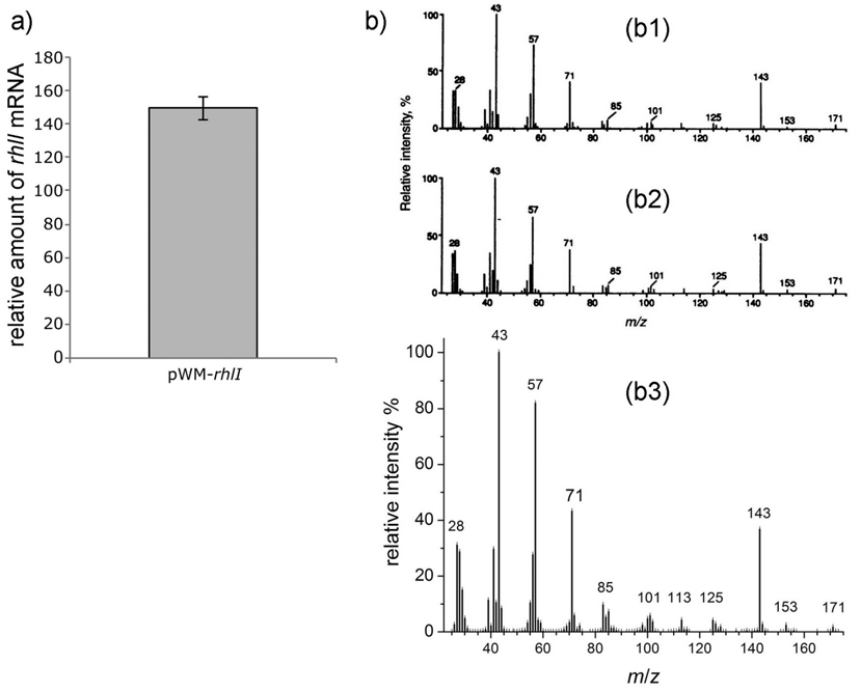

Figure S4. a) Histogram reporting the relative amount of *rhlI* RNA inside SCs containing pWM-*rhlI* plasmid with respect to SCs containing pWM-EV. The average of five independent experiments is reported with standard deviations. b) *m/z* spectrum obtained by GC-MS analysis. (b1) *m/z* spectrum of C4-HSL obtained by *P. aeruginosa* culture medium [15]); (b2) *m/z* spectrum of synthetic C4-HSL [15]; (b3) *m/z* spectrum of C4-HSL produced in this study. In particular, C4-HSL was extracted with ethyl acetate from SCs containing the PURE system, 0.5 mM SAM, 2 mM C4-CoA and pWM-*rhlI* plasmid. Panels (b1) and (b2) have been reproduced from [15] (Copyright 1995 National Academy of Sciences).

2.5 Release of C4-HSL from C4-HSL-filled GVs

The droplet transfer method allows achieving high entrapment yields. Typically, GVs contain between 50 and 70% of solutes in the sum of their inner volumes. We therefore prepared two samples (100 μL each) from the O-solution supernatant and pellet. According to scenario "a" of Fig. S5 corresponding to a low/moderate diffusion

rate of C4-HSL through the lipid membrane, the supernatant (S) should contain the "released" fraction of C4-HSL (*i.e.*, released during the droplet transfer), whereas the resuspended pellet (P) should contain a larger amount of C4-HSL mainly derived from the GVs lumen (Fig. S5a). When co-incubated with RepC_4*lux*, all C4-HSL becomes available, due to GVs lysis, and therefore its overall concentration could be determined as previously shown. In contrary, according to scenario "b" (Fig. S5b), corresponding to a high diffusion rate of C4-HSL through the lipid membrane, S and P should contain a very similar amount of C4-HSL, and correspondingly, produce similar biological response.

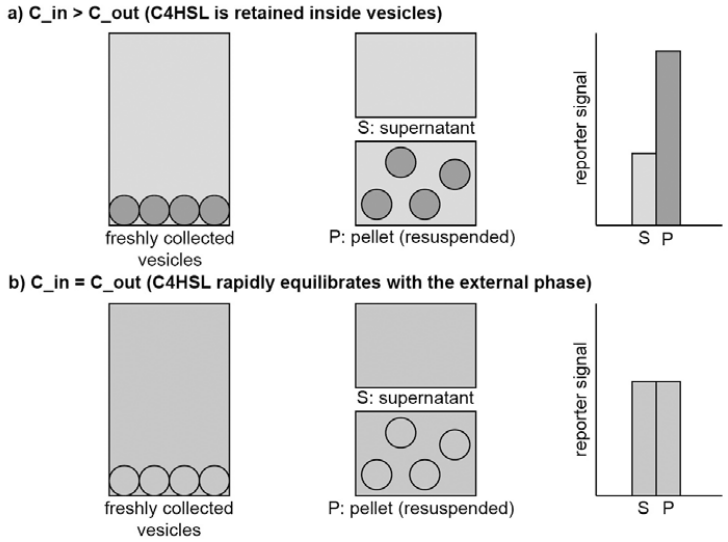

Figure S5. Experimental strategy to show the extent of C4-HSL encapsulation inside GVs. "C_in" means the intra-vesicle concentration of a certain solute, "C_out" means the external concentration. Grey colours of different darkness have been used to represent concentrations. Experimental results are shown in Fig. S6, confirming hypothesis "b".

Experimental results are shown in Fig. S6, for different incubation times and different C4-HSL concentrations. Note that S and P are run in duplicate, and sodium cholate (2.5 mM) has been added to the duplicate in order to release all C4-HSL from GVs or other small vesicles present in the supernatant, thus allowing bacteria to be exposed from the beginning to the total amount of C4-HSL in the samples. Note that sodium cholate has a minor effect on the RepC4lux response (depending on incubation time, bacterial density, and bioluminescence), but, as detailed in the experimental methods, this lies within –13 and +12%. The data support the scenario "b" of Fig. S5, in full agreement with the facile diffusion of C4-HSL through the membrane (equilibration time around 30 seconds [16]). Note that the preparation of GVs takes 10-15 minutes.

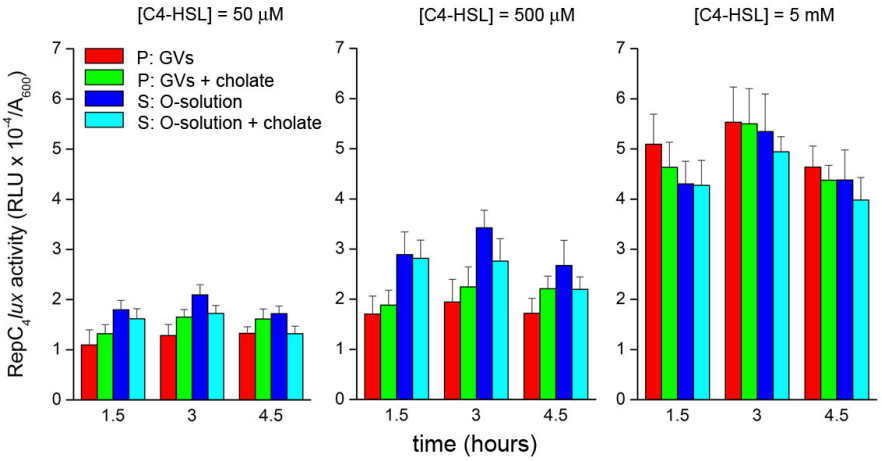

Figure S6. Experimental data referring to three different experiments, each run in duplicate (n = 2), on C4-HSL filled GVs prepared by the droplet transfer method. The pelletted GVs (P) and the supernatant (S), as derived from the droplet transfer method, have been co-incubated with $RepC_4$*lux* in order to determine the amount of C4-HSL present in each sample. Moreover, each sample was treated with 2.5 mM sodium cholate to release all C4-HSL. Please note that sodium cholate has a minor effect on the bacterial response (depending on incubation time, bacterial density, bioluminescence values) but this lies within a –13% to +12% range. In no case an enrichment of C4-HSL can be observed in the pelletted GVs, suggesting that C4-HSL rapidly equilibrates across the lipid membrane.

2.6 SCs stability in LB and in a bacterial culture (*P. aeruginosa, E. coli*)

The synthetic-to-natural communication system presented above consisted of two sequential steps: *i)* C4-HSL was produced by SCs in the absence of bacteria; *ii) P. aeruginosa* reporter cells were incubated with SCs. Ideally, the same result could be obtained in a one-step process, in which C4-HSL synthesized by SCs directly activates a response in neighbouring natural cells. This requires the co-incubation of SCs and *P. aeruginosa,* hence SCs should be stable in a bacterial growth medium and in the presence of *P. aeruginosa* cells. Therefore, we assessed SCs functionality and stability in the standard bacterial growth medium LB. To this aim, SCs containing the PURE system and the pWM-*egfp* plasmid, for the T7 RNA polymerase-dependent expression of the eGFP protein, were generated by the droplet transfer method, and the SCs pellet was immediately suspended in LB. Confocal microscope imaging of the sample revealed that SCs are stable in LB at 37°C for at least 3 h. Moreover, the eGFP protein was clearly expressed, ensuring that functional proteins can be produced inside SCs suspended in this bacterial growth medium (Fig. S7a). A spectral analysis of the emitted fluorescence confirmed eGFP identity (Fig. S7b).

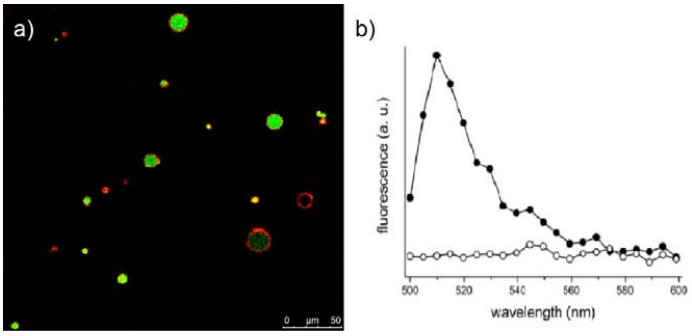

Figure S7. a) Confocal microscope image of synthetic cells expressing eGFP (green signal) in LB. The synthetic cells membrane was stained with Nile Red (red signal). b) Fluorescence emission spectrum of the green synthetic cells shown in a) as recorded by microspectrofluorimetry (expected eGFP λmax: 512 nm).

Synthetic cells stability was also assessed in more quantitative way by means of classic calcein test [10] using calcein-loaded synthetic cells as a model system. At high concentration inside SCs, calcein is self-quenched and emits low fluorescence signal; if it is released in the medium, for example as a consequence of SCs lysis, calcein is diluted, and its fluorescence emission increases (Fig. S8a). On these bases, SCs containing calcein were generated, and incubated for 3 h in LB or in a bacterial culture of the RepC4*lux* strain grown in LB. Fluorescence of the samples was measured at different time points, and sodium cholate was added to control samples to induce synthetic cells lysis; fluorescence emission from the sodium cholate containing samples was considered as 100% lysis, while basal fluorescence measured at the beginning of the experiment was considered as 0% lysis. As shown in Fig. S8b, low-fluorescence emission was measured from samples containing calcein-loaded SCs incubated in LB (maximal lysis ca. 8%), indicating that SCs are stable in LB, confirming the initial confocal microscopy qualitative observations. Conversely, the fluorescence signal increased when the calcein-loaded SCs were incubated with the RepC4*lux* culture, indicating that *P. aeruginosa* induces SCs lysis. In particular, ca. 50% lysis was induced in the first 2 h, while fluorescence emission was stable in the following hour of incubation. SCs instability induced by bacteria could be due to the crowding of *P. aeruginosa* cells in proximity of SCs (Fig. S8c).

However, the non-lysed SCs reached a steady level of ca. 50% in the bacterial culture (Fig. S8b), and such an amount of SCs could be sufficient to produce enough C4-HSL to induce a response in the bacteria. Moreover, the number of lysed synthetic cells steadily increases during the first 2 h of incubation (Fig. S8b), therefore more synthetic cells could be active in synthesizing C4-HSL in the first period of co-incubation with *P. aeruginosa*. Overall, the experiments reported above provide the indications that SCs are stable and functional in a bacterial growth medium, such as the widely used LB, while they are not entirely stable in the presence of a *P. aeruginosa* culture. However, as an alternative strategy it has been reasoned that the bacteria/SCs co-incubation could be realisable by immobilizing SCs and bacteria in a gel matrix, instead of a liquid medium. This should limit their direct contact, possibly decreasing the destabilization of SCs induced by bacteria. Moreover, the use of a low-diffusion gel matrix should lead to the local accumulation of the signal molecule nearby producer SCs, and natural receiver cells trapped in their close proximity should be exposed to high signal molecule concentration.

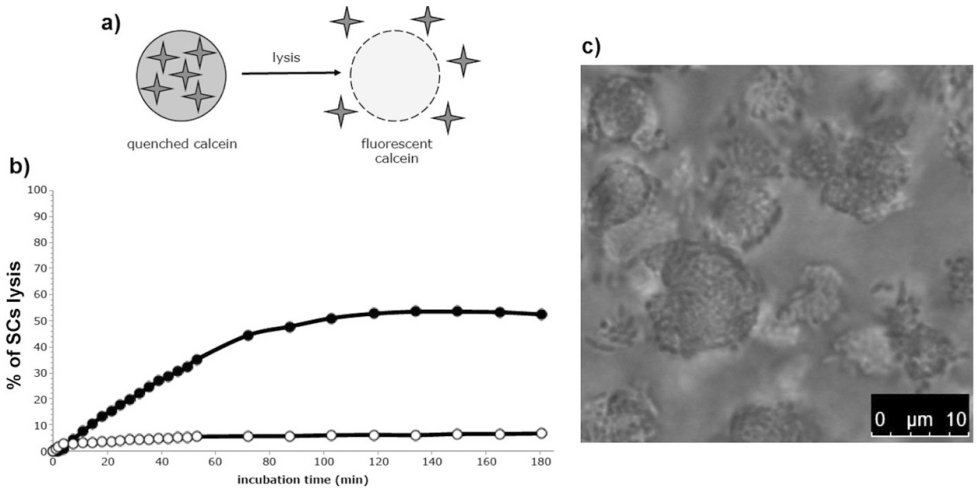

Figure S8. a) Schematic representation of the 'calcein dequenching test' used to assess SCs stability. b) Graph reporting the percentage of SCs lysis, as a function of calcein release, when incubated in LB (open circles) or in an LB-grown RepC4*lux* culture (filled circles). Maximal fluorescent emission measured upon sodium cholate addition is considered as 100% lysis. c) Bright field image, obtained by confocal microscope, of SCs containing the PURE system, co-incubated with the RepC4*lux* biosensor. Bacteria form aggregates that cover the synthetic cells.

We have also checked the stability of GVs in the presence of other bacteria, *e.g. E. coli*. As shown in Figure S9, GVs are stable, as already reported by Lentini *et al.*, 2017 [17]. Notably, the latter study also mention that *V. fischeri* and *V. harveyi*, in addition to *E. coli*, do not degrade vesicles. Therefore, we reasoned that *P. aeruginosa* behave differently due to its specific biological nature, and we ask what would be the factor determining its behavior when co-incubated with GVs (Figure S8c).

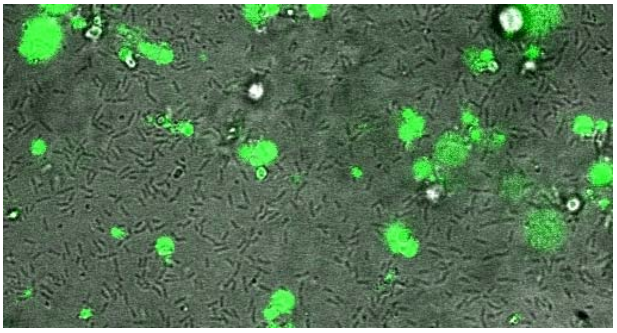

Figure S9. Calcein-containing giant liposomes (green circular particles) coincubated with *E. coli* (black rod-like particles). No vesicle/bacteria physical interaction.

A detailed investigation on the molecular basis of this interaction, although interesting, lies out of the scope of this work. However, we provide here a discussion based on available knowledge.

Rhamnolipids (Figure S10) are the best-characterized bacterial surfactants. They are produced and secreted by *P. aeruginosa* in order to make hydrophobic substances bioavailable for bacterial metabolism. These

biosurfactants are capable of emulsifying/solubilizing lipids [18, 19], justifying the disruption of GVs. Moreover, their haemolytic activity can lead to solute release from GVs (without GVs disruption). Bioavailable lipids released from GVs as a consequence of rhamnolipids activity may also act as attractants for the motile *P. aeruginosa* cells, causing crowding.

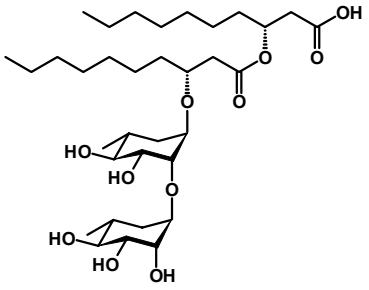

Figure S10. A typical di-rhamnolipid.

We have carried out experiments to verify whether vesicles are attacked by *P. aeruginosa* even when they do not contain the TX-TL protein-rich kit.

- We have observed that simple calcein-containing giant vesicles are also degraded by *P. aeruginosa*. This excludes a major role played by TX-TL components.

- Then we have also checked that sucrose (included inside the GVs for technical reasons) does not sustain or stimulate *P. aeruginosa* growth.

- Similarly, it can be excluded that residual oil (traces) present in GVs membrane (derived by our preparation method) is a relevant factor, because Lentini *et al.* [17] (who used an oil-free preparation method) also reported vesicles lysis by *P. aeruginosa*.

It can be concluded, in absence of further information, that crowding and lysis of GVs are specific properties of *P. aeruginosa*, likely connected to the presence of bioavailable phospholipids and involving rhamnolipids production. Indeed, it is plausible that rhamnolipids-mediated release of phospholipids from GVs activates the *P. aeruginosa* chemotactic response, causing crowding of the bacteria on GVs and their lysis.

SCs stabilization in gel

Preliminary experiments showed that SCs are stable until 4 h in a gel formed by LB growth medium in which agar is added at a concentration of 15 gr/L (LB-agar) (up to one week, if stored at 4°C, data not shown). When incubated with *P. aeruginosa* culture, recurrent is the observation in LB-agar of aggregated and/or deformed SCs. Despite this partial instability, it has been clearly demonstrated that SCs are capable of synthesizing a protein (eGFP) when co-incubated in LB-agar gel in the presence of *P. aeruginosa* (Fig. S11).

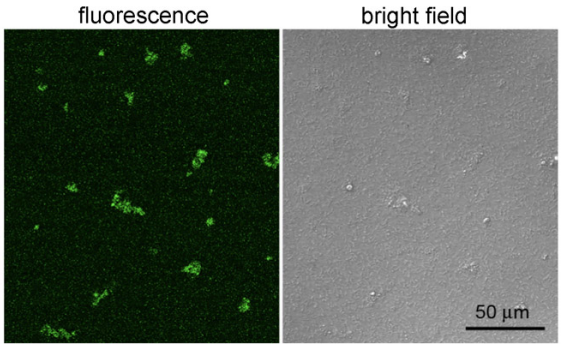

Figure S11. Image obtained by confocal microscopy of SCs capable of expressing the eGFP protein from the PURE system and the pWM-*egfp* plasmid, co-incubated with a *P. aeruginosa* culture in LB-agar.

2.7 Construction and validation of the RepC4*red* reporter strain

The immobilization of SCs and natural cells in a gel matrix described above should allow imaging of both SCs and bacteria at the single cell level, possibly increasing the detection of those bacteria activated by the signal molecule produced by the SCs. Moreover, since this approach should allow time-lapse imaging of the same natural cell, it could be useful to demonstrate the SCs to natural cells communication system "in action" at the single cell level.

