Surface sensing triggers a broad-spectrum antimicrobial response in *Pseudomonas aeruginosa*

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Summary

Interspecies bacterial competition may occur via cell-associated or secreted determinants and is key to successful niche colonization. We previously evolved *Pseudomonas aeruginosa* in the presence of *Staphylococcus aureus* and identified mutations in the Wsp surface-sensing signalling system. Surprisingly, a ΔwspF mutant, characterized by increased c-di-GMP levels and biofilm formation capacity, showed potent killing activity towards *S. aureus* in its culture supernatant. Here, we used an unbiased metabolomic analysis of culture supernatants to identify rhamnolipids, alkyl quinoline N-oxides and two siderophores as members of four chemical clusters, which were more abundant in the ΔwspF mutant supernatants. Killing activities were quorum-sensing controlled but independent of c-di-GMP levels. Based on the metabolomic analysis, we formulated a synthetic cocktail of four compounds, showing broad-spectrum anti-bacterial killing, including both Gram-positive and Gram-negative bacteria. The combination of quorum-sensing-controlled killing and Wsp-system mediated biofilm formation endows *P. aeruginosa* with capacities essential for niche establishment and host colonization.

Introduction

Whether in the environment or during host infection, bacteria have to compete with established microbial communities to gain access to nutrients and space. Typical examples for polymicrobial infections are burn wounds and cystic fibrosis (CF) lungs, where the dominant pathogenic species are *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Although *S. aureus* is prevalent in young CF patients, *P. aeruginosa* occurs more frequently in adult respiratory samples (Folkesson et al., 2012). This switch in colonization frequency might result from host immune responses, antibiotic treatments and/or from direct competition between the two microorganisms (Limoli and Hoffman, 2019). Focusing on the latter hypothesis, we have previously established an *in vitro* model in which we evolved *P. aeruginosa* for 150 generations in the presence and absence of *S. aureus* to assess how *P. aeruginosa* adapts to the presence of a pre-established niche competitor (Tognon et al., 2017). By comparing the genomes of ancestor and evolved populations, we observed emergence of mutations in the *P. aeruginosa* Wsp (wrinkly spreader phenotype) signal transduction system (D’Argenio et al., 2002; Hickman et al., 2005). The mutations occurred in the wspF gene, resulting in constitutive activation of the Wsp signalling cascade. Surprisingly, the wspF mutants showed increased killing activity against *S. aureus* in comparison to the ancestor strain PA14.

The Wsp signal transduction system of *P. aeruginosa* is similar to the chemosensory system of *Escherichia coli* and uses methylation and demethylation of the WspA transmembrane sensor by WspE and WspF, respectively, to adapt to variations of external stimuli (D’Argenio et al., 2002; Hickman et al., 2005). The Wsp system in *P. aeruginosa* was reported to respond to surface attachment (Guvener and Harwood, 2007; O’Connor et al., 2012; Song et al., 2018) and to changes in membrane composition (Blanka et al., 2015). These stimuli ultimately translate into the generation of the intracellular signalling molecule c-di-GMP, via the cognate di-guanylate cyclase WspR, entailing increased polysaccharide production, cell aggregation and biofilm formation (Kulasakara et al., 2006; Valentini and Filloux, 2016). Loss of function mutations in the methyl esterase WspF results in constitutive activation...
of the Wsp signalling cascade and hence increased c-di-GMP levels (Hickman et al., 2005). Compared to the wild type, wspF mutants are less motile and display a typical wrinkly colony morphology and reduced colony size, a phenotype reminiscent of the wrinkly spreaders described initially in P. fluorescens (Spiers et al., 2002). The secondary messenger c-di-GMP plays a major role in the switch between the two main lifestyles of bacteria: the motile, planktonic lifestyle, associated with low c-di-GMP levels, and the sessile biofilm mode of growth triggered by high intracellular levels of c-di-GMP (Valentini and Filloux, 2016). Wsp mutants emerge in vitro upon exposure of P. aeruginosa to sub-lethal concentrations of hydrogen peroxide (Chua et al., 2016), but also in vivo during chronic lung infection in CF patients and in burn wounds (Smith et al., 2006; Starkey et al., 2009; Marvig et al., 2015; Gloag et al., 2019).

The observation that P. aeruginosa, programmed for the sessile lifestyle, also displays antibacterial activity, prompted us to analyse the supernatants of a wspF mutant, using metabolomics analysis. We show that metabolite clusters comprising rhamnolipids, alkyl quinoline N-oxides and the siderophores pyochelin and pyoverdin are the most upregulated chemical species in the wspF supernatant compared to the ancestor PA14. We observed a coordinated antibacterial action of these compounds and were able to reconstitute an artificial cocktail showing a broad-spectrum antibacterial activity against Gram-positive and Gram-negative species. Hence, switching-on the Wsp signalling system during biofilm formation also protects against invading or established competitors.

Results

Metabolomic analysis reveals overproduction of rhamnolipids, siderophores and N-oxide quinolones in ΔwspF mutant supernatant

We have previously shown that a P. aeruginosa ΔwspF mutant supernatant presents killing activity against S. aureus (Tognon et al., 2017). To identify secreted bacterial compounds in an unbiased and global approach, we profiled supernatants of the PA14 reference strain and a ΔwspF mutant by Ultra-High Performance Liquid Chromatography high-resolution tandem mass spectrometry (UHPLC-HR/MSMS). The HR/MSMS datasets were organized as a molecular network (MN), which provides tandem fragmentation spectra according to their spectral similarity (Wang et al., 2016). The experimental fragmentation dataset in the MN was annotated by comparison with a database of simulated spectra of natural products obtained by in silico MS/MS fragmentation (Allard et al., 2016) and following a taxonomically informed metabolite annotation process (Rutz et al., 2019). In a MN, a cluster of nodes is generally indicative of a family of structurally related molecules. We further integrated in the MN the relative intensities of all detected metabolites in the supernatants. We reasoned that compounds with potential killing activity were more abundant in supernatants of a ΔwspF mutant (red colour in nodes) than in those of the PA14 wild type strain (blue colour in nodes). Based on this assumption, we identified four distinct clusters belonging to alkyl quinoline N-oxides (AQNOs), rhamnolipids, pyoverdine and pyochelin families (Figs 1 