To pursue this objective, a *P. aeruginosa*-based reporter strain in which C4-HSL induces the expression of the fluorescent protein mCherry was generated. Indeed, the RepC_4*lux* reporter previously described is based on light emission, a phenotype that is not possible to measure at the single cell level. Therefore, a transcriptional fusion between the P*rhlA* promoter region and the mCherry gene was generated, and it was inserted in single copy in a neutral site of the chromosome of the *P. aeruginosa rhlI* mutant strain (see Table S1 and Table S2). The resulting RepC4*red* reporter strain should emit a red fluorescence signal in response to the QS signal molecule C4-HSL. As shown in Fig. S12, this phenotype was verified by confocal microscope imaging, ensuring the functionality of the RepC4*red* reporter strain.

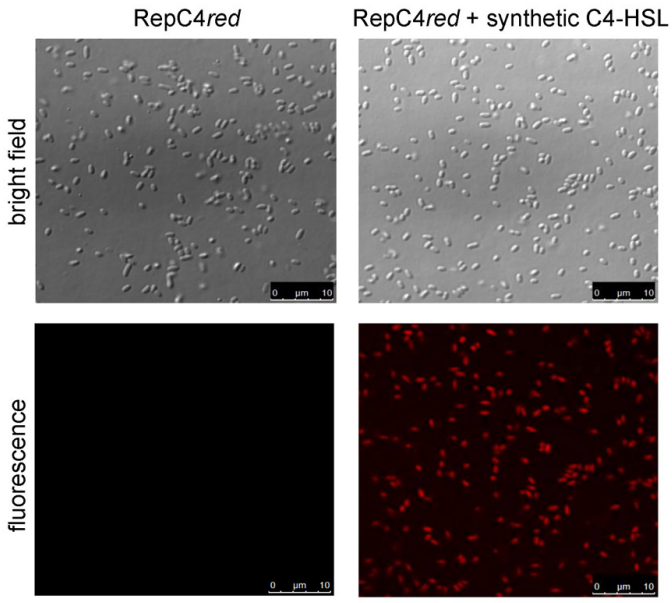

Figure S12. Confocal microscope images showing C4-HSL-induced red fluorescence emission by the RepC4*red* strain. RepC4*red* was grown in LB (left panels) or in LB supplemented with 20 μM synthetic C4-HSL (right panels).

Finally, the fluorescence properties of intra-bacterial mCherry protein (for example in experiments as those described in the main text, Fig. 3c) were verified by microspectrofluorimetry. In particular, regions hosting bacteria were submitted to λ-scan experiment in order to record the emission spectrum. As shown in Fig. S13, a remarkable agreement is observed between the emission spectra recorded in bulk (panel *a*) and in the microscope slide (panel *b*).

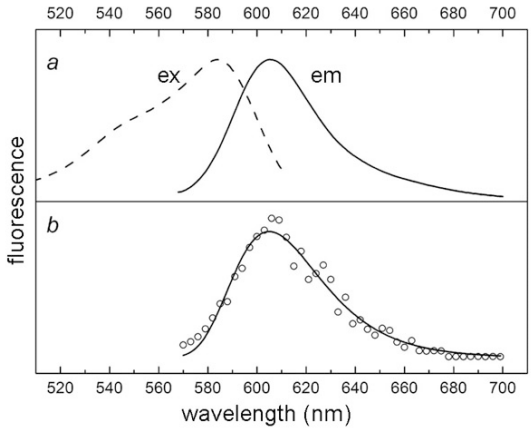

Figure S13. Fluorescence properties of mCherry. a) Excitation and emission spectra of mCherry extracted from *P. aeruginosa* treated with synthetic C4-HSL (the spectra were recorded on conventional the fluorimeter Jasco FP-6200), note that λex = 584 nm and λem = 605 nm. b) Emission spectrum of mCherry inside *P. aeruginosa* RepC4*red* in the gel matrix as reported in the main text, Fig. 3c.

2.8 Discussion on the potential development of SCs for nanomedicine

Our results, as well as those presented by other groups, elicit the question whether, and at what extent, SCs can be used as tools in nanomedicine, for example – in the context of SCs-bacteria communication, to interfere with bacterial communication, or to behave as "intelligent" drug delivery systems capable of interfacing with biological cells, feel/send signal, and behaves correspondingly. In particular, an open question is the *in vivo* stability of SCs.

Liposomes are already used in internationally approved commercial formulations for the delivery of anti-tumour and anti-fungal drugs (*e.g.*, Doxil, Ambisome), or many other formulations are in phase I, II, and III. A huge amount of studies have been devoted to understanding the fate of liposomes in vivo, in laboratory animals [20].

The factors affecting liposome stability *in vivo* (in the blood stream), are well known: (1) vesicle size (max size up to 100-150 nm), (2) lipid composition (cholesterol improves stability), (3) presence of few % PEG-ylated lipids (increase stability by reducing liposome opsonization), (4) vesicle charge (cationic lipids decrease liposome stability).

SCs are liposomes filled with macromolecules. As far as the external liposome surface does not present proteins or other biomolecules, it is expected that SCs behave as "simple" drug-containing liposomes.

Importantly, our SCs, based on GVs, cannot be directly used for *in vivo* applications, because they are too large. Future developments in this field must be based on conventional submicrometer vesicles (100-150 nm), as miniaturized versions of our SCs. This elicits more questions:

- Is it possible to build conventional submicrometer vesicles encapsulating TX-TL systems, and producing proteins? Yes, as confirmed by an already published report [21].
- Is it possible to build vesicles encapsulating TX-TL systems, whose lipids include PEGylated lipids and/or cholesterol? Yes, as confirmed by already published reports, for example [22,23].
- Is it possible to tune the lipid composition, and therefore the electrostatic charge, of vesicles encapsulating TX-TL systems? Yes, [as confirmed by already published reports, for example [24].

In conclusion, available knowledge provides motivated and well-documented reasons to state that small SCs (100-150 nm), not presenting proteins on their surface, would be stable in plasma in comparable way to current commercially available liposome formulations for drug delivery.

Our study deals with GVs only for demonstrative purposes, and because GVs can be easily studied by conventional light microscopy. GVs cannot be used for *in vivo* applications based on pulmonary or intravenous administration (but could be used, for example, for topical administration).

3. Acknowledgements and Authors Contribution

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Author Contribution statement. P. Stano conceived the idea of chemical communication between synthetic and natural cells. G. Rampioni defined an experimental research project based on the use of *P. aeruginosa* and quorum sensing molecules such as C4-HSL. P. Stano, G. Rampioni, F. D'Angelo and L. Leoni further elaborated the experimental approach (F. D'angelo, "*Studio delle basi molecolari per lo sviluppo di sistemi di comunicazione tra batteri e cellule semi-sintetiche*", Thesis for the Bachelor Degree in Biology, University of Roma Tre, February 2012). F. D'Angelo, A. Zennaro, M. Messina, G. Rampioni and P. Stano carried out chemical communication experiments by using SCs and *P. aeruginosa*. Y. Kuruma provided the PURE system and carried out experiments on cell-free RhlI synthesis. D. Tofani identified C4-HSL by GC-MS. P. Stano, G. Rampioni, L. Leoni and F. D'Angelo wrote the paper; all authors commented and revised the paper.

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Chapter 5

A coculture-based approach for screening campaigns aimed at identifying novel *Pseudomonas aeruginosa* quorum sensing inhibitors

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Preface to Chapter 5

Rapid emergence of antibiotic-resistant pathogens worldwide calls for the development of alternative antibacterial approaches. An innovative strategy to treat infections caused by antibiotic-resistant pathogens relies on the use of antivirulence drugs inhibiting their adaptability to the host environment, rather than growth. Since quorum sensing (QS) controls coordinated expression of virulence determinants in many bacterial pathogens, this communication system is considered a promising target for the development of antivirulence drugs.

In the last decades, different experimental approaches have been used to identify molecules interfering with QS systems controlling virulence in different human pathogens, including *Pseudomonas aeruginosa*. The methodological paper reported in this Chapter describes a fast and cost-effective high throughput screening system for the identification of novel inhibitors of the *las* QS system of *P. aeruginosa*. This approach has been proven successful for the identification of a novel *las* QS inhibitor, the anthelmintic drug niclosamide. A similar coculture-bases screening system has been also employed to identify FDA-approved drugs inhibiting the *pqs* QS systems of *P. aeruginosa*, as it will be discussed in Chapter 6.

Chapter 22

A Coculture-Based Approach for Screening Campaigns Aimed at Identifying Novel *Pseudomonas aeruginosa* Quorum Sensing Inhibitors

Giordano Rampioni, Giulia Giallonardi, Francesca D'Angelo, and Livia Leoni

Abstract

Quorum sensing (QS) is recognized as a promising target for the identification of anti-virulence drugs hampering *Pseudomonas aeruginosa* adaptability to the host environment and pathogenicity. Consequently, a number of studies in the last decade focused on the identification of small molecules or proteins with anti-QS activity, mainly targeting the *las* QS system, which is based on *N*-3-oxododecanoyl-homoserine lactone ($3OC_{12}$-HSL) as signal molecule. Different experimental approaches have been successfully used to identify QS blockers interfering with the activity/stability of the $3OC_{12}$-HSL receptor LasR, with the functionality of the $3OC_{12}$-HSL synthase LasI, or with the stability/bioavailability of the $3OC_{12}$-HSL signal molecule itself.

Here we describe the use of a high-throughput screening system for the identification of novel *las* QS inhibitors based on the cocultivation of *P. aeruginosa* wild type and the *P. aeruginosa*-derived biosensor strain PA14-R3, in which light emission relies on the ability of the wild type strain to synthesize $3OC_{12}$-HSL and of the biosensor strain to perceive this signal molecule. With respect to other screening systems, this method has the advantage of being cost-effective and allowing the identification of compounds targeting, besides $3OC_{12}$-HSL reception, any cellular process critical for the functionality of the *las* QS system, including $3OC_{12}$-HSL synthesis and secretion.

Key words Quorum sensing inhibitors, Screening, Whole-cell biosensors, Anti-virulence drugs, Niclosamide, *Pseudomonas aeruginosa*, *lasR*, *lasI*

1 Introduction

The introduction of antibiotics into clinical practice at the middle of the twentieth century is a milestone in the history of medicine. However, the original expectation that all bacterial infections could be defeated by antibiotics has been soon disregarded by the emergence of antibiotic-resistant strains. As traditional antibiotic research appears to be helpless in coping with the emergence of

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antibiotic-resistant strains, novel approaches should be undertaken in order to identify new drugs [1, 2].

An innovative strategy to combat bacterial infections relies on specific inhibition of bacterial virulence, hence the ability to cause disease rather than bacterial growth. The use of anti-virulence drugs is expected to reduce bacterial adaptability to the host environment, facilitating the host immune system to resolve the infection, and to diminish the strong selective pressure exerted by conventional antibiotics, although this is not yet supported by direct clinical evidence [3–5].

Since in many bacteria pathogenicity is controlled and coordinated by quorum sensing (QS), this communication system is considered one of the most promising targets for anti-virulence therapies [5, 6].

The Gram-negative bacterium *Pseudomonas aeruginosa* is one of the most dreaded opportunistic pathogens, and represents a prototype of multidrug resistant bug for which effective therapeutic options are limited. The ability of *P. aeruginosa* to cause a wide range of both community- and hospital-acquired infections in humans is linked to its capacity to produce a large repertoire of virulence factors, form antibiotic-tolerant biofilms and, ultimately, respond and adapt to environmental fluctuations, including host immune responses and antibiotic treatments. For these reasons, *P. aeruginosa* infections are generally characterized by high morbidity and mortality rates [7, 8].

The pathogenic potential of *P. aeruginosa* relies on the coordinated expression of a large array of virulence factors, the majority of which are positively controlled by QS [9, 10]. The *P. aeruginosa* QS network consists of at least three different QS systems, *las*, *rhl*, and *pqs*, based on the production and perception of the signal molecules *N*-3-oxododecanoyl-homoserine lactone ($3OC_{12}$-HSL), *N*-butanoyl-homoserine lactone (C_4-HSL), and 2-heptyl-3-hydroxy-4-quinolone (PQS), respectively. *P. aeruginosa* QS is hierarchically organized, since the *las* QS system is required for optimal activation of the *rhl* and *pqs* QS systems. Overall, QS controls the expression of nearly 10% of the *P. aeruginosa* genome, including genes for secreted virulence factors, biofilm formation, and immune-modulatory and pro-inflammatory agents [10, 11].

QS signal molecules can be detected in clinical samples, proving that QS is active during *P. aeruginosa* infections. Moreover, QS-defective mutants show strongly impaired virulence in several animal models of infection, corroborating the importance of QS for *P. aeruginosa* pathogenicity and its suitability as a target for the development of anti-*Pseudomonas* drugs [12, 13].

On these bases, a number of studies focused on the identification of small molecules or proteins with anti-QS activity, mainly targeting the *P. aeruginosa las* QS system. Different experimental approaches have been successfully used to identify small molecules

interfering with the activity/stability of the $3OC_{12}$-HSL receptor LasR or with the functionality of the $3OC_{12}$-HSL synthase LasI. Also the identification of enzymes that inactivate $3OC_{12}$-HSL and the development of antibodies that limit the bioavailability of this signal molecule have been reported [14, 15].

Here we describe a convenient strategy for the identification of compounds affecting the *P. aeruginosa las* QS system at multiple levels: (1) expression/activity of the signal receptor LasR; (2) activity/availability of the signal molecule $3OC_{12}$-HSL; (3) expression/activity of the signal synthase LasI [16]. This strategy is defined by a primary assay, suitable for high-throughput screening of chemical compounds, and by secondary assays, used to confirm the specific activity of the hit compounds selected in the primary assay. This approach has been successfully employed for the identification of a novel *las* QS inhibitor, the FDA-approved anthelmintic drug niclosamide [17].

As a general remark, it should be pointed out that, while we chose to illustrate a screening strategy linked to the activity of the *las* QS system in *P. aeruginosa*, some of the techniques presented here can be applied to a variety of different biological systems. For instance, reporter-based assays modelled on the one described here can be devised for the *rhl* and *pqs* QS systems of *P. aeruginosa*, as well as for different QS systems in other Gram-negatives or in Gram-positive bacteria.

2 Materials

1. Bacterial strains: *P. aeruginosa* PA14 [18] and PA14-R3 biosensor [16].
2. Growth media: Luria-Bertani broth (LB: 10 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract); Luria-Bertani agar (LA, as LB plus 15 g/l agar).
3. 1 M MOPS Buffer: 83.7 g/l 3-(*N*-morpholino) propanesulfonic acid (MOPS), 13.6 g/l sodium acetate trihydrate, 3.7 g/l ethylenediaminetetraacetic acid (EDTA) disodium salt, pH 7.0.
4. Protease Buffer: 100 mM Tris, 1 mM $CaCl_2$, pH 7.5.
5. Synthetic *N*-3-oxododecanoyl-homoserine lactone ($3OC_{12}$-HSL).
6. Elastin-Congo Red.
7. Chloroform.
8. 0.2 N HCl.
9. Black clear-bottom 96-wells microtiter plates.

10. Automated luminometer-spectrometer plate reader.
11. UV/Vis spectrophotometer.
12. Plastic microcuvettes for UV/Vis spectrophotometer.

3 Methods

3.1 Rationale of the Primary Screening

The screening system for inhibitors of *P. aeruginosa* QS is based on a biosensor strain (PA14-R3) able to detect the QS signal molecule $3OC_{12}$-HSL [16]. The PA14-R3 biosensor is available from the authors upon request.

The PA14-R3 strain carries a nonfunctional allele of the *lasI* gene, and is thus unable to synthesize $3OC_{12}$-HSL; however, it can respond to exogenous $3OC_{12}$-HSL provided either through supply of the purified molecule or by cocultivation with a wild type *P. aeruginosa* proficient in $3OC_{12}$-HSL production, such as the PA14 strain. The screening system is detailed in Fig. 1. The $3OC_{12}$-HSL signal synthesized by the wild type PA14 diffuses into the PA14-R3 biosensor and induces bioluminescence emission. The addition of a molecule with inhibitory activity towards any process related to the $3OC_{12}$-HSL-dependent QS system, namely, $3OC_{12}$-HSL synthesis, transport, and perception, will reduce light emission by the biosensor with respect to a control coculture grown in the absence of any chemical compound.

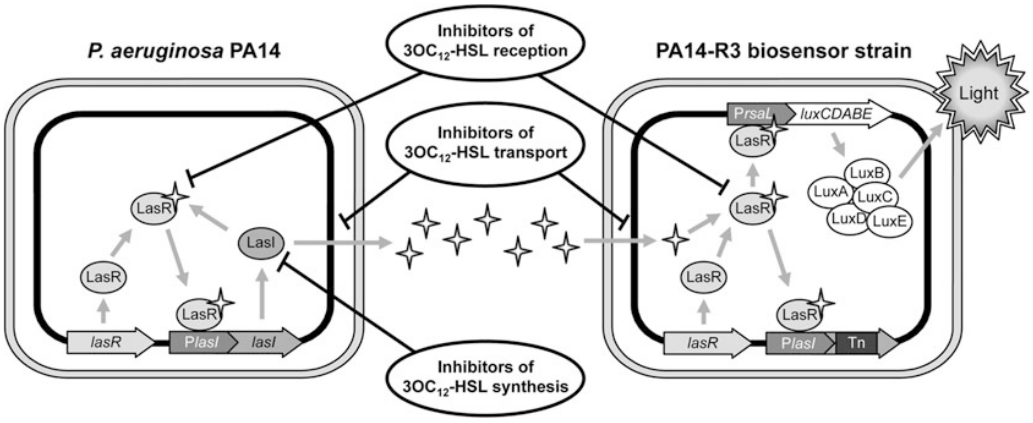

Fig. 1 Schematic representation of the PA14/PA14-R3 cocultivation screening system. The wild type PA14 produces $3OC_{12}$-HSL signal molecules that induce bioluminescence emission in the biosensor strain PA14-R3. PA14-R3 is a PA14 derivative in which a transcriptional fusion between the LasR-dependent *rsaL* promoter (P*rsaL*) and the *luxCDABE* operon was integrated at the *attB* neutral site of the chromosome. In addition, in order to avoid self-activation of the reporter strain, the *lasI* gene, encoding the $3OC_{12}$-HSL synthase LasI, was inactivated by transposon insertion (Tn). Molecules interfering with different steps of the *las* QS system are expected to cause a reduction in bioluminescence in comparison to the untreated control. Modified from [16]

3.2 Primary Screening Procedure

1. Grow *P. aeruginosa* PA14 wild type and the $3OC_{12}$-HSL reporter strain PA14-R3 (*see* **Note 1**) overnight at 37 °C on LA plates.
2. Scrape bacteria from LA plate surfaces and dilute in 1 ml of LB supplemented with 100 mM MOPS Buffer to an absorbance at 600 nm wavelength (A_{600}) of 0.09 and 0.03 for PA14-R3 and PA14, respectively (3:1 reporter:wild type ratio). Mix isovolumes of the PA14-R3 and PA14 diluted cultures, to obtain a coculture in which the A_{600} of PA14-R3 and PA14 is 0.045 and 0.015, respectively.
3. Aliquot 100 μl per well of the coculture in a 96-well microtiter black plates with clear bottom.
4. As untreated control, add 100 μl of LB in six wells containing the coculture.
5. Set up serial dilutions of the compounds to 2× the final concentrations to be tested. For example: chemical compounds to be used in the screening assays are dissolved in 10 mM DMSO and then diluted to 400 μM, 40 μM, and 4 μM in LB medium (to obtain 200 μM, 20 μM, and 2 μM final concentrations in the assay, respectively). Aliquot 100 μl of each compound in the microtiter wells containing the coculture.
6. Incubate the microtiter plate at 37 °C for 4 h with gentle shaking.
7. Measure A_{600} and light counts per second (LCPS), simultaneously with an automated luminometer-spectrometer plate reader (*see* **Note 2**).
8. Average the A_{600} and LCPS measurements of the untreated control samples. For all samples, normalize the LCPS to the A_{600} to obtain PA14-R3 reporter activity. Compare PA14-R3 reporter activity of the treated samples to one of the untreated controls (*see* **Note 3**).

3.3 Rational of the Secondary Assays: $3OC_{12}$-HSL, Elastase, and Pyocyanin Production

As previously described, compounds reducing light emission in the primary screening could hamper the *las* QS system at different levels, including $3OC_{12}$-HSL synthesis, transport, and perception. Inhibition of any of these steps would limit $3OC_{12}$-HSL synthesis in PA14 wild type, as well as the production of $3OC_{12}$-HSL-dependent virulence factors such as elastase and pyocyanin.

Elastase is a *P. aeruginosa* secreted protease that mainly targets mammalian elastin and plays a key role in virulence [19]. Transcription of the elastase gene, *lasB*, is strongly activated by the *las* QS system [20].

Pyocyanin is a redox-active phenazine responsible for the blue-green color characteristic of *P. aeruginosa* cultures. Besides its role in virulence, pyocyanin has been also recognized as a signalling

molecule, as an electron shuttle for bacterial respiration, and as an antibacterial and antifungal agent [21, 22].

Therefore, a convenient method to validate the *las* inhibitory activity of a hit compound is to measure its effect on $3OC_{12}$-HSL, elastase, and pyocyanin production, in comparison with an untreated control.

The assay for $3OC_{12}$-HSL production by PA14 wild type is based on the use of the same PA14-R3 biosensor strain used in the primary screening [16].

The elastase assay is based on the Elastin-Congo Red reagent, a water-insoluble powder in which elastin is bound to the Congo Red dye. Hydrolysis of elastin causes the release of the dye in the aqueous phase. The amount of released (soluble) dye is proportional to the level of hydrolyzed elastin and, ultimately, to the level of elastase present in a *P. aeruginosa* culture supernatant. The method here described is modified from ref. 23.

Pyocyanin can be easily quantified in chloroform extracts of *P. aeruginosa* culture supernatants by spectrophotometric analysis. The method here described is modified from ref. 24.

3.4 Quantification of $3OC_{12}$-HSL

1. Inoculate *P. aeruginosa* PA14 in 5 ml of LB and grow the culture overnight at 37 °C with 200 rpm shaking.
2. Refresh the overnight culture to an A_{600} of 0.015 in 30 ml of LB supplemented with 50 mM MOPS Buffer, in the presence of increasing concentrations of the test compound. Incubate at 37 °C with 200 rpm shaking.
3. Withdraw 7 ml of bacterial cultures every 3 h, up to 9 h. Measure A_{600} of the samples, harvest the cells by centrifugation, and recover the culture supernatants (*see* **Notes 4** and **5**).
4. Scrape bacteria from the surfaces of an LA plate of the biosensor strain PA14-R3 and dilute in 2 ml of LB. Measure the A_{600} of this bacterial suspension and use it to prepare an inoculum of the biosensor strain in LB supplemented with 50 mM MOPS Buffer to an A_{600} of 0.045.
5. Aliquot 195 μl per well of the biosensor culture in a 96-well microtiter black plates with clear bottom.
6. Add 5 μl of each culture supernatant from **step 3** in three wells containing the reporter culture. As untreated control, add 5 μl of LB in six wells containing the reporter culture.
7. For the calibration curve, set up 1:3 serial dilutions of synthetic $3OC_{12}$-HSL in LB, from a maximal concentration of 120 μM to a minimum concentration of ~18 nM. Add 5 μl of each diluted $3OC_{12}$-HSL sample in three wells containing the reporter culture.
8. Incubate the microtiter plate at 37 °C for 4 h with gentle shaking.

9. Measure A_{600} and LCPS, simultaneously (*see* **Note 2**). Average the A_{600} and LCPS measurements of the replicates. Normalize the averaged LCPS to the averaged A_{600} to obtain PA14-R3 reporter activity. Extrapolate $3OC_{12}$-HSL concentration in the treated and untreated supernatants based on the values obtained for the calibration curve (*see* **Note 6**).

3.5 Elastase Assay Procedure

1. Set up 1.5 ml tubes each one containing 20 mg of Elastin-Congo Red and 1 ml of Protease Buffer (*see* **Note 7**).
2. Add 100 μl of culture supernatant collected in Subheading 3.4, **step 3** (*see* **Notes 5** and **8**) to the tube containing the Elastin-Congo Red suspension. Prepare a control sample (blank) by adding 100 μl of sterile LB instead of the culture supernatant.
3. Incubate 2 h with gentle shacking at 37 °C.
4. Centrifuge for 5 min at 11,000 × *g* at room temperature.
5. Measure absorbance at 495 nm wavelength (A_{495}) of the clear supernatants in plastic microcuvettes, using as blank the control sample (see above). Normalize with respect to the A_{600} of the corresponding culture measured in Subheading 3.4, **step 3**.

3.6 Pyocyanin Assay Procedure

1. Add 3 ml of chloroform to 15 ml conical tubes containing 5 ml of the supernatants collected in Subheading 3.4, **step 3** (*see* **Notes 5** and **8**). Mix vigorously by vortexing for 10 s. As control sample (blank), use 5 ml of sterile LB in place of the bacterial supernatant.
2. Centrifuge the tubes at 3,000 × *g* for 5 min.
3. Transfer 2 ml of the lower organic phase in clean 15 ml conical tubes (the lower organic phase is blue if pyocyanin is present) and add 1 ml of 0.2 N HCl. Mix vigorously by vortexing for 10 s.
4. Centrifuge the tubes at 3,000 × *g* for 5 min.
5. Transfer 800 μl of the upper aqueous phase in plastic microcuvettes (the upper aqueous phase is pink if pyocyanin is present).
6. Measure the absorbance at 520 nm wavelength (A_{520}), using as blank the control sample (see above). Normalize with respect to the A_{600} of the corresponding culture measured in Subheading 3.4, **step 3**.

4 Notes

1. By using bacterial biosensors in which light emission is induced by exogenous C_4-HSL, PQS, or other QS molecules different from $3OC_{12}$-HSL, similar coculture-based approaches can be designed to identify inhibitors of other QS systems. Please

consider that growth of biosensor strains different from PA14-R3 may require LB supplementation with antibiotics for plasmid selection. Moreover, the use of other biosensor strains may require preliminary optimization of experimental parameters, including wild type/biosensor ratio, A_{600} of the coculture at t_0, and incubation time of the coculture at 37 °C.

2. We routinely use a Wallac 1420 Victor^{3V} multiplate reader (Perkin-Elmer) as automated luminometer-spectrometer plate reader. For the Wallac 1420 Victor^{3V} multiplate reader relevant parameters for bioluminescence measurement are: emission aperture, large; counting time, 1 s. Relevant parameters for absorbance measurement are: filter 595/60; excitation aperture, normal; reading time, 0.1 s.
3. The criteria used for the selection of hit compounds in [17] were: (a) ≥50% inhibition of PA14-R3 reporter activity; (b) ≤10% reduction of growth with respect to the untreated controls. The latter criterion was aimed at avoiding any unspecific effect of impaired growth on the QS response.
4. This step is just for harvesting cells, so speed and times for centrifugation can vary. Our standard conditions are 6,300 × *g* for 5 min at room temperature.
5. For the $3OC_{12}$-HSL quantification assay, the supernatants can be stored at −20 °C. Conversely, for elastase and pyocyanin assays it is recommended to process supernatants as soon as possible.
6. The same method can be used to quantify the amount of $3OC_{12}$-HSL produced by different strains of *P. aeruginosa* (*e.g.*, to compare a wild type and a mutant) or of *P. aeruginosa* grown in different media. Also quantification of $3OC_{12}$-HSL in *P. aeruginosa* clinical isolates has been described [16].
7. Avoid the preparation of a stock suspension of Elastin-Congo Red. This powder is highly insoluble and aliquots of a suspension could contain different amounts of the reagent. We have observed that aliquoting the powder in each sample tube enhances the assay reliability.
8. Under these conditions, *P. aeruginosa* usually starts to produce detectable levels of elastase and pyocyanin at around $A_{600} \approx 3.0$, therefore consider using only supernatants collected after 6 and 9 h incubation in Subheading 3.4, **step 3**.

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Chapter 6

Identification of antivirulence drugs targeting the *pqs* quorum sensing system of *Pseudomonas aeruginosa*

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Preface to Chapter 6

The identification of new drugs to be used in humans is a challenging task. This decade-long process has high-costs and low probability of yielding safe molecules with favourable pharmacological properties to be used in clinical settings. Especially for what concerns the search for new antibiotics, rapid emergence of antibiotics-resistant pathogens further discourage investments in this field. The identification of off-target activities in drugs already approved for use in humans is a promising strategy that could reduce the time and costs generally associated with standard drug discovery processes, with high probability of yielding bioavailable and safe compounds which can move straight into clinical trials.

In this Chapter an experimental work aimed at repurposing FDA-approved drugs as antivirulence agents to treat bacterial infections is presented. In particular, a coculture-based screening method similar to the one described in Chapter 5 has been used to identify new inhibitors of the *pqs* quorum sensing (QS) system of *Pseudomonas aeruginosa*. Promising hits have been selected decreasing *P. aeruginosa* pathogenic potential both *in vitro* and *in vivo*. Preliminary analysis indicate that these drugs hamper *P. aeruginosa* virulence by targeting the QS receptor PqsR. Notably, the most potent inhibitor identified in this study, clofoctol, is also active against *P. aeruginosa* clinical isolate from cystic fibrosis (CF) patients. The pharmacological properties of clofoctol support its use for treatment of *P. aeruginosa* CF lung infections.

Identification of antivirulence drugs targeting the *pqs* quorum sensing system of *Pseudomonas aeruginosa*

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Running Head: New FDA-approved inhibitors of the *pqs* QS system

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ABSTRACT

The long-term use of antibiotics has led to the emergence of multi-drug-resistant bacteria. A promising strategy to combat infections caused by these pathogens aims at hampering their adaptability to the host environment without affecting their growth. In this context, the intercellular communication system quorum sensing (QS), which controls virulence factors production and biofilm formation in several human pathogens, is considered an ideal target.

Here we describe the identification of new inhibitors of the *pqs* QS system of the human pathogen *Pseudomonas aeruginosa*, by screening a library of 1,600 FDA-approved drugs. Phenotypic characterization of *ad hoc* engineered strains and *in silico* molecular docking demonstrated that the antifungal drugs clotrimazole and miconazole, and the antibacterial compound active against Gram-positive pathogens clofoctol inhibit the *pqs* system, likely by targeting the transcriptional regulator PqsR. The most active inhibitor, clofoctol, specifically inhibits the expression of *pqs*-controlled virulence traits in *P. aeruginosa*, such as pyocyanin production, swarming motility, biofilm formation, and expression of genes involved in siderophores production. Moreover, clofoctol protects *Galleria mellonella* larvae from *P. aeruginosa* infection and hampers the *pqs* QS system in *P. aeruginosa* isolates from cystic fibrosis patients. Notably, clofoctol is already approved for clinical treatment of pulmonary infections caused by Gram-positive bacterial pathogens, hence this drug has high clinical plausibility as an antivirulence agent to treat *P. aeruginosa* lung infections.

INTRODUCTION

The development of new drugs to be used in humans is a challenging task that usually requires decade-long laboratory experimentation followed by clinical trials. This process is time consuming and necessitates high economic investments, with high-risk of failure due to poor pharmacological properties of newly identified bioactive molecules. This is particularly discouraging for the search of new antibiotics, since the economic investment required for their development could not be adequately rewarded due to the fast rate at which antibiotic resistant bacterial pathogens emerge (Fernandes and Martens, 2017). As a consequence, while the emergence of antibiotic resistant pathogens is accelerating at an unprecedented pace, the pipeline for the discovery of new antibiotics is running dry, with 15 big pharmaceutical companies out of 18 abandoning antibacterial discovery programmes in the last decade (Ventola, 2015; Mohr, 2016).

The search for off-target activities in drugs already approved for use in humans is a promising strategy that could reduce the time and costs generally associated with standard drug discovery processes, with high probability of yielding bioavailable and safe compounds which can more easily move into clinical trials (Ashburn and Thor, 2004; Mullard, 2012).

A number of studies have shown the promise of drug repurposing strategies for the identification of new antibacterial drugs (Rangel-Vega *et al*., 2015; Savoia, 2016). Examples are gallium nitrate and 5-fluorouracil, conventionally used for the treatment of hypercalcemia and cancer, respectively, which display growth-inhibitory activity against some Gram-negative and Gram-positive pathogens (Gieringer *et al*., 1986; Minandri *et al*., 2014). An alternative approach to the development of new antimicrobials is the inhibition of bacterial virulence, rather than growth (Rasko and Sperandio, 2010). Recently, antivirulence effects have been identified in drugs already approved for use in humans (Rampioni *et al*., 2017a). As an example, the antifungal compound 5-fluorocytosine inhibits virulence factors production in the Gram-negative human pathogen *Pseudomonas aeruginosa* both *in vitro* and in a mouse model of lung infection (Imperi *et al*., 2013a). Since antivirulence drugs disarm rather than kill pathogens they should in principle combat bacterial infections without exerting the strong selective pressure for resistance imposed by conventional antibiotics (Rasko and Sperandio, 2010). Emergence of resistance is less likely to occur for drugs targeting social behaviours, such as the production of secreted virulence factors. Indeed, resistant mutants expressing extracellular factors that are shared by the members of the entire bacterial population should not experience a fitness advantage relative to susceptible clones (Allen *et al*., 2014). In this context, quorum sensing (QS) is considered as a promising target for the identification and development of antivirulence drugs, since this intercellular communication system positively controls the expression of virulence factors in

73 different human pathogens, including *P. aeruginosa* (Brannon and Hadjifrangiskou, 2016; Maura
74 *et al*., 2016).
75 *P. aeruginosa* is one of the most dreaded human pathogens in industrialized countries, since it
76 can cause a variety of severe infections, especially among hospitalized and immunocompromised
77 patients (Boucher *et al*., 2009; Pendleton *et al*., 2013). These infections are difficult to treat due
78 to the intrinsic and acquired antibiotic resistance of *P. aeruginosa* (Aloush *et al*., 2006),
79 strengthened by its ability to form antibiotic tolerant biofilms (Ciofu *et al.*, 2015). *P. aeruginosa*
80 is the main cause of morbidity and mortality in cystic fibrosis (CF) patients, in which it can form
81 biofilm and establish chronic lung infections impossible to eradicate by antibiotic treatment
82 (Lund-Palau *et al*., 2016). The necessity for new therapeutic options to treat *P. aeruginosa*
83 infections is testified by a recent release from the World Health Organization, in which this
84 bacterium is top ranked among pathogens for which new antibiotics are urgently needed (Priority
85 1 - Critical; www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/).
86 Due to its relevance as a human pathogen, *P. aeruginosa* is also a model organism for QS
87 studies. This bacterium is endowed with a complex QS circuitry constituted by four
88 interconnected systems (*i.e. las*, *rhl*, *pqs* and *iqs*), which collectively control social behaviours
89 and the expression of virulence determinants, such as secreted virulence factors, swarming
90 motility and biofilm formation (Williams and Cámara, 2009; Lee and Zhang, 2015). As a
91 consequence, in the last decade various compounds interfering with the *P. aeruginosa* QS circuit
92 have been identified, and their effectiveness as antivirulence drugs both *in vitro* and *in vivo* has
93 boosted the research in the field (Rampioni *et al*., 2014). Unfortunately, most of the drugs
94 identified so far are cytotoxic or display unfavourable pharmacological properties, thus limiting
95 their transfer to the clinical practice (Maura *et al*., 2016).
96 To combine the advantages of drug-repurposing with the antivirulence approach, we
97 previously showed that the anthelmintic drug niclosamide has a potent antivirulence activity
98 against *P. aeruginosa* (Imperi *et al*., 2013b). Niclosamide targets the *las* QS system, thereby
99 decreasing the expression of *las*-controlled virulence factors and protecting *Galleria mellonella*
100 larvae from *P. aeruginosa* infection.
101 In the present study we searched for inhibitors of the *pqs* QS system of *P. aeruginosa* among
102 drugs already approved for use in humans.
103 The *pqs* QS system of *P. aeruginosa* is based on 2-alkyl-4-quinolones (AQs) as signal
104 molecules, namely 2-heptyl-3-hydroxy-4-quinolone (PQS), and its immediate precursor 2-
105 heptyl-4-hydroxyquinoline (HHQ). Both HHQ and PQS can bind to and activate the
106 transcriptional regulator PqsR (also known as MvfR). The PqsR/HHQ and PqsR/PQS complexes
107 bind the P*pqsA* promoter region and trigger the transcription of the *pqsABCDE* operon, coding

108 for the enzymes required for the synthesis of HHQ. HHQ is in turn oxidized to PQS by the
109 monooxygenase PqsH. Therefore, like for other QS systems, the HHQ and PQS molecules act as
110 autoinducers by generating a feedback loop that accelerates their synthesis (Heeb *et al*., 2011;
111 Dulcey *et al*., 2013; Drees and Fetzner, 2015).

112 While HHQ only activates the expression of the *pqsABCDE* operon, PQS has additional
113 activities. Indeed, PQS is an iron chelator, participates in the formation of outer membrane
114 vesicles and controls the expression of virulence genes *via* a PqsR-independent pathway
115 (Bredenbruch *et al*., 2005; Mashburn and Whiteley, 2005; Diggle *et al*., 2007; Rampioni *et al*.,
116 2016).

117 The mechanism of action of the protein coded by the last gene of the *pqsABCDE* operon,
118 PqsE, is still poorly understood. PqsE is a pathway-specific thioesterase, which contributes to the
119 synthesis of HHQ, despite its function can be complemented by other thioesterases in a *pqsE*
120 mutant strain (Drees and Fetzner, 2015). Notably, PqsE positively controls the expression of
121 multiple virulence factors also in a *P. aeruginosa* genetic background in which it cannot
122 participate to AQs biosynthesis, indicating that this protein has additional functions (Hazan *et*
123 *al*., 2010; Rampioni *et al*., 2010 and 2016).

124 Overall, *P. aeruginosa* mutants defective in AQs synthesis/reception or in PqsE are severely
125 attenuated in different plant and animal experimental models of infection (Cao *et al*., 2001;
126 Déziel *et al*., 2005; Xiao *et al*., 2006; Lesic *et al*., 2007; Rampioni *et al*., 2010; Dubern *et al*.,
127 2015). Moreover, AQs are detectable in sputum, blood and urine of individuals with CF and their
128 presence correlates with clinical status (Barr *et al*., 2015).

129 In this study, a convenient screening system has been developed and used to select for FDA-
130 approved drugs targeting the *pqs* QS system at multiple levels. This screening campaign led to
131 the identification of the antifungal drugs clotrimazole and miconazole, and of clofoctol, an
132 antimicrobial compound commonly used to treat lung infections caused by Gram-positive
133 bacteria, as inhibitors of *pqs* signaling, plausibly targeting the PqsR receptor protein. Phenotypic
134 analyses performed in the laboratory strain PAO1 and in *P. aeruginosa* isolates from CF patients
135 support the antivirulence potential of the most active inhibitor, clofoctol.

136 **RESULTS**

137 **Development of a coculture-based system for monitoring *pqs* signaling activity**

138 A reporter system for monitoring the activity of the *pqs* QS system has been developed. This
139 is based on the coculture between wild type *P. aeruginosa* PAO1 (herein referred to as PAO1)
140 and the AQs biosensor strain *P. aeruginosa* Δ*pqsA* P*pqsA*::*luxCDABE* (herein referred to as Rep-
141 AQs; Table S1). Rep-AQs cannot synthesize AQs due to deletion of the *pqsA* biosynthetic gene,
142 and emits light in response to exogenously provided AQs due to PqsR-dependent activation of
143 the P*pqsA*::*luxCDABE* transcriptional fusion integrated in a neutral site of its genome (Diggle *et*
144 *al.,* 2007). Therefore, in the PAO1/Rep-AQs coculture system the AQ signal molecules produced
145 by PAO1 induce bioluminescence emission by Rep-AQs. Therefore, *pqs* inhibitors interfering
146 with each step of the *pqs* signaling circuit, including AQs synthesis or reception, should reduce
147 the bioluminescence signal in the PAO1/Rep-AQs coculture (Fig. 1A).

148 Preliminary experiments aimed at setting-up the screening system revealed that maximal
149 response of Rep-AQs to exogenous PQS was obtained after 5 h incubation in microtiter plates
150 (Fig. S1A), when this biosensor strain was inoculated at an absorbance at 600 nm wavelength
151 (A_{600}) of 0.1 (Fig. S1B). Cocultivation of Rep-AQs and PAO1 at different ratios and in different
152 culture conditions showed that the highest bioluminescence signal was registered when Rep-AQs
153 and PAO1 were inoculated in a ~ 3:1 ratio (A_{600} of Rep-AQs and PAO1 of 0.1 and 0.03,
154 respectively) (Fig. S1C), and the resulting coculture was incubated at 37°C with shaking (Fig.
155 S1D). Therefore, the screening campaign has been set up under the above conditions to
156 maximize the biosensor responsiveness to AQs, and possibly to drugs interfering with AQs
157 signaling.

158 The functionality of the PAO1/Rep-AQs coculture system for the identification of anti-*pqs*
159 drugs has been assessed by using the commercially available compounds methyl anthranilate and
160 farnesol. Methyl anthranilate has been proven to inhibit AQs synthesis by competing with the
161 HHQ precursor anthranilate for binding to PqsA (Calfee *et al*., 2001), while farnesol has been
162 shown to decrease the expression of HHQ biosynthetic genes *via* an unknown mechanism
163 (Cugini *et al*., 2007). As expected, both methyl anthranilate and farnesol reduced
164 bioluminescence emission from the PAO1/Rep-AQs coculture in a dose-dependent manner, with
165 an IC_{50} of ca. 1 mM (Fig. 1B), in accordance with literature data (Calfee *et al*., 2001; Cugini *et*
166 *al*., 2007).

167

168 **Identification of new anti-*pqs* drugs**

169 The PAO1/Rep-AQs coculture system has been used to screen a library of 1,600 FDA-
170 approved compounds with known biological activities selected for their high chemical and

171 pharmacological diversity and safety in humans (PHARMAKON). In the primary screening,
172 each drug was tested at two different concentrations, 20 μM and 200 μM, for the ability to
173 reduce bioluminescence in the PAO1/Rep-AQs coculture. Since compounds of the library are
174 dissolved in dimethyl sulfoxide (DMSO), untreated samples containing the same amount of
175 DMSO of the treated samples were used as controls. Cell density and bioluminescence emission
176 of the untreated samples were considered as 100%, and the criteria for selection of anti-*pqs* drugs
177 were: *i*) inhibition of bioluminescence emission ≥ 20% at 20 μM; *ii*) inhibition of
178 bioluminescence emission ≥ 60% at 200 μM; *iii*) reduction of cell density ≤ 10% at both 20 μM
179 and 200 μM. This primary screening led to the selection of seventeen hits meeting these criteria
180 (Fig. S2A), hence possibly endowed with *pqs* inhibitory activity.

181 However, reduced bioluminescence in the samples treated with the selected drugs could be
182 due to their effects on the enzymes involved in light emission or on ATP levels (Hakkila *et al*.,
183 2002; Jansson, 2003). Since inhibition of P*pqsA* promoter activity in *P. aeruginosa* should
184 decrease the production of the AQs HHQ and PQS, a secondary screening to test the ability of
185 the seventeen hits to reduce AQs production in PAO1 was performed. In this case, AQs levels
186 were measured by means of the Rep-AQs biosensor strain in spent media of PAO1 cultures
187 grown for 16 h in Luria-Bertani Broth (LB) supplemented with the selected hits at 20 μM or 200
188 μM concentration, or with corresponding amounts of DMSO. This analysis revealed that only
189 three drugs specifically reduced the production of AQs in PAO1: clotrimazole, clofoctol and
190 miconazole (I-3, I-9 and I-14 in Fig. S2B, respectively). Two of the identified drugs,
191 clotrimazole and miconazole, are antifungal compounds (Clayton and Connor, 1973; Sawyer *et*
192 *al*., 1975; Shellow, 1982; Ahmed, *et al*., 2012), while clofoctol is an antibacterial drug with
193 activity against Gram-positive bacteria infecting human lungs (Buogo, 1981; Yablonsky and
194 Simonnet, 1982; Danesi *et al*., 1988) (Table 1).

195 To confirm the results of the primary and secondary screenings, clotrimazole, clofoctol and
196 miconazole were purchased from an alternative supplier (Sigma-Aldrich). The tested drugs did
197 not inhibit PAO1 growth in Muller-Hinton Broth and LB even at the highest concentration
198 achievable in solution (*i.e.* MIC clotrimazole > 1.6 mM; MIC miconazole and clofoctol > 6.4
199 mM). Moreover, these drugs did not alter PAO1 growth profile up to the maximal concentration
200 used in the primary and secondary screenings (*i.e.* 200 μM; Fig. S3).

201 The effect of the purchased hits has been then retested on bioluminescence emission in the
202 PAO1/Rep-AQs coculture system. Dose-response inhibition of P*pqsA* promoter activity was
203 observed for the three drugs (Fig. 2A), with IC_{50} values of 39 μM, 20 μM and 27 μM for
204 clotrimazole, clofoctol and miconazole, respectively (Table 1). The three hits had no effect on
205 bioluminescence emission in a *P. aeruginosa* strain in which the expression of the *luxCDABE*

206 operon for light emission is independent on the activity of the *pqs* signaling system (Fig. S4),
207 ruling out the possibility that the inhibitory activity on the PAO1/Rep-AQs coculture was due to
208 unspecific inhibition of bioluminescence. Moreover, the three drugs confirmed their ability to
209 decrease AQs production in PAO1 in a dose-dependent manner (Fig. 2B), in accordance with the
210 repressive effect exerted on the P*pqsA* promoter.
211 Overall, these data confirm that clotrimazole, clofoctol and miconazole exert an anti-*pqs*
212 activity without altering *P. aeruginosa* growth.
213
214 **Characterization of the mechanism of action of the newly identified *pqs* inhibitors**
215 The inhibition of P*pqsA* activity in the PAO1/Rep-AQs coculture system (Fig. 2A) could be
216 due to inactivation of AQs biosynthesis in the PAO1 strain or of AQs reception in both PAO1
217 and Rep-AQs strains (Fig. 1A). Also reduced AQs levels in PAO1 (Fig. 2B) could be due to
218 inhibition of AQs synthesis or of AQs reception, due to the PqsR-dependent regulatory loop
219 governing transcription of the HHQ biosynthesis enzymes (Wade *et al*., 2005; Xiao *et al*., 2006).
220 To discriminate between these two possibilities, the effect of the three drugs on AQs
221 production has been tested in a PAO1 Δ*pqsA*Δ*pqsH* double mutant strain (Δ*pqsAH*; Table S1)
222 carrying the pFD-*pqsABCD* plasmid for constitutive expression of the HHQ biosynthetic
223 enzymes. In this genetic background, in which AQs production does not depend on the ability of
224 AQs to activate P*pqsA via* PqsR, the inhibitors did not reduce AQs levels, demonstrating that
225 they do not affect the functionality of the enzymes required for HHQ synthesis (Fig. 3A).
226 Moreover, the inhibitors were effective in reducing bioluminescence emission by the Rep-AQs
227 biosensor strain grown in the presence of synthetic PQS (Fig. 3B), suggesting that the inhibitors
228 target AQs reception rather than AQs biosynthesis.
229 To validate this hypothesis, we investigated the effect of the hits on the levels of *pqsR* mRNA
230 and PqsR protein. As shown in Fig. 4A, Real Time RT-PCR analysis revealed that the hits do not
231 affect *pqsR* mRNA level. Moreover, western immunoblotting showed that the inhibitors do not
232 decrease PqsR protein level in a PAO1 Δ*pqsA*Δ*pqsH*Δ*pqsR* triple mutant strain (Δ*pqsAHR*; Table
233 S1) carrying the pPqsR-6H plasmid for IPTG-inducible expression of a 6xHis-tagged variant of
234 PqsR (Fig. 4B; Ilangovan *et al*., 2013). Clotrimazole increased PqsR level, indicating that this
235 drug has a positive effect on the translation of the *pqsR* mRNA or on PqsR stability. However,
236 experiments performed in PAO1 Δ*pqsAHR*(pPqsR-6H) carrying the transcriptional fusion
237 P*pqsA*::*luxCDABE* grown in the presence of exogenous PQS revealed that the inhibitory effect
238 exerted by the hits on P*pqsA* activity decreases in parallel with the increase of PqsR levels,
239 obtained by increasing the concentration of IPTG in the medium (Fig. 4C), indicating that all the
240 hits act downstream of PqsR expression, likely targeting PqsR functionality.

241 To support the hypothesis of a direct interaction of the inhibitors with PqsR, molecular
242 docking simulations were performed based on the crystal structure of the PqsR co-inducer
243 binding domain (CBD) in the apo form (PDB ID: 4JVC; Ilangovan *et al*., 2013). To increase
244 simulations reliability, the docking search space encompassed the entire CDB of PqsR, *i.e.* a
245 "blind" docking procedure was carried out. Residues previously reported to be involved in PqsR
246 binding to the natural ligand 2-nonyl-4-hydroxy-quinoline (NHQ; Ilangovan *et al*., 2013) were
247 considered flexible (see Materials and Methods for details). This analyses indicated that the three
248 hits bind with high affinity to PqsR in the same site as the natural ligand NHQ (Fig. 5), predicted
249 ΔG values for binding of clotrimazole, clofoctol and miconazole being -8.4, -9.8 and -8.5
250 kcal/mol, respectively. Interestingly these values are lower than the predicted ΔG value for
251 binding of NHQ (-7.9 kcal/mol; Table 1). Similar results were obtained when using the PqsR
252 CDB structure bound to NHQ (PDB ID: 4JVD; Ilangovan *et al*., 2013), from which the ligand
253 was removed. In this latter case, ΔG values for binding of clotrimazole, clofoctol, miconazole
254 and NHQ were -9.4, -9.9, -8.1 and -8.1 kcal/mol, respectively. Finally, also keeping all the CDB
255 residues fixed yielded very similar results (data not shown). Interestingly, in all cases the
256 predicted affinity of the hits for PqsR parallels their efficacy as *pqs* inhibitors (Table 1).
257 Overall, these data suggest that the newly identified inhibitors could be endowed with a
258 similar mechanism of action, that is to hamper PqsR functionality by competing with HHQ and
259 PQS for PqsR binding. Moreover, both activity assays and *in silico* predictions indicate that
260 clofoctol is endowed with higher inhibitory activity relative to miconazole and clotrimazole
261 (Table 1). To support competitive binding of PQS and clofoctol to PqsR, the ability of this drug
262 to repress P*pqsA* activity was evaluated in the Rep-AQs biosensor grown in the presence of
263 different concentrations of the native PqsR agonist PQS. This competition assay revealed
264 reduced ability of clofoctol to inhibit P*pqsA* activity in the presence of increasing concentrations
265 of PQS (Fig. S5), in line with the possible effect of clofoctol as a competitor antagonist of the
266 PQS receptor protein PqsR.
267
268 **Clofoctol inhibits the expression of *pqs*-controlled virulence phenotypes**
269 By hampering the ability of PqsR to activate the transcription of the *pqsABCDE* operon,
270 clofoctol is expected to decrease the expression of virulence traits controlled by both PQS and
271 PqsE in *P. aeruginosa*. First of all, since the assays previously performed to assess the effect of
272 clofoctol on AQs production did not allow discriminating between HHQ and PQS, these QS
273 signal molecules have been quantified by liquid chromatography - tandem mass spectrometry
274 (LC-MS/MS) analysis on spent media of PAO1 cultures treated with different concentrations of
275 clofoctol. As shown in Fig. 6A, this analysis confirmed that clofoctol inhibits AQs production in

276 *P. aeruginosa*, with both HHQ and PQS concentration significantly reduced in the presence of
277 the drug.

278 Concerning the effect of clofoctol on PQS- and PqsE-controlled virulence determinants,
279 phenotypic analyses revealed that 100 µM clofoctol leads to > 80% reduction of pyocyanin
280 production (Fig. 6B), and considerably decreased swarming motility in PAO1 (Fig. 6C).
281 Moreover, 100 µM clofoctol significantly reduced biofilm formation in a PAO1 strain
282 constitutively expressing GFP *via* the pMRP9-1 plasmid (Davies *et al*., 1998) (Fig. 6D).
283 Notably, the effect of clofoctol on the tested phenotypes in PAO1 mimicked deletion of the *pqsR*
284 gene (Δ*pqsR*; Fig. 6B-D), in line with the hypothesis that PqsR is the target of clofoctol.

285 Subsequently, Real Time RT-PCR analyses were performed to examine the effect of clofoctol
286 on the expression of *pqs*-controlled virulence genes (Rampioni *et al*., 2016). The PQS-dependent
287 *pvdS* and *pchR* genes code for the PvdS and PchR regulatory proteins required for the synthesis
288 of the siderophores pyoverdine and pyochelin, respectively (Visca *et al.,* 2002; Rampioni *et al*.,
289 2016); the PqsE-dependent *lecA* gene codes for the LecA lectin involved in the formation of
290 antibiotic-resistant biofilm (Diggle *et al.,* 2006; Rampioni *et al*., 2016). As a control, also the
291 mRNA level of *pqsA* was measured. Real Time RT-PCR analyses showed that clofoctol
292 significantly decreased the mRNA level of all the tested genes, in agreement with the down-
293 regulation observed in a PAO1 Δ*pqsR* mutant strain (Fig. 6E). The negative effect exerted by
294 clofoctol on *lecA* transcription was also confirmed by promoter activity assay showing reduced
295 activity of the P*lecA*::*luxCDABE* transcriptional fusion in PAO1 cultures treated with clofoctol
296 (Fig. S6).

297 Overall, these data support clofoctol as an antivirulence agent active against *P. aeruginosa*.

298

299 **Clofoctol protects *Galleria mellonella* larvae from *P. aeruginosa* infection and inhibits the**
300 ***pqs* QS system also in *P. aeruginosa* CF clinical isolates**

301 The antivirulence activity of clofoctol was tested in *G. mellonella* larvae, an insect model of
302 infection, which well correlates with the mouse model (Jander *et al*., 2000). Firstly, *G.*
303 *mellonella* larvae were infected with ca. 10 cells of *P. aeruginosa* PAO1 or of the isogenic
304 Δ*pqsR* mutant and incubated at 37°C for 120 h. As shown in Fig. 7A, *pqsR* mutation
305 significantly reduced the ability of *P. aeruginosa* to kill *G. mellonella* larvae, demonstrating the
306 suitability of this insect model to investigate the antivirulence potential of drugs targeting PqsR.

307 Since the average weight of *G. mellonella* larvae was ca. 500 mg, and arbitrarily assuming
308 uniform dispersal of injected bacteria and clofoctol in 500 µL of larval volume (Rampioni *et al*.,
309 2009 and 2017b), 10 µL of saline containing 5 mM clofoctol were injected to reach 100 µM
310 clofoctol in each larva. Preliminarily, we verified that the injection of 10 µL of saline containing

311 5 mM clofoctol did not affect the survival of uninfected larvae, and that 2 h incubation of *P.*
312 *aeruginosa* with 5 mM clofoctol did not affect *P. aeruginosa* growth and viability (data not
313 shown). Then, *G. mellonella* larvae were inoculated with *P. aeruginosa* PAO1 in the absence or
314 in the presence of clofoctol. The treatment with clofoctol led to a survival percentage of 87%,
315 similar to that observed with the Δ*pqsR* mutant (83%), while only 50% of untreated *G.*
316 *mellonella* larvae survived PAO1 infection (Fig. 7A). Overall, these data demonstrate that
317 clofoctol attenuates *P. aeruginosa* PAO1 lethality in *G. mellonella*.
318 To verify if clofoctol is active also against clinical *P. aeruginosa* strains, its ability to reduce
319 AQs production was evaluated in a collection of 20 *P. aeruginosa* isolates from the lungs of CF
320 patients, grouped in four categories with respect to the stage of infection (Table S2). A
321 preliminary analysis revealed that only 2 strains isolated from patients with more than 15 years
322 of chronic infection (chronic late group) did not produce detectable levels of AQs (Table S2),
323 hence these strains should be considered resistant to the antivirulence effect of clofoctol. The
324 remaining 18 clinical isolates were grown in LB for 24 h in the absence and in the presence of
325 100 μM clofoctol, and AQs concentration was determined in the corresponding spent media by
326 using the Rep-AQs biosensor. Residual AQs production was estimated for each treated isolate
327 relative to the amount of AQs detected in the corresponding untreated sample, considered as
328 100%. Notably, clofoctol decreased AQs production in all the tested clinical isolates, with a
329 reduction ranging from 12.7% to 88.4% (Fig. 7B). The median reduction of AQs production in
330 the tested isolates was 68.6%, hence comparable to the decrease of AQs level measured in PAO1
331 treated with 100 μM clofoctol in the same conditions (65.7%; Fig. 7B). Differences in the
332 median reduction values among the analysed groups were not statistically significant. Moreover,
333 differences in the median reduction of AQs production were not significant also when grouping
334 the isolates according to their antibiotic resistance profile (Table S2). Indeed, the median
335 reduction of AQs level was 71.6% and 67.4% in 4 antibiotic susceptible and in 12 antibiotic
336 resistant strains, respectively (Fig. S7). Also the 2 multidrug resistant (MDR) or extensively drug
337 resistant (XDR) strains analysed in this study were susceptible to clofoctol, with a reduction of
338 AQs levels of 56.5% and 88.4%, respectively (Fig. S7). Although performed on a limited
339 number of clinical isolates, this analysis indicates that clofoctol is effective in hampering the *pqs*
340 QS system in CF strains, irrespective of their adaptation to the host environment during long
341 lasting chronic lung infection and of their antibiotic resistance profile.

342 **DISCUSSION**

343 As a consequence of the widespread antibiotic resistance, inhibition of virulence rather than
344 growth has become a viable approach to combat bacterial infections with lower selective
345 pressure for emergence of resistance (Rasko and Sperandio, 2010). In particular, *in vitro*
346 evolution experiments suggest that resistant mutants will not emerge for drugs targeting public
347 goods, such as virulence factors that are secreted and shared between individuals (Mellbye and
348 Schuster, 2011). Moreover, since antivirulence drugs target specific bacterial functions required
349 for the infection, these molecules are not expected to affect the beneficial resident microbiota
350 relative to antibiotics (Allen *et al*., 2014; Rampioni *et al.*, 2017a).

351 In many bacterial pathogens QS positively controls the expression of secreted virulence
352 factors, hence this communication system is considered a promising target for the development
353 of antivirulence agents (LaSarre and Federle, 2013; Rampioni *et al.,* 2014). Since *P. aeruginosa*
354 has four interconnected QS systems that positively control the production of virulence factors
355 and biofilm formation, most of the research on QS inhibition has been focused on this bacterium
356 as a model system. Indeed, several molecules inhibiting the *las* QS system of *P. aeruginosa* have
357 been identified (LaSarre and Federle, 2013; Rampioni *et al.,* 2014). Recently, different studies
358 focused on the inhibition of the *pqs* QS system. The *pqs* system positively controls the
359 expression of multiple virulence determinants, including secreted virulence factors and biofilm
360 formation, and *pqs* mutant strains display attenuated virulence in plant and animal models of
361 infection (Cao *et al*., 2001; Déziel *et al.*, 2005; Hazan *et al*., 2010; Rampioni *et al*., 2010; Dubern
362 *et al*., 2015). Moreover, the *pqs* system is active during *P. aeruginosa* infections in humans
363 (Machan *et al*., 1992; Collier *et al*., 2002; Barr *et al.,* 2015).

364 Inhibitors of the *pqs* system were previously identified among analogs of anthranilate, the
365 substrate of PqsA in the first step of the biosynthetic route leading to AQs production (Calfee *et*
366 *al.,* 2001; Lesic *et al.,* 2007). Subsequently, different molecules binding to the AQ-biosynthetic
367 enzyme PqsD were shown to act as potent *pqs* inhibitors, with IC_{50} values in the low µM range
368 (from 1 to 14 µM; Storz *et al.,* 2012; Weidel *et al.,* 2013). The possibility of interfering with the
369 *pqs* system *via* enzymatic degradation of the AQ signals, rather than *via* small molecules
370 targeting their biosynthesis, was also explored, and PQS degrading activity has been described in
371 *Arthrobacter nitroguajacolicus* and *Achromobacter xylosoxidans* (Pustelny *et al*., 2009; Soh *et*
372 *al*., 2015). The majority of anti-*pqs* molecules identified so far are competitive inhibitors of the
373 PqsR transcriptional regulator. Potent PqsR antagonists with IC_{50} values ranging from 0.4 to 38.5
374 µM have been found among analogs of the natural agonists HHQ and PQS (Klein *et al.,* 2012;
375 Ilangovan *et al.,* 2013; Zender *et al*., 2013; Lu *et al.,* 2014). A whole-cell high-throughput
376 screening and structure-activity relationship analysis led to the identification of benzamide-

377 benzimidazole PqsR inhibitors with low IC_{50} values (lower than 1 μM), and some of these
378 molecules inhibited the PqsBC complex in addition to PqsR (Starkey *et al.*, 2014; Maura *et al.*,
379 2017; Maura and Rahme, 2017). Also 2-sulfonylpyrimidines were identified hampering both
380 AQs reception and biosynthesis (Thomann *et al*., 2016). Overall, a number of reports validated
381 the antivirulence potential of anti-*pqs* molecules, showing their ability to reduce the expression
382 of *pqs*-controlled virulence traits both *in vitro* and in animal models of infection. Despite the
383 promise of anti-*pqs* agents for the treatment of *P. aeruginosa* infections, to the best of our
384 knowledge none of these molecules entered clinical trials so far. This is probably due to the poor
385 pharmacological properties of the inhibitors, including possible cytotoxicity, and to the lack of
386 ADME-TOX studies required for their experimentation in humans. In this context, searching for
387 off-target activities in drugs already approved for use in humans represents a potential shortcut to
388 develop new anti-*pqs* molecules that could move straight into clinical trials.

389 In this study, a drug-repurposing approach led to the identification of three promising anti-*pqs*
390 drugs already used in humans, by screening a library of 1,600 FDA-approved compounds (Table
391 1; Fig. S2). Data about acute and chronic toxicity are already available for these drugs, as well as
392 information on their pharmacokinetics. Clotrimazole and miconazole are antifungal drugs used
393 in humans to treat ring worm, pityriasis versicolor, vaginal and oral candidiasis and skin yeast
394 infections (Clayton and Connor, 1973; Sawyer *et al*., 1975; De Cremer *et al.,* 2015; Zhang *et al.,*
395 2016). They both alter the permeability of the fungal cell wall by binding to the phospholipids
396 and inhibiting the biosynthesis of ergosterol and other sterols required for fungal cell membrane
397 integrity (Fothergill, 2006; Crowley and Gallagher, 2014). Miconazole displays its function also
398 by inhibiting fungal peroxidases, which results in peroxide-mediated cell death (Fothergill,
399 2006). Both these drugs are mainly administered as creams or ointments, thus their current
400 formulations could be particularly suitable for topic treatment of chronic wound infections
401 caused by *P. aeruginosa* (Osmon *et al.,* 2004; Driscoll *et al.*, 2007). However, this opportunistic
402 pathogen is a main cause of infections in lungs, especially in CF patients, where it establishes
403 chronic infections that can last for decades (Lyczak *et al*., 2000). The use of clotrimazole and
404 miconazole to treat *P. aeruginosa* lung infections would require their reformulation as inhalable
405 nanosuspensions, an approach that has been recently proven valuable for repurposing the
406 anthelmintic drug niclosamide as an anti-QS agent against *P. aeruginosa* (Imperi *et al.,* 2013b;
407 Costabile *et al.,* 2015).

408 Out of the 1,600 compounds tested in this screening campaign, the most promising anti-*pqs*
409 drug was clofoctol, an antimicrobial used for the treatment of acute and chronic upper respiratory
410 tract infections and for tracheobronchial infections caused by Gram-positive pathogens,
411 especially staphylococci, pneumococci and streptococci (Buogo, 1981; Danesi *et al*., 1988).

412 Clofoctol is also used in preventive and curative treatment of otolaryngology and stomatology
413 (Danesi and Del Tacca, 1985). The mechanism of action of this drug as an antimicrobial is still
414 poorly understood, but a detrimental effect of clofoctol on membrane and cell wall biosynthesis
415 in Gram-positives has been reported (Yablonsky and Simonnet, 1982; Yablonsky, 1983).
416 Clofoctol is usually administered as suppositories, it is well absorbed through the rectal mucosa
417 and it rapidly spreads across all tissues, reaching the highest concentrations in the respiratory
418 system (Del Tacca *et al*., 1987). The fact that clofoctol mainly acts in the airways is extremely
419 valuable for future treatment of *P. aeruginosa* lung infections. Notably, clofoctol is also used to
420 treat infections in infants, and this is another advantageous feature if considering that in CF
421 patients *P. aeruginosa* lung infection is established since the paediatric age (Lyczak *et al*., 2002).
422 Overall, despite less potent than other *pqs* inhibitors described so far, the anti-*pqs* drugs
423 identified in this study are endowed with great potential for use in humans, and could be directly
424 tested in clinical trials or serve as scaffold for future drug-optimization programmes.
425 Concerning the mechanism of action of the identified inhibitors, they all affect PqsR
426 functionality, likely by competing with the natural ligands HHQ and PQS for PqsR binding
427 (Figs. 3, 4 and S5). This hypothesis is supported by docking simulations, which predict that all
428 three compounds bind to the PqsR co-inducer binding domain in the same binding site of the
429 natural ligand NHQ (Fig. 5). This result was somehow unexpected, since the PAO1/Rep-AQs
430 coculture used in the screening campaign should primarily identify molecules affecting both
431 AQs biosynthesis and AQs reception (Fig. 1A). Indeed, this coculture-based reporter system was
432 functional in identifying the PqsA-inhibitor methyl anthranilate (Fig. 1B). Intriguingly, the side
433 anti-QS activity of the anthelmintic drug niclosamide was discovered by using a coculture-based
434 reporter system similar to the one deployed in this work, and also niclosamide hampered QS
435 signal molecule reception rather than synthesis (Massai *et al*., 2011; Imperi *et al.,* 2013b).
436 Therefore, the selection of drugs targeting QS receptors could be a bias intrinsic to the screening
437 systems used. In fact, in coculture-based screening systems, drugs interfering with QS signal
438 molecules reception would have a dual effect, since they would directly hamper the receptor
439 protein both in *P. aeruginosa* wild type, thus decreasing its ability to synthesize the signal
440 molecules, and in the reporter strain, thus reducing its ability to respond to them. Conversely, an
441 inhibitor of QS signal molecules synthesis would only affect functionality of the *P. aeruginosa*
442 wild type strain. Hence, the PAO1/Rep-AQs coculture system could be more sensitive to PqsR
443 inhibitors than to drugs affecting AQs biosynthesis, so that only drugs targeting PqsR met the
444 selection criteria of the primary screening.
445 Since all the hits identified in this study likely display the same mechanism of action, we
446 focused our attention on the most potent inhibitor, clofoctol (Table 1). It has recently been

447 clarified that different elements of the *pqs* QS system control distinct virulence traits. In
448 particular, the PQS signal molecule drives the expression of genes required for the biosynthesis
449 of siderophores and of genes coding for PrpL and AprX proteases, and exotoxin S, while PqsE is
450 required for the production of pyocyanin, LecA and LecB lectins, hydrogen cyanide,
451 rhamnolipids and ChiC chitinase (Rampioni *et al*., 2016). With regard to pleiotropic virulence
452 phenotypes such as swarming motility and biofilm formation, these seem to be regulated by both
453 PQS and PqsE (Rampioni *et al*., 2010). Coherent with the activity of clofoctol as a PqsR
454 inhibitor, the expression of both PQS-controlled virulence traits, such as the expression of genes
455 required for siderophores biosynthesis (Fig. 6E), and of PqsE-dependent phenotypes, including
456 pyocyanin production (Fig. 6B) and expression of the *lecA* gene (Fig. 6E), were inhibited.
457 Moreover, clofoctol reduced swarming motility and biofilm formation (Figs. 6C and 6D).
458 Notably, clofoctol exerted an antivirulence effect also *in vivo*, since this drug alleviated *P.*
459 *aeruginosa* infection in *G. mellonella* larvae (Fig. 7A).
460 A main concern on the use of anti-QS drugs for treatment of CF pulmonary infection
461 originates from the evolutionary process increasing *P. aeruginosa* adaptation to the CF lung.
462 Indeed, during chronic infection CF isolates accumulate mutations that reduce the production of
463 virulence factors, lead to the formation of mucoid biofilms, increase antibiotic resistance mainly
464 as a consequence of efflux pumps over-expression, and in some cases inactivate QS (Winstanley
465 and Fothergill, 2009; Folkesson *et al.*, 2012; Kamath *et al*., 2016; Winstanley *et al*., 2016). Since
466 *P. aeruginosa* QS-defective mutants should be considered resistant to anti-QS drugs, the
467 suitability of QS-inhibition for CF therapy is under debate. However, most studies focused on
468 the inactivation of the *las* QS system in chronic CF isolates, while little attention has so far been
469 given to the *pqs* QS system (Hoffman *et al*., 2009; Bjarnsholt *et al.*, 2010; Jiricny *et al*., 2014;
470 Feltner *et al.,* 2016). The evidence that AQs have been identified in the sputum of CF patients
471 with both intermittent and chronic *P. aeruginosa* infections indicate that the *pqs* QS system is
472 active in the CF lung (Machan *et al*., 1992; Collier *et al*., 2002; Guina *et al*., 2003; Jiricny *et al*.,
473 2014). In particular, a recent work showed that AQs were detectable in the sputum, plasma and
474 urine of ca. 80% of CF patients suffering *P. aeruginosa* chronic lung infection, and that the
475 levels of the AQ molecule NHQ increased at the start of a pulmonary exacerbation and positively
476 correlated with quantitative measures of *P. aeruginosa* cells in the lung (Barr *et al*., 2015). This
477 evidence is in line with the results obtained in this study, since only 2 out of the 20 clinical
478 isolates tested did not produce detectable levels of AQs (Table S2). Notably, clofoctol reduced
479 functionality of the *pqs* QS system in all the *pqs*-proficient CF isolates, irrespective of their
480 antibiotic resistance (Fig. 7B and Fig. S7).
481 Future analyses performed on larger panel of *P. aeruginosa* clinical isolates from both CF and

482 chronic wound patients are required to better assess the suitability of clofoctol, clotrimazole and
483 miconazole for the treatment of *P. aeruginosa* chronic infections. However, the results of this
484 work should encourage further preclinical studies to transfer the newly identified *pqs* inhibitors
485 from the laboratory to the clinical practice.

486 **MATERIALS AND METHODS**

487 **Bacterial strains, media and chemicals**

488 The bacterial strains, clinical isolates, plasmids and oligonucleotides used in this study are
489 listed in Table S1, S2, S3 and S4, respectively. Bacterial strains were routinely grown at 37°C in
490 Luria-Bertani Broth (LB) with aeration and, when necessary, antibiotics were added at the
491 following concentrations: tetracycline (Tc), 200 µg/mL; carbenicillin (Cb), 150 µg/mL;
492 gentamicin (Gm), 100 µg/mL. When necessary, isopropyl β-D-1-thiogalactopyranoside (IPTG)
493 was added at the concentrations indicated in the text. Muller-Hinton Broth (MHB) and M9
494 minimal medium supplemented with 200 mM glucose as carbon source were used in the MIC
495 assay (Clinical and Laboratory Standards Institute, CLSI) and in the biofilm assay, respectively.
496 Synthetic HHQ and PQS stock solutions were prepared in MeOH. Clotrimazole, clofoctol and
497 miconazole were purchased from Sigma-Aldrich and dissolved in DMSO.

498

499 **Primary screening for the identification of *pqs* inhibitors**

500 *P. aeruginosa* PAO1 and the Rep-AQs biosensor strain (PAO1 Δ*pqsA* P*pqsA::luxCDABE*)
501 were grown overnight at 37°C on LB agar plates. Bacteria were scraped from plate surfaces and
502 diluted in LB to an absorbance at 600 nm wavelength (A_{600}) of 0.1 and 0.03 for the biosensor
503 and PAO1 strains, respectively (procedure modified from Massai *et al*., 2011). Two-hundred µL
504 aliquots of the coculture were grown at 37°C in 96-well microtiter plates in LB supplemented
505 with each compound of the PHARMAKON library (20 µM and 200 µM). The A_{600} and relative
506 light units (RLU) were measured after 5 h incubation by using a Wallac 1420 Victor^{3}V
507 multilabel plate reader (PerkinElmer). Eight samples grown in the presence of DMSO (0.2% or
508 2%) were used as controls in each microtiter plate. Reporter activity was determined as
509 RLU/A_{600} for each sample. Residual reported activity was determined in treated sample relative
510 to the control samples grown in the presence of DMSO, considered as 100%.

511

512 **Quantification of AQs**

513 Levels of AQ signal molecules in treated-*P. aeruginosa* PAO1 culture supernatants were
514 determined by using the reporter strain Rep-AQs, as previously described (Fletcher *et al*., 2007).
515 Bacterial cultures were grown in 96-well microtiter plates at 37°C with shaking. Supernatants
516 were collected after 16 h for the experiments shown in in Figs. 2B, 3A and S2, or after 24 h for
517 experiments shown in Figs. 7B and S7, to allow optimal AQs production in slow-growing
518 clinical isolates. Briefly, 10 µL of culture supernatant was added to 190 µL of LB inoculated
519 with Rep-AQs biosensor (final A_{600} = 0.1) in 96-well microtiter plates. Microtiter plates were
520 incubated at 37°C with gentle shaking, and the A_{600} and RLU were measured after 5 h of

521 incubation. A calibration curve was generated by growing the Rep-AQs biosensor in the
522 presence of increasing concentrations of synthetic HHQ or PQS; the resulting dose-response
523 curve was used to calculate the concentration of the AQ signals in each culture supernatant.
524 AQs produced by *P. aeruginosa* PAO1 were also quantified in by LC-MS/MS analysis, as
525 previously described (Ortori *et al.,* 2011). Briefly, PAO1 was inoculated into 5 mL of LB in the
526 absence or in the presence of 100 µM clofoctol. After 16 h incubation at 37°C with shaking, cell
527 density of the culture was recorded and the supernatants were filter-sterilized. Supernatants were
528 solvent extracted with ethyl acetate, dried under vacuum and re-dissolved in MeOH prior to
529 quantitative analysis by LC-MS/MS. For each sample, a supernatant concentration of HHQ and
530 PQS was calculated by comparing analytic peak areas with a matched calibration line.
531
532 **Pyocyanin production, swarming motility and biofilm formation assays**
533 Pyocyanin was extracted and quantified from *P. aeruginosa* PAO1 and Δ*pqsR* grown in LB
534 supplemented with 100 µM clofoctol or with DMSO as a control, as previously described (Essar
535 *et al.,* 1990). Swarming motility assay were performed on swarming plates [0.8% (wt/vol)
536 nutrient broth N.2, 0.5% (wt/vol) glucose, 0.5% (wt/vol) bacteriological agar]. Plates were
537 supplemented or not with 100 µM clofoctol. After 16 h of growth at 37°C, swarming motility
538 was directly observed at the air-agar interface.
539 For microscopic visualization of biofilm, *P. aeruginosa* PAO1 or Δ*pqsR* constitutively
540 expressing GFP *via* the pMRP9-1 plasmid (Davies *et al*., 1998) were grown in an 8-well
541 chamber slide, as previously described (Jurcisek *et al.,* 2011), with minor modifications. Briefly,
542 bacterial cells were inoculated at an A_{600} of 0.02 in 700 µL of M9 minimal medium
543 supplemented with 20 mM glucose as carbon source, in the absence or in the presence of 100
544 µM clofoctol. Cultures were incubated at 30°C for 24 h to allow the adhesion of the bacterial
545 cells on the glass surface. To maintain bacterial viability, the medium was changed every 24 h.
546 Biofilms formation was examined after 3 days incubation by using the Leica TCS SP5 confocal
547 microscope.
548
549 **Western immunoblotting**
550 Protein crude extracts were collected from the *P. aeruginosa* PAO1 *pqsA*Δ*pqsH*Δ*pqsR* triple
551 mutant strain carrying the pPqsR-6H plasmid grown in LB supplemented with 10 µM PQS and
552 20 µM IPTG, in the absence or in the presence of 100 µM clotrimazole, clofoctol or miconazole.
553 *P. aeruginosa* PAO1 *pqsA*Δ*pqsH*Δ*pqsR* strain carrying the pME6032 empty vector was used as a
554 control. Bradford assay (Bradford, 1976) was used to quantify and normalize total protein
555 content in the samples. Western immunoblotting was performed by using a standard technique

556 (Sambrook *et al*., 1989) with mouse anti-6His antibody (1:5,000; Sigma-Aldrich) and goat anti-
557 mouse IgG HRP-conjugate as secondary antibody (1:6,000; Bio-Rad Laboratories). Final
558 development was performed with the Amersham ECL chemiluminescent reagents (Amersham
559 Biosciences). A C-DiGit blot scanner (LI-COR Biosciences) was used for data acquisition.
560

561 **RNA extraction and Real Time RT-PCR analysis**

562 *P. aeruginosa* PAO1 and Δ*pqsR* were inoculated at an A_{600} of 0.02 in 5 mL of LB in the
563 absence or in the presence of 100 μM clotrimazole, clofoctol or miconazole. Cultures were
564 grown at 37°C with vigorous shaking until they reached an A_{600} of 2.0, and then 1 mL of cells
565 was harvested by centrifugation and resuspended in 2 mL of RNAprotect Bacteria Reagent
566 (Qiagen). Total RNA extraction was performed with the RNeasy Mini Columns Kit (Qiagen)
567 according to the manufacturer's instructions, including the on-column DNase I digestion step. In
568 addition, eluted RNA was treated for 1 h at 37°C with DNase TURBO (0.2 U per μg of RNA;
569 Ambion) and with SUPERase-In (0.4 U per μg of RNA; Ambion). DNase I was removed by
570 suing the RNeasy Column Purification kit (Qiagen). Purified RNA was quantified by using the
571 NanoDrop 2000 spectrophotometer (Thermo-Fisher Scientific). Absence of genomic DNA in the
572 RNA samples was verified by PCR performed with primers FWP*pqsL* and RVP*pqsL* (Table S4).
573 cDNA synthesis was performed with the iScript Reverse Transcription Supermix for RT-qPCR
574 kit (Bio-Rad Laboratories) according to manufacturer's instructions, and quantified with
575 NanoDrop 2000. Real Time RT-PCRs were performed using iTaq™ Universal SYBR® Green
576 Supermix kit (Bio-Rad Laboratories), according of the manufacturer's instructions, and the Rotor
577 Gene 6000 thermocycler (Corbett Research). Primers employed in Real Time RT-PCR analysis
578 were designed using the Primer-blast software (www.ncbi.nlm.nih.gov/tools/primer-blast) and
579 are listed in Table S4. The reaction procedure involved incubation at 95°C for 1 min and 40
580 cycles of amplification at 95°C for 10 sec and 60°C for 45 sec. Fluorescence was registered in
581 the last 15 sec of the 60°C step. 16S ribosomal RNA was chosen as an internal control
582 (housekeeping gene) to normalize the Real Time RT-PCR data in each single run, and to
583 calculate the relative fold change in gene expression by using the $2^{-\Delta\Delta Ct}$ method. The average
584 data and standard deviations were calculated from three independent experiments.
585

586 ***Galleria mellonella* killing assays**

587 The *G. mellonella* killing assay was performed as previously described (Jander *et al.,* 2000;
588 Rampioni *et al*., 2017b), with minor modifications. Briefly, *G. mellonella* caterpillars in the final
589 instar larval stage (average weight, 486 ± 67 mg) were infected with 10 μL of saline containing
590 about 10 bacterial cells, in the absence or in the presence of 5 mM clofoctol. Although PAO1

591 cells were incubated in the presence of clofoctol for less than 5 min before injection, preliminary
592 assays showed that 5 mM clofoctol treatment (for up to 24 h) does not significantly affect PAO1
593 cell viability (data not shown). *G. mellonella* larvae were incubated at 37°C in petri dishes (ten
594 larvae *per* dish) and monitored for 120 h. Larvae were considered dead when they did not
595 respond to gentle prodding. At least 30 larvae *per* condition were used in four independent
596 experiments. Survival curves for the *G. mellonella* killing assay were generated by the Kaplan-
597 Meier method.
598
599 **Molecular docking simulations**
600 Molecular docking simulations were carried out using DockingApp (Di Muzio *et al.*, 2017), a
601 user friendly interface for the docking program AutoDock Vina (Trott and Olson, 2010). In all
602 simulations, the search space (docking grid) included the whole PqsR co-inducer binding domain
603 (CDB) structure, in order to carry out "blind" predictions of the hits binding site.
604 Simulations were carried out on the apo (PDB ID: 4JVC; Ilangovan *et al*., 2013) and olo
605 (PDB ID: 4JVD; Ilangovan *et al*., 2013) forms of the protein both by keeping all protein residues
606 rigid and by allowing flexibility only of the residues previously reported to be involved in PqsR
607 binding to the natural ligand NHQ (*i.e.* ILE 149, ALA 168, VAL 170, ILE 186, LEU 189, LEU
608 207, LEU 208, PHE 221, ILE 236, TYR 258, ASP 264, THR 265; Ilangovan *et al*., 2013).
609
610 **Statistical analysis**
611 Statistical analysis was performed with the software GraphPad Prism 5, using one-way
612 analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests.
613 Differences having a p value < 0.05 were considered statistically significant.

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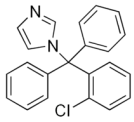
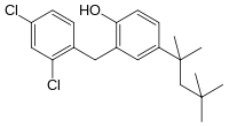
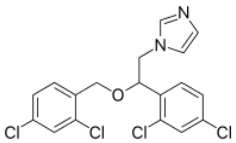
923 **Table 1.** Anti-*pqs* compounds identified by screening the PHARMAKON library of FDA-
924 approved drugs.

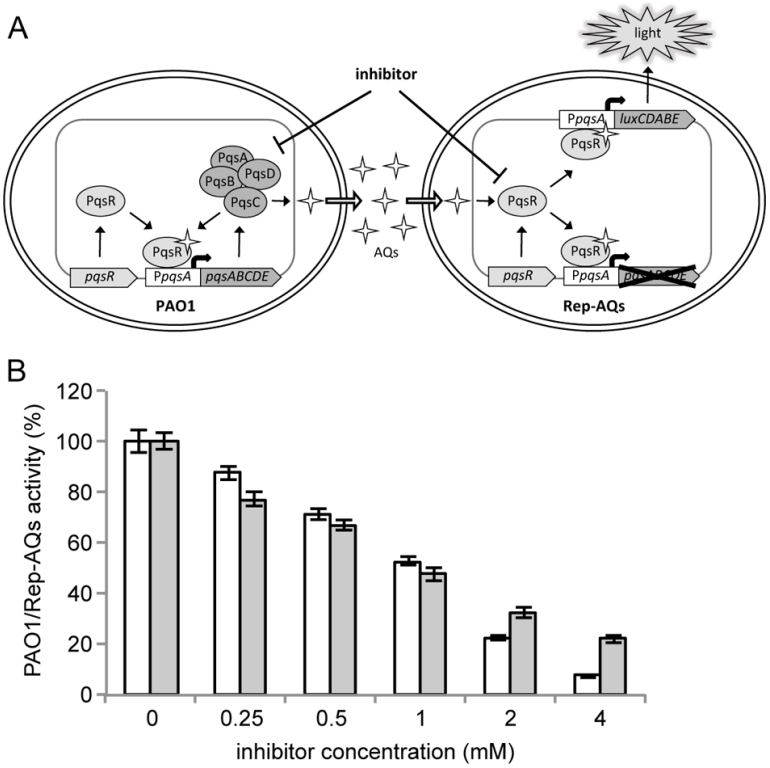
Drug name	Property	Structure	IC_{50}[a]	ΔG
Clotrimazole	Antifungal		39	-8.4
Clofoctol	Antibacterial		20	-9.8
Miconazole	Antifungal		27	-8.5

925

926 [a] The IC_{50} values (μM) were determined by using the PAO1/Rep-AQs coculture system.
927 [b] ΔG values (kcal/mol) for drugs binding to the PqsR CDB apo form (PDB ID: 4JVC; Ilangovan
928 *et al*., 2013) predicted by molecular docking simulations.

929 **Figure 1**

930



931

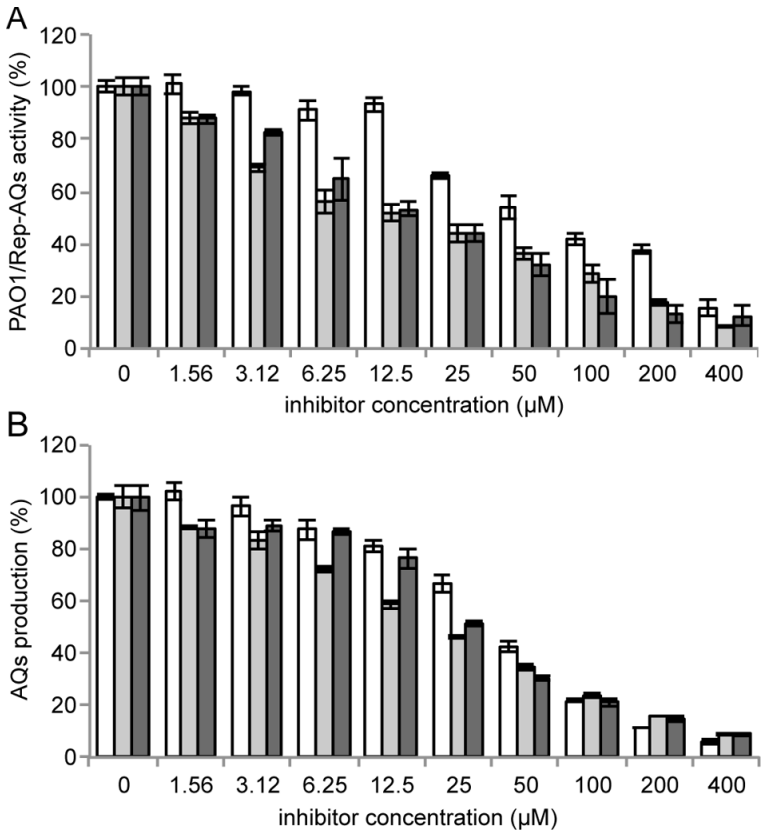
932

933 **Figure 1. Validation of the screening system.**

934 **(A)** Schematic representation of the coculture-based reporter system developed in this study. The
935 *P. aeruginosa* PAO1 strain (PAO1) produces the AQs signal molecules which activate
936 P*pqsA*::*luxCDABE* transcription, hence bioluminescence emission, in the biosensor strain Rep-
937 AQs. Drugs interfering with AQs synthesis or reception are expected to reduce bioluminescence
938 in the PAO1/Rep-AQs coculture, relative to the untreated samples. **(B)** Activity of the
939 PAO1/Rep-AQs coculture system treated with indicated concentrations of the *pqs* inhibitors
940 methyl anthranilate (white bars) or farnesol (grey bars). Bioluminescence of the untreated
941 PAO1/Rep-AQs coculture normalized to cell density is considered as 100%.

942 **Figure 2**

943



944

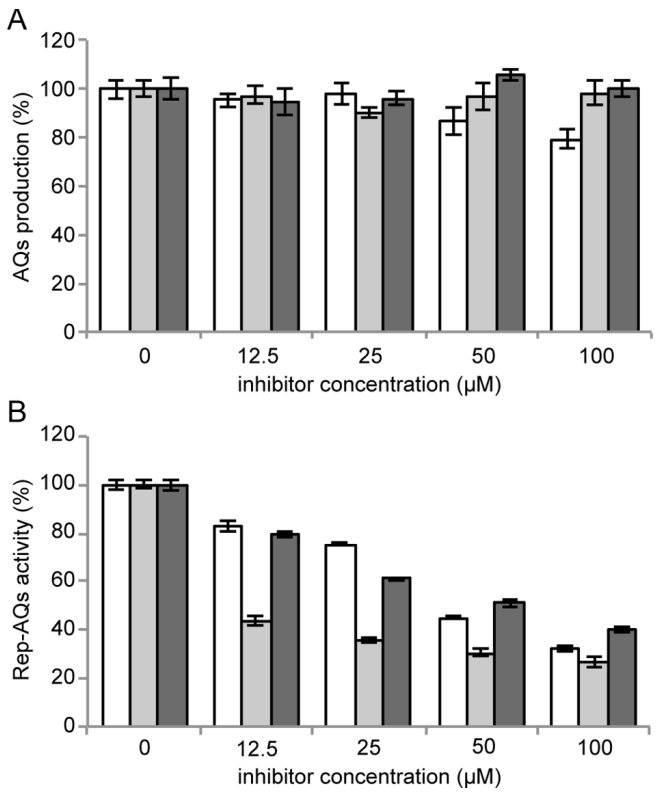
945

946 **Figure 2. Clotrimazole, clofoctol and miconazole inhibit P*pqsA* activity and AQs**
947 **production.**

948 Effect of clotrimazole (white bars), clofoctol (light-grey bars) and miconazole (dark-grey bars)
949 on the PAO1/RepAQs coculture system. Bioluminescence of the untreated PAO1/Rep-AQs
950 coculture normalized to cell density is considered as 100%. **(B)** Effect of clotrimazole (white
951 bars), clofoctol (light-grey bars) and miconazole (dark-grey bars) on AQs production in PAO1.
952 The level of AQs produced by untreated PAO1 is considered as 100%. For both **(A)** and **(B)**, the
953 average of at least three independent experiments is reported with SD.

954 **Figure 3**

955



956

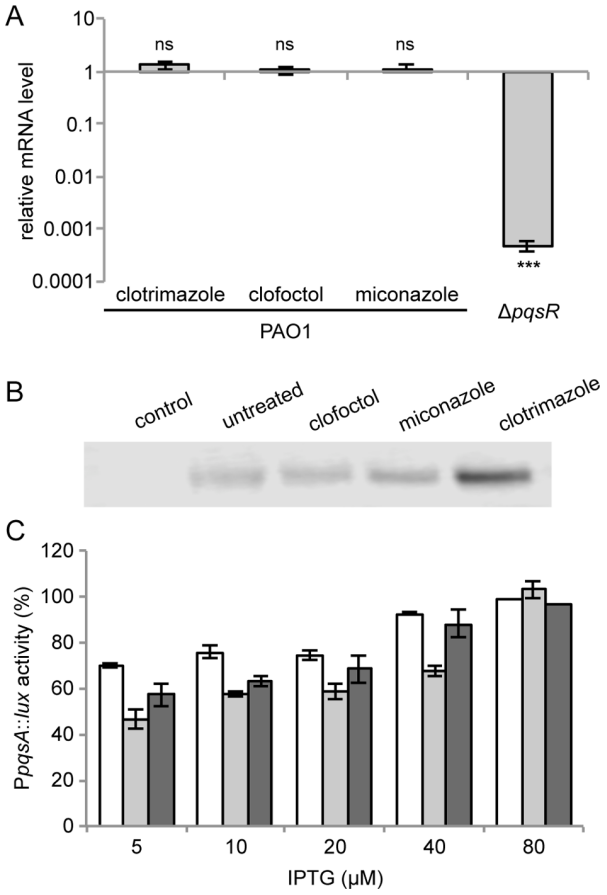
957

958 **Figure 3. Clotrimazole, clofoctol and miconazole hamper AQs reception.**

959 **(A)** Production of AQs in *P. aeruginosa* PAO1 Δ*pqsAH*(pFD-*pqsABCD*) grown for 16 h in LB
960 in the absence or in the presence of clotrimazole (white bars), clofoctol (light-grey bars) and
961 miconazole (dark-grey bars). The level of AQs measured in the untreated sample is considered as
962 100%. **(B)** Activity of the Rep-AQs biosensor strain grown in LB supplemented with 10 μM
963 synthetic PQS and clotrimazole (white bars), clofoctol (light-grey bars) or miconazole (dark-grey
964 bars). Bioluminescence of the untreated Rep-AQs biosensor normalized to its cell density is
965 considered as 100%. For both **(A)** and **(B)**, the average of at least three independent experiments
966 is reported with SD.

967
968

Figure 4



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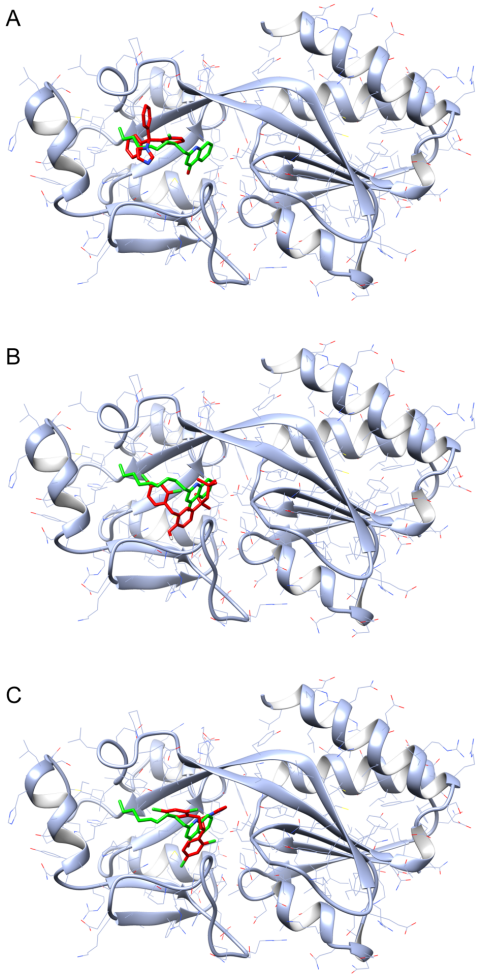
970
971 **Figure 4. Clotrimazole, clofoctol and miconazole hamper PqsR functionality.**
972 **(A)** Real Time RT-PCR analysis showing the mRNA level of *pqsR* in PAO1 cultures treated
973 with 100 μM of the indicated drugs relative to untreated PAO1 culture. The PAO1 Δ*pqsR* strain
974 has been used as negative control. The average of three independent experiments is reported with
975 SD. ns, non-significant difference; ***, $p < 0.001$ (ANOVA). **(B)** Western immunoblotting
976 performed with anti-6xHis antibody on protein crude extracts of PAO1 Δ*pqsAHR*(pPqsR-6H)
977 grown in LB supplemented with 10 μM PQS and 20 μM IPTG, in the absence (untreated) or in
978 the presence of the indicated drugs (100 μM). The PAO1 Δ*pqsAHR* strain carrying the empty
979 vector pME6032 was used as a control. The picture is representative of three independent
980 experiments. **(C)** Effect of 100 μM clotrimazole (white bars), clofoctol (light-grey bars) and
981 miconazole (dark-grey bars) on P*pqsA*::*lux* activity in the PAO1 Δ*pqsA*Δ*pqsH*Δ*pqsR* mutant
982 carrying the pPqsR-6H plasmid, grown in LB supplemented with 10 μM PQS and different
983 concentrations of IPTG, indicated in the graph. The average of three independent experiments is
984 reported with SD.

985 **Figure 5**

986



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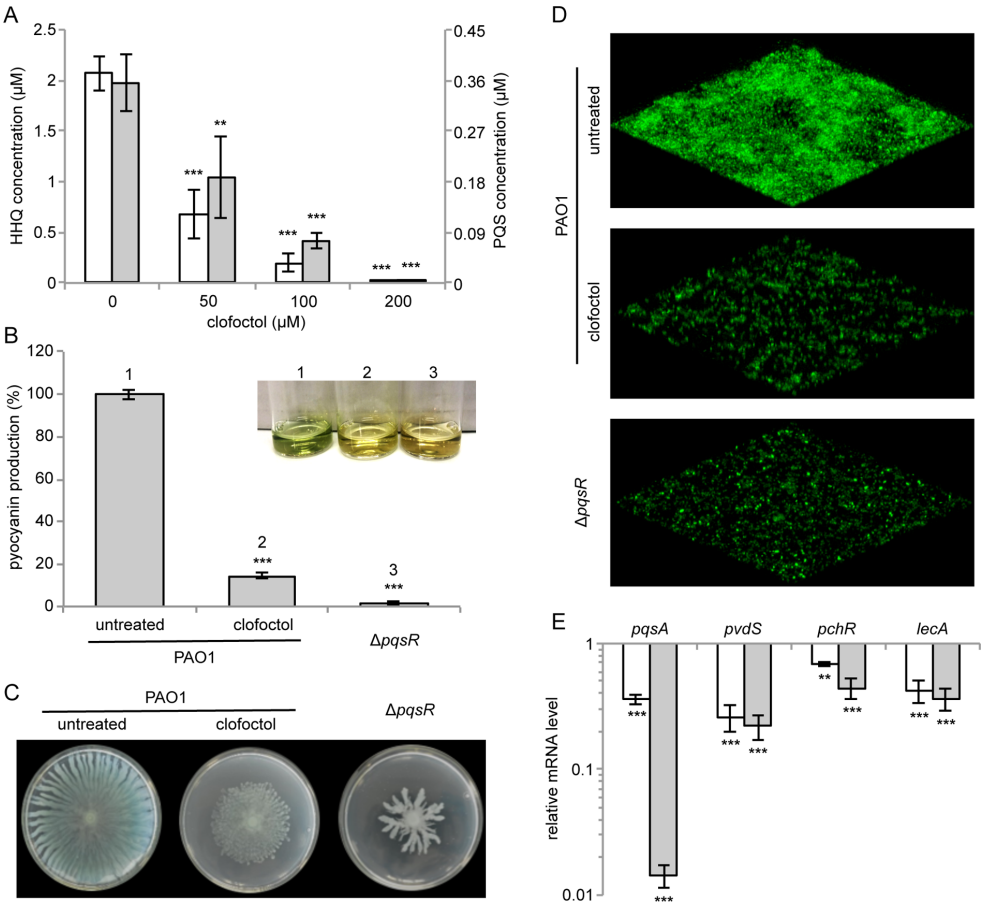
989 **Figure 5. Putative complexes formed by clotrimazole, clofoctol and miconazole with PqsR.**
990 Schematic representation of the complexes formed by clotrimazole **(A)**, clofoctol **(B)** and
991 miconazole **(C)** with the PqsR co-inducer binding domain (CDB), obtained by molecular
992 docking simulations (see Materials and Methods for details). The three drugs are represented in
993 red, while the natural ligand NHQ is represented is green.

994 **Figure 6**

995



996

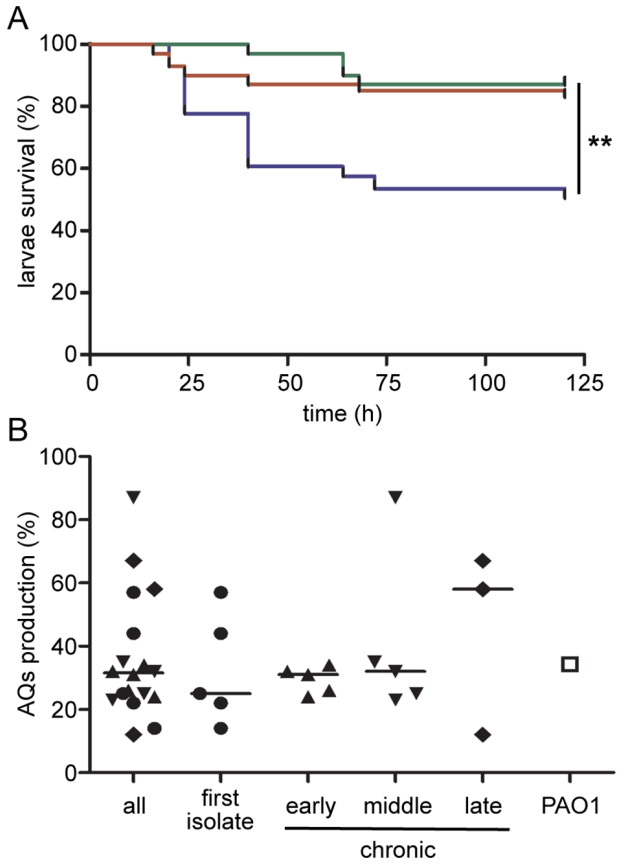
997

998 **Figure 6. Clofoctol inhibits the expression of *pqs*-controlled virulence traits.**

999 **(A)** Levels of HHQ (white bars) and PQS (grey bars) measured by LC-MS/MS on supernatants
1000 of PAO1 cultures grown for 16 h in LB in the absence or in the presence of clofoctol at the
1001 indicated concentrations. The average of three independent experiments is reported with SD. **,
1002 $p = 0.0062$; ***, $p < 0.001$ (ANOVA). **(B)** Effect of 100 µM clofoctol on pyocyanin production,
1003 **(C)** swarming motility, and **(D)** biofilm formation in PAO1. The same phenotypes were
1004 evaluated in the Δ*pqsR* mutant as a control. For pyocyanin production **(B)**, the average of three
1005 independent experiments is reported with SD and representative supernatants are shown in the
1006 inset picture. ***, $p < 0.001$ (ANOVA). For swarming motility **(C)** and biofilm formation **(D)**,
1007 representative pictures of three independent experiments are shown. **(E)** Real Time RT-PCR
1008 analysis showing mRNA level of the indicated genes in PAO1 treated with 100 µM clofoctol
1009 (white bars) and in Δ*pqsR* (grey bars) relative to untreated PAO1. The average of three
1010 independent experiments is reported with SD. **, $p = 0.0012$; ***, $p < 0.001$ (ANOVA).

1011 **Figure 7**

1012



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1015 **Figure 7. Clofoctol displays an antivirulence effect *in vivo* and inhibits the *pqs* QS system in**
1016 ***P. aeruginosa* CF clinical isolates.**

1017 **(A)** Kaplan-Meier plot showing the survival percentage of *G. mellonella* larvae inoculated with
1018 *P. aeruginosa* PAO1 (blue line), with PAO1 and clofoctol at final concentration 100 µM (red
1019 line), or with Δ*pqsR* (green line). The mean survival rate calculated from four independent
1020 experiments performed on at least 30 larvae *per* condition is reported. **, $p = 0.0033$ for PAO1
1021 vs. PAO1 plus clofoctol; $p = 0.0016$ for PAO1 vs. Δ*pqsR* (ANOVA). **(B)** Dot plot showing the
1022 inhibition of AQs production in *P. aeruginosa* CF isolates (filled symbols) and *P. aeruginosa*
1023 PAO1 (open square) treated with 100 µM clofoctol, relative to the untreated samples considered
1024 as 100%. Black lines represent the median values: all, 31.4%; first isolate, 25.2%; early chronic,
1025 31.1%; middle chronic, 32.1%; late chronic, 57.8%. AQs production in treated PAO1 was 34.3%
1026 relative to untreated PAO1. Differences between the median values are not statistically
1027 significant. Mean results of three independent experiments are reported.

Identification of antivirulence drugs targeting the *pqs* quorum sensing system of *Pseudomonas aeruginosa*

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SUPPLEMENTAL MATERIAL

Table S1. Bacterial strain used in this study.
Table S2. Clinical isolates used in this study.
Table S3. Plasmids used in this study.
Table S4. Oligonucleotides used in this study.
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Figure S3. Effect of the *pqs* inhibitors on PAO1 growth.
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Figure S5. Competition assay between clofoctol and PQS for binding to PqsR.
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Figure S7. Effect of clofoctol on AQs production in *P. aeruginosa* CF isolates grouped according to their antibiotic resistance profile.

Table S1. Bacterial strain used in this study.

Strains	Characteristics	References
E. coli		
S17.1λ*pir*	conjugative strain for suicide plasmids.	Simon *et al*., 1983
P. aeruginosa		
PAO1	Nottingham collection wild type strain.	
Δ*pqsR*	PAO1 mutant strain with in frame clear deletion of the *pqsR* gene.	Rampioni *et al*., 2010
PAO1 P*pqsA*::*lux*	PAO1 wild type strain carrying chromosomal insertion of the P*pqsA*::*lux* transcriptional fusion; Tc^R.	Fletcher *et al*., 2007
PAO1 mini-CTX::*lux*	PAO1 wild type strain carrying chromosomal insertion of the mini-CTX::*lux* empty vector; Tc^R.	Fletcher *et al*., 2007
Δ*pqsA* P*pqsA*::*lux*	PAO1 mutant strain deleted in *pqsA* gene carrying chromosomal insertion of the P*pqsA*::*lux* transcriptional fusion; Tc^R.	Diggle *et al*., 2007
Δ*pqsAH* P*pqsA*::*lux*	PAO1 double mutant strain deleted in *pqsA* and *pqsH* genes carrying chromosomal insertion of the P*pqsA*::*lux* transcriptional fusion; Tc^R.	Fletcher *et al*., 2007
Δ*pqsAHR* P*pqsA*::*lux*	PAO1 triple mutant strain deleted in *pqsA, pqsH* and *pqsR* genes carrying chromosomal insertion of the P*pqsA*::*lux* transcriptional fusion; Tc^R.	Ilangovan *et al*., 2013
PAO1 P*lecA*::*lux*	PAO1 wild type strain carrying chromosomal insertion of the P*lecA*::*lux* transcriptional fusion; Tc^R.	Winzer *et al*., 2000
Δ*pqsR* P*lecA*::*lux*	PAO1 mutant strain deleted in *pqsA* gene carrying chromosomal insertion of the P*lecA*::*lux* transcriptional fusion; Tc^R.	This study

Table S2. Clinical isolates used in this study.

Isolate name[a]	Colonization[b]	Years of colonization	Phenotypic characteristics[c]	Antibiotics susceptibility[d]	Residual AQs production
BG 4	first isolate	-	frayed	R	22
BG 5	chronic early	2	frayed	R	31
BG 6	chronic middle	6	frayed	R	35
BG 7	first isolate	-	circular	S	25
BG 8	chronic early	2	circular	R	26
BG 10	first isolate	-	frayed	S	14
BG 11	chronic early	3	frayed	R	44
BG 12	chronic middle	5	frayed	S	32
BG 13	first isolate	-	frayed	MDR	44
BG 17[e]	chronic early	2	circular	R	24
BG 18[e]	chronic middle	6	circular	R	25
BG 36	chronic early	3	rugose	S	32
BG 56	first isolate	-	small	R	57
BG 76	chronic middle	7	mucoid	R	23
BG 80[e]	chronic middle	5	circular	R	87
BG 92[e]	chronic late	≥15	small	XDR	12
BG 93	chronic late	≥15	rugose	MDR	np
BG 96	chronic late	≥15	circular	R	58
BG 97	chronic late	≥15	frayed	XDR	np
BG 100[e]	chronic late	≥15	small	R	67

[a] CF clinical isolates from the collection of the Bambino Gesú hospital, Rome, Italy.

[b] Different categories depending on the year of infection of the clinical isolates in the lung of cystic fibrosis patients: first isolate; chronic early (from 2 to 3 years); chronic middle (from 5 to 7 years); chronic late (equal or more than 15 years).

[c] Characteristics observed when clinical isolates were grown as colony biofilms.

[d] Criteria to define multi-drug resistant (MDR) and extensively-drug resistant (XDR) bacteria have been taken from European Centre for Diseases Prevention and Control (ECDC) web site (http://ecdc.europa.eu/en/Pages/home.aspx): MDR, resistant to one or more antibiotics belonging to at least three different classes; XDR, resistant to one or more antibiotics belonging to all classes except two or less; S, susceptible to all classes of antibiotics; R, resistant to one or more antibiotics belonging to less than three different classes.

[e] Residual production of AQs in samples treated with 100 μM clofoctol relative to untreated samples, considered as 100%. np, strains that do not produce detectable levels of AQs.

Table S3. Plasmids used in this study.

Plasmids	Characteristics	References
pME6032	pVS1-p15A shuttle expression (IPTG-inducible) vector; Tc^R.	Heeb *et al*., 2002
pPqsR-6H	pME6032 derivative for IPTG-inducible expression of the PqsR protein fused with a 6xHis tag; Tc^R.	Ilangovan *et al*., 2013
pBBR1MCS-5	shuttle vector for constitutive expression; Gm^R.	Kovach *et al*., 1995
pBBR-*pqsABCD*	pBBR1MCS-2 derivative for constitutive expression of PqsA, PqsB, PqsC and PqsD proteins in *P. aeruginosa*; Km^R.	Niewerth *et al*., 2011
pFD-*pqsABCD*	pBBR1MCS-5 derivative for constitutive expression of PqsA, PqsB, PqsC and PqsD proteins in *P. aeruginosa*; Gm^R. This plasmid was obtained by cloning into pBBR1MCS-5 the SalI-SacI *pqsABCD* fragment extracted from pBBR-*pqsABCD*.	This study
pMRP9-1	pMRP9 derivative for constitutive expression of GFP in *P. aeruginosa*; Ap^R/Cb^R.	Davies *et al.,* 1998

Table S4. Oligonucleotides used in this study.

Name	Sequence (5'-3')
FW*pqsA*	GACCGCGAAGGACACACTAT
FW*pqsA*	TGAACAGATCGTCTTCCCGC
FW*lecA*	CAGGGCAGGTAACGTCGATT
RV*lecA*	CAACCCGGTATTGACCGGAA
FW*pchR*	CTCAGCGCACAGTTCCTTTC
RV*pchR*	CGAACACCTTGCGAAAGCC
FW*pqsR*	AACATGTTCCTCCAGGTCATCG
RV*pqsR*	TGCGCATGTAAGGGATCAGG
FW*pvdS*	GGAACAACTGTCTACCCGCA
RV*pvdS*	GTAGCTGAGCTGTGCCTTGA
FW16S	GAGAGTTTGATCCTGGCTCAG
RV16S	CTACGGCTACCTTGTTACGA
FWP*pqsL*	TCCGCTCGAGGATCGTCACCGTCAACTG
RVP*pqsL*	TAACTGCAGCGTCATGGATGAGTCTCCG

References not included in the main text:

- Heeb S, Blumer C, Haas D. 2002. Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. J Bacteriol 184:1046-1056.
- Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM 2nd, Peterson KM. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166:175-176.
- Niewerth H, Bergander K, Chhabra SR, Williams P, Fetzner S. 2011. Synthesis and biotransformation of 2-alkyl-4(1H)-quinolones by recombinant *Pseudomonas putida KT2440*. Appl Microbiol Biotechnol 91:1399-1408.
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- Winzer K, Falconer C, Garber NC, Diggle SP, Cámara M, Williams P. 2000. The *Pseudomonas aeruginosa* lectins PA-IL and PA-IIL are controlled by quorum sensing and by RpoS. J Bacteriol 182:6401-6411.

Figure S1

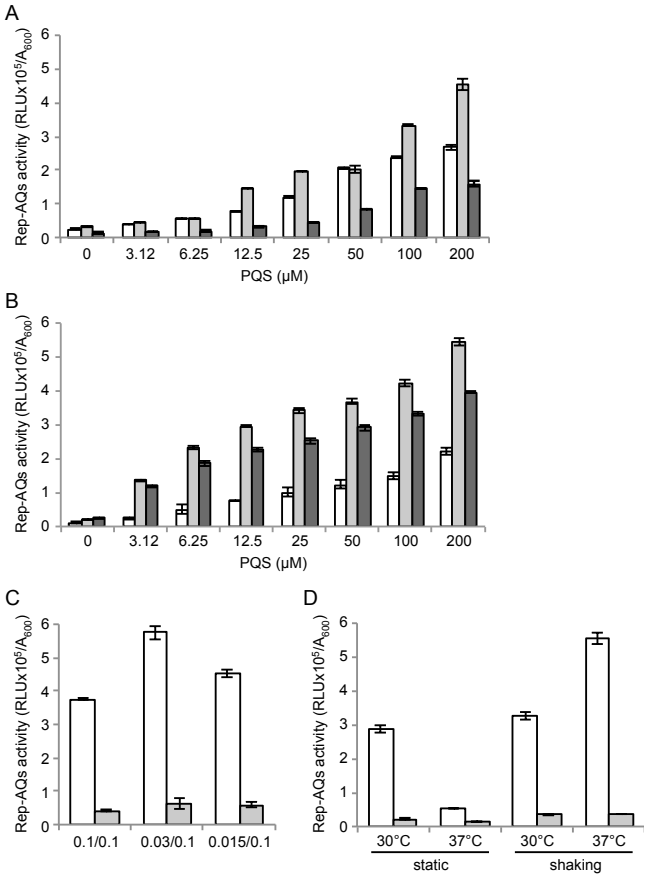

Figure S1. Set up of the PAO1/Rep-AQs coculture system.

(A) Response of the Rep-AQs biosensor after 3 h (white bars), 5 h (light-grey bars) and 7 h (dark-grey bars) incubation in LB supplemented with the indicated concentrations of PQS. **(B)** Activity of the Rep-AQs biosensor inoculated at starting absorbance (A_{600}) of 0.4 (white bars), 0.1 (light-grey bars) and 0.025 (dark-grey bars) after 5 h incubation in LB supplemented with the indicated concentrations of PQS. **(C)** Activity of the Rep-AQs biosensor after 5 h co-incubation with PAO1 (white bars) or Δ*pqsA* (grey bars) strains at the indicated starting absorbance (A_{600}). The first value refers to the PAO1 or Δ*pqsA* strains (A_{600} from 0.1 to 0.015), the second to the Rep-AQs biosensor (A_{600} = 0.1). **(D)** Activity of the PAO1/Rep-AQs (white bars) and Δ*pqsA*/Rep-AQs (grey bars) cocultures after 5 h incubation at 30°C or 37°C in static or shaking (200 rpm) conditions. Staring A_{600} was 0.1 for the Rep-AQs biosensor and 0.03 for the PAO1 and Δ*pqsA* strains. For **(A)-(D)**, biosensor activity is reported as relative light units (RLU) normalized to cell density (A_{600}); the average of three independent experiments is reported with SD.

Figure S2

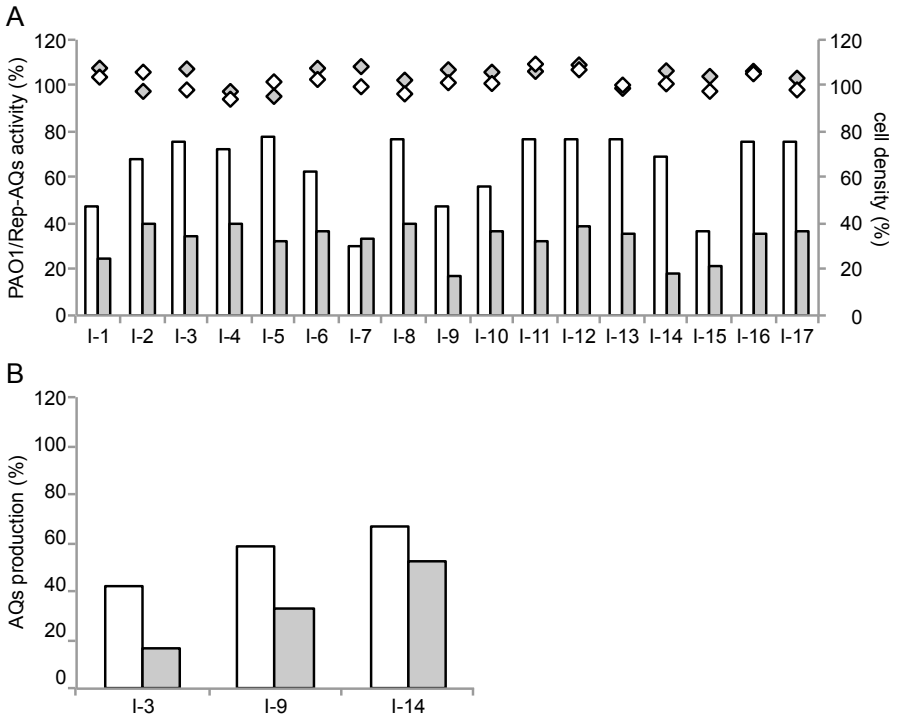

Figure S2. Primary and secondary screenings of the PHARMAKON library.

(A) PAO1/Rep-AQs activity (bars) and cell density (diamonds) measured after 5 h incubation at 37°C in shaking conditions in LB supplemented with molecules of the PHARMAKON library, indicated with codes from inhibitor 1 (I-1) to inhibitor 17 (I-17), at 20 µM (white bars and diamonds) and 200 µM (grey bars and diamonds) concentration. PAO1/Rep-AQs activity and cell density measured in the presence of 0.2% and 2% DMSO were considered as 100%. **(B)** AQs production measured in supernatants of the PAO1 strain treated with the molecules of the PHARMAKON library clotrimazole (I-3), clofoctol (I-9) and miconazole (I-14) at 20 µM (white bars) and 200 µM (grey bars) concentration. AQs were quantified by using the Rep-AQs biosensor strain.

Figure S3

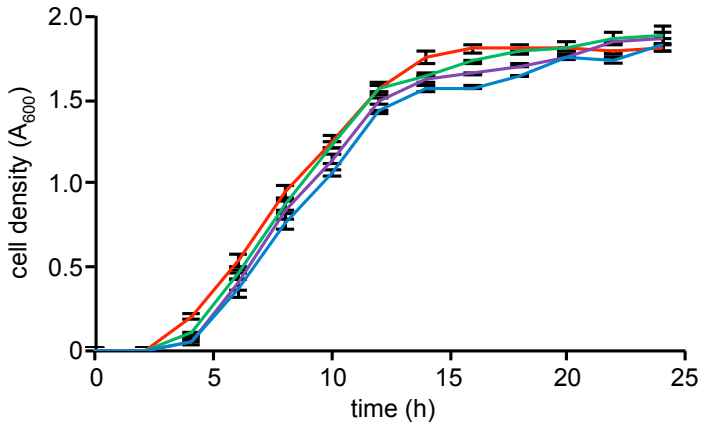

Figure S3. Effect of the *pqs* inhibitors on PAO1 growth.

Growth curves of PAO1 incubated at 37°C in shaking conditions in LB supplements with 200 µM clotrimazole (blue line), clofoctol (green line), miconazole (purple line), or with the corresponding amount of DMSO (red line). The average of three independent experiments is reported with SD.

Figure S4

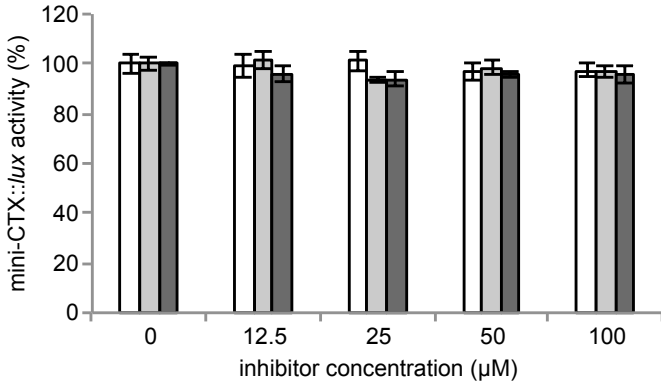

Figure S4. Effect of the *pqs* inhibitors on bioluminescence emission.

Percentage of light emitted by the PAO1 strain carrying the mini-CTX::*lux* empty vector grown at 37°C in shaking conditions in LB supplements with 200 µM clotrimazole (white bars), clofoctol (light-grey bars), or miconazole (dark-grey bars). Bioluminescence of the same strain grown in the presence of DMSO was considered as 100%. The average of three independent experiments is reported with SD.

Figure S5

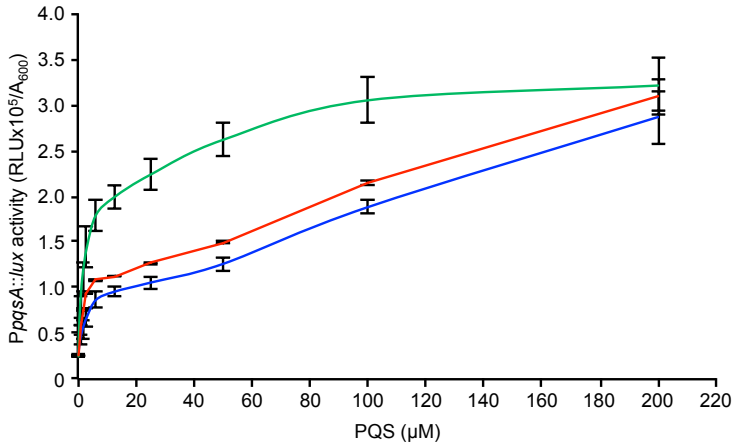

Figure S5. Competition assay between clofoctol and PQS for binding to PqsR.

P*pqsA*::*lux* activity measured in the Rep-AQs biosensor grown in LB supplemented with different concentrations of PQS in the absence (green line) or in the presence of 12.5 µM (red line) or 50 µM (blue line) clofoctol. Promoter activity is reported as relative light units (RLU) normalized to cell density (A_{600}).

Figure S6

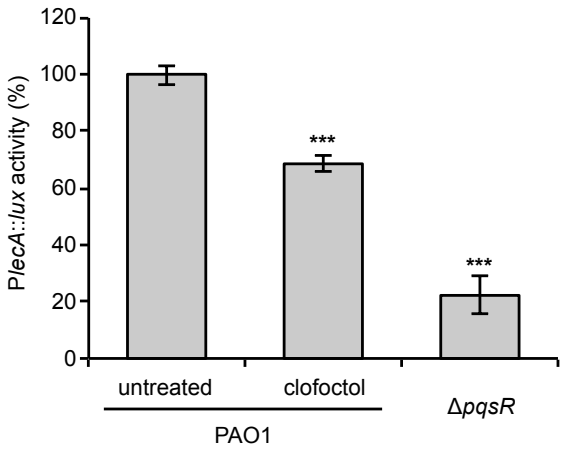

Figure S6. Effect of clofoctol on P*lecA* activity.

Activity of the P*lecA* promoter in PAO1 cultures grown in LB supplements with DMSO (untreated) or with 100 µM clofoctol, and in the Δ*pqsR* culture grown in LB supplements with DMSO. P*lecA* activity in untreated PAO1 is considered as 100%. The average of three independent experiments is reported with SD. ***, $p < 0.001$ (ANOVA).

Figure S7

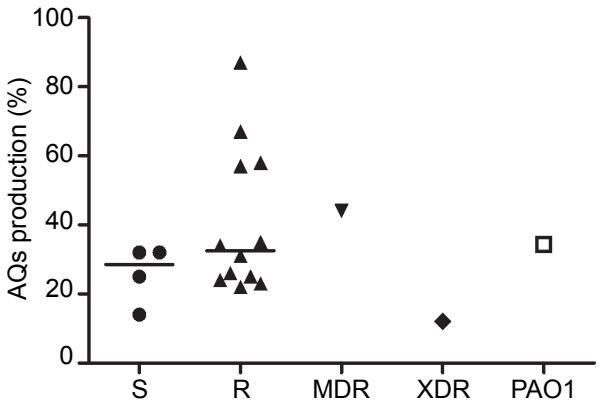

Figure S7. Effect of clofoctol on AQs production in *P. aeruginosa* CF isolates grouped according to their antibiotic resistance profile.

Dot plot showing the inhibition of AQs production in *P. aeruginosa* CF isolates (filled symbols) and *P. aeruginosa* PAO1 (open square) treated with 100 µM clofoctol, relative to the untreated samples considered as 100%. Black lines represent the median values: S, 28.4%; R, 32.6%. AQs production in the MDR, XDR and PAO1 strains were 43.5%, 11.6% and 34.3%, respectively. Differences between the median values are not statistically significant. Mean results of three independent experiments are reported.

Chapter 7

Concluding remarks

Since the term quorum sensing (QS) was coined in 1994 (Fuqua *et al*., 1994), the number of publications on the topic has steadily risen. The interest of scientists on QS over the last decades witnesses the promising advances it represents in basic science and biotechnological fields. For many researchers, untangling complex QS networks governing bacterial social behaviours is an engaging puzzle to solve, for others QS circuits are useful tools to be exploited in synthetic biology applications, or ideal targets for new therapeutic approaches. Despite their specific interest, the broad and multidisciplinary group of scientists involved in QS research has produced an impressive amount of knowledge that allowed fine understanding of bacterial communication processes, from detailed characterization of their molecular constituents and mechanism of action, to the complex social interactions they allow at the intraspecies, interspecies and interkingdom level.

This PhD thesis builds up on a considerable amount of previous works investigating QS functionality and inhibition in the human opportunistic pathogen *Pseudomonas aeruginosa* to deploy biotechnological applications with relevance in the biomedical field. In particular, as described in the previous Chapters, this PhD thesis deals with *i)* the investigation of regulatory properties emerging from the network motif governing the *las* QS system in *P. aeruginosa*, *ii)* the exploitation of *P. aeruginosa* QS systems for the generation of synthetic cells interfacing with natural cells, and *iii)* the identification of antivirulence drugs targeting *P. aeruginosa* QS.

The network motif known as type 1 incoherent feedforward loop (IFFL-1), in which a transcriptional regulator triggers the expression of both an output gene and of its transcriptional repressor, is conserved in all living organisms investigated so far, from bacteria to plants and humans (Alon, 2007; Hart and Alon, 2013). Wet-lab experiments and *in silico* simulations revealed that the IFFL-1 confers peculiar properties to the expression of its output gene(s), including pulse generation, acceleration of response time and fold-change detection capacity (Mangan *et al*., 2006; Kaplan *et al*., 2008; Goentoro *et al*., 2009). In this context, in Chapter 2 an experimental work has been presented investigating new regulatory properties conferred by the QS *las* IFFL-1 to the expression of virulence genes in *P. aeruginosa*. Our work demonstrated that the IFFL-1 constituted by LasR and RsaL confers robustness with respect to fluctuations in the levels of LasR to phenotypes controlled by both these transcriptional regulators. In contrast, other virulence-related traits controlled by LasR but not by RsaL are sensitive to changes in LasR level. Thus, the LasR regulon is split in two distinct sub-regulons with different robustness with respect to LasR fluctuations (Bondí *et al*., 2017). This characteristic highlights *P. aeruginosa* phenotypic plasticity and contributes to clarify some aspects underlying *P. aeruginosa* adaptation to the host environment.

P. aeruginosa can establish both acute and chronic infections. Acute infections are carried out by planktonic bacterial expressing high levels of virulence factors, while chronic infections, as in the lung of CF patients, are characterized by sessile bacteria forming biofilm and expressing lower levels of some virulence factors, such as elastase and proteases. However, high levels of pyocyanin have been recovered in the sputum of CF patients with *P. aeruginosa* chronic lung infection (Furukawa *et al*., 2006; Folkesson *et al*., 2012; Winstanley *et al*., 2016). Since the majority of virulence phenotypes expressed by *P. aeruginosa* during both acute and chronic infections are positively controlled by QS, it is unclear how *P. aeruginosa* can modulate the differential expression of QS-dependent virulence genes under these two conditions. Our results suggest that differential expression of virulence factors controlled by LasR in acute and chronic infection could be due to the peculiar regulatory properties of the *las* multi-output IFFL-1. Indeed, virulence phenotypes involved in acute infection, such as elastase and proteases production, are only controlled by LasR, hence their expression could be rapidly adjusted in the transition from acute to chronic infection depending on variations in LasR level. Conversely, other virulence phenotypes, that like pyocyanin production are activated by LasR and repressed by RsaL, could be steadily expressed in both acute and chronic infections, irrespective of fluctuations in LasR level.

The importance of the *las* multi-output IFFL-1 in *P. aeruginosa* pathogenicity *in vivo* is supported by the observation that a *P. aeruginosa* Δ*rsaL* mutant strain is impaired in the establishment of chronic lung infection in mice (Bondí *et al*., 2014), and that *P. aeruginosa* strains with mutations in *lasR* but not in *rsaL* are generally isolates from CF lungs (Hoffman *et al*., 2009; Winstanley and Fothergill, 2009; Bjarnsholt *et al*., 2010; Feltner *et al*., 2016).

Notably, regulatory networks with a topological architecture resembling the *las* IFFL-1 have been described in QS systems in several bacterial species, suggesting that the robustness conferred by this kind of network motif to the expression of QS genes could be a common feature of bacterial communication systems (Venturi *et al.,* 2011; Rampioni *et al.,* 2012).

Beside its contribution to elucidate the function and properties of the *las* QS system in *P. aeruginosa*, this work has a perspective applicative relevance. Indeed, multi-output IFFL-1 network motifs could be exploited in the future for the generation of engineered bacteria or synthetic cells expressing multiple phenotypes with different responsiveness to the concentration of their transcriptional regulator, or to the external stimuli they could be programmed to respond to.

The second part of my PhD thesis was focused on the exploitation of QS for the generation of synthetic cells able to communicate with bacterial cells. Briefly, the paper presented in Chapter 4 describes the development of liposome-based synthetic cells able to synthesize a QS signal molecule that is then perceived by *P. aeruginosa* cells (Rampioni *et al*., 2018a). This achievement

provides the proof of concept that man-made synthetic cells endowed with the ability of interfacing with natural cells can be generated *via* a bottom-up constructive approach.

Remarkably, the methodological approach used to reach this goal follows the main synthetic biology principles of standardization, modularity and orthogonality. Indeed, the synthetic cells described in Chapter 4 are built by trapping inside lipid vesicles only: *i)* a plasmid coding for a QS signal synthase gene; *ii)* the QS signal molecule precursors; *iii)* the PURE system, *i.e.* a well-characterized minimal kit for cell-free transcription-translation. In other terms, the synthetic cells based on the PURE system are molecular devices made of few modular standard parts, whose behaviour and reciprocal interactions are predictable and quantifiable. Notably, a previous *in silico* modelling study allowed predicting the level of QS signal molecule produced by synthetic cells (Rampioni *et al*., 2014a), and similar QS signal molecule concentration has been measured in the experimental work reported in Chapter 4 (Rampioni *et al*., 2018a). Also the genetic construct driving QS signal molecule production can be seen as a standard part *per se*, that confers to a synthetic cell the ability to activate a response in a natural cell. This genetic standard part could be used in the future for the modular engineering of more complex synthetic cells that, beside sending a message to natural cells, could be endowed with other functions, likely by hosting additional genetic circuits. Since all the parts of the system are known and properly defined, it should be possible to predict their behaviour, so to avoid undesired and detrimental process to occur. As an example, a plasmid for the constitutive expression of a signal molecule receptor, if properly interlinked with an *ad hoc* designed variant of the QS signal synthesis plasmid, could lead to the generation of synthetic cells that, upon stimulus perception, are able to respond *via* signal molecules production to chemical messages sent by natural cells. This would allow establishing a bidirectional communication system between synthetic and natural cells. In this context, the generation of synthetic cells able to perceive QS signal molecules has been recently described (Lentini *et al*., 2017).

Obviously, the development and the integration of multiple functional parts and devices in synthetic cells can be envisaged in the future, despite the fact that current technology is only at its infancy. Future synthetic cells could be functionalized to synthesize products of industrial interest, to degrade pollutants in contaminated sites, to compute external stimuli in an increasingly complex way as bio-computers, or to produce/release a therapeutic compound inside the human body only upon perception of a signal molecule produced by a pathogen or by cancer cells, so providing new therapeutic alternatives and paving the way for future intelligent drug-delivery approaches (Leduc *et al*., 2007). Some of these applications have been discussed in Chapter 3 (Stano *et al*., 2016).

One of the main limitations for future biotechnological applications based on synthetic cells relies on our still limited ability to standardize their production, hence to generate a uniform population of synthetic cells containing all the molecular components required for their functionality at the desired concentration. It is expectable that this technical limitation will be readily overcame in the next years, also thanks to the fast growing microfluidic technology. The potential of microfluidic devices in the synthetic cells field has already been considered (Matosevic and Paegel, 2011).

Moreover, present-day synthetic cells are fragile objects, whose stability and functionality are strongly dependent on external agents. However, future synthetic cells could be possibly implemented with complex functional behaviours, such as the capability of coping with perturbing environments and to display adaptive behaviours. The possible future exploitation of this ambitious goal, tending to confer to synthetic cells an autonomous behaviour proper of natural cells, would incredibly increase their prospective application fields. However, the increased autonomy of synthetic cells would contrast with the need of their predictable behaviour, so opening an issue of appropriately balancing their autonomy and controllability.

Overall, synthetic cells studies will likely continue to flourish in the next years, resulting in the achievement of scientific and technological advancements far beyond our imagination. It is foreseeable that the generation of synthetic cells interacting with natural cells will significantly contribute to this process.

The last part of this PhD thesis focuses on exploitation of QS as a target for new antivirulence drugs. Indeed, the emergence of antibiotic resistant bacterial pathogens calls for the development of therapeutic approaches alternative to antibiotics, and in this context molecules decreasing bacterial pathogenicity rather than growth have a great promise for future biomedical applications (Rasko and Sperandio, 2010). Since QS controls the expression of virulence traits in many bacterial pathogens, several studies in the last 15 years aimed at identifying synthetic or natural molecules interfering with QS communications systems (Rampioni *et al*., 2014b). Probably due to cytotoxicity of the majority of the molecules identified to date, to the best of my knowledge garlic extract is the only QS inhibitor that has been tested in humans so far. Although the results were not statistically significant, a trend toward improvement of the clinical outcome of *P. aeruginosa*-infected CF patients after oral garlic extract administration was observed (Smyth *et al*., 2010). Following work identified ajoene as the most active QS inhibitor in garlic extract, but ajoene alone was less active than the crude garlic extract in hampering *P. aeruginosa* virulence both *in vitro* and *in vivo* (Jakobsen *et al*., 2012). Thus, despite the huge efforts made to date in the field, clinical application of QS inhibitors remains far away (Maura *et al*., 2016).

The problem of unfavourable pharmacological properties of QS inhibitors can be circumvented by exploring the anti-QS activity of drugs already approved for use in humans. This approach, known as drug repurposing, has higher probability to identify safe molecules that can be easily transferred to the clinical practice compared to conventional drug discovery programmes (Rampioni *et al*., 2017). Repurposing of the anthelmintic drug niclosamide to treat *P. aeruginosa* infections has been proposed, since this molecule was found to hamper the *las* QS system of this pathogen, hence lowering *P. aeruginosa* pathogenicity (Imperi *et al*., 2013). In Chapter 5, the coculture-based screening methodology used to identify niclosamide as an anti-QS drug has been described (Rampioni *et al*., 2018b). Notably, this coculture-based screening system is prone to optimization for the identification of drugs targeting other QS systems in *P. aeruginosa* and in different pathogens.

A main concern on the use of anti-QS drugs for treatment of CF pulmonary infections caused by *P. aeruginosa* relies on the frequent isolation of QS defective mutants in the CF lung (Hoffman *et al*., 2009; Bjarnsholt *et al*., 2010; Jiricny *et al*., 2014; Feltner *et al*., 2016; Winstanley *et al*., 2016). It is possible that QS mutants are non-cooperating cheaters that exploit resources produced by a QS-proficient sub-population of co-infecting clones, since QS cheaters emerge in a population of wild type *P. aeruginosa* both *in vitro* and *in vivo* (Sandoz *et al*., 2007; Rumbaugh *et al*., 2009). Anyway, QS-deficient strains should be considered as resistant to anti-QS drugs, thus the use of anti-QS agents against *P. aeruginosa* CF lung infection is still debated. Recent reports demonstrated that a considerable fraction of *P. aeruginosa* CF isolates is indeed resistant to the anti-QS molecule furanone C-30 (García-Contreras *et al*., 2013; García-Contreras *et al*., 2015). However, most studies focused on the inactivation of the *las* QS system in chronic CF isolates, while little attention has been given to the *pqs* QS system. It has been demonstrated that signal molecules of the *pqs* QS system are detectable in the sputum, plasma and urine of ca. 80% of CF patients suffering *P. aeruginosa* chronic lung infection, and that their level positively correlates with quantitative measures of *P. aeruginosa* cells in the lung (Barr *et al*., 2015). In this context, the *pqs* QS system could represent a target more suitable than the *las* QS system for the treatment of *P. aeruginosa* infections in CF patients.

On these bases, Chapters 6 of this PhD thesis focuses on the identification of antivirulence drugs *via* a drug repurposing approach in which the coculture screening system described in Chapter 5 has been modified to select for FDA-approved drugs targeting the *pqs* QS system of *P. aeruginosa*. This strategy led to the identification of three drugs inhibiting the *pqs* receptor PqsR, hence hampering the *pqs* QS system, clofoctol, clotrimazole and miconazole (D'Angelo *et al*., manuscript in preparation). Clofoctol is an antibiotic used to treat pulmonary infections caused by Gram-

positive bacteria, while clotrimazole and miconazole are antifungal drugs used to treat vaginal and oral candidiasis and skin yeast infections (Clayton and Connor, 1973; Sawyer *et al.,* 1975; Buogo, 1981; Del Tacca *et al*., 1987; Danesi *et al*., 1988; De Cremer *et al*., 2015; Zhang *et al*., 2016).

As clofoctol was the most potent *pqs* QS inhibitor identified in our study, experiments were performed showing that this drug inhibits the expression of PqsR-dependent virulence factors in *P. aeruginosa*, including biofilm formation. Moreover, clofoctol protected *Galleria mellonella* larvae from *P. aeruginosa* infection and hampered the functionality of the *pqs* QS system also in *P. aeruginosa* clinical isolates from CF patients. Due to its current formulation and pharmacodynamic properties, clofoctol is particularly promising to treat *P. aeruginosa* CF lung infection. Indeed, this drug reaches the highest concentration in the respiratory system when administered as suppositories. Moreover, clofoctol is especially used in children, and this is important if considering that *P. aeruginosa* establishes intermittent infections in the lungs of CF patients since the paediatric age (Lyczak *et al*., 2002).

For what concerns clotrimazole and miconazole, the antivirulence potential of these PqsR inhibitors should be supported by future experiments assessing their effect on the expression of *P. aeruginosa* virulence phenotypes. These drugs could be particularly suitable to treat chronic wound infections caused by *P. aeruginosa*, since they are currently formulated as ointments for topic treatment of fungal infections.

Overall, despite additional work is need to assess the potential antivirulence effect of the newly identified anti-*pqs* drugs both *in vitro* and *in vivo* (*e.g.* by using mice model systems of chronic lung and wound infections), clofoctol, clotrimazole and miconazole are promising agents for future anti-*P. aeruginosa* therapeutic approaches. In a more general perspective, the work described in this PhD thesis could boost the use of antivirulence approaches and drug repurposing strategies for future identification of new therapeutic agents active against other antibiotic-resistant pathogens.

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List of publications presented in this PhD thesis

1. **D'Angelo F**, Baldelli V, Halliday N, Polticelli F, Fiscarelli E, Williams P, Visca P, Leoni L and Rampioni G. Identification of antivirulence drugs targeting the *pqs* quorum sensing system of *Pseudomonas aeruginosa.* Manuscript in preparation for submission to *Antimicrobial Agents and Chemotherapy*.
2. Rampioni G, **D'Angelo F**, Messina M, Zennaro A, Kuruma Y, Tofani D, Leoni L and Stano P (2018a) Synthetic cells produce a quorum sensing chemical signal perceived by *Pseudomonas aeruginosa*. *Chem Commun*. doi:10.1039/C7CC09678J.
3. Rampioni G, Giallonardi G, **D'Angelo F** and Leoni L (2018b) A coculture-based approach for screening campaigns aimed at identifying novel *Pseudomonas aeruginosa* quorum sensing inhibitors. *Methods Mol Biol* 1673:287-296.
4. Bondí R, Longo F, Messina M, **D'Angelo F**, Visca P, Leoni L and Rampioni G (2017) The multi-output incoherent feedforward loop constituted by the transcriptional regulators LasR and RsaL confers robustness to a subset of quorum sensing genes in *Pseudomonas aeruginosa*. *Mol Biosyst* 13:1080-1089.
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Other publications not included in this PhD thesis

6. **D'Angelo F**, Baldelli V, Pavoncello V, Visca P, Rampioni G and Leoni L. Identification of FDA-approved antivirulence drugs targeting PqsE protein of *Pseudomonas aeruginosa.* Manuscript in preparation.
7. Rampioni G, Mavelli F, Damiano L, **D'Angelo F**, Messina M, Leoni L and Stano P (2014) A synthetic biology approach to bio-chem-ICT: first moves towards chemical communication between synthetic and natural cells. *Nat Comput* 13:333-349.
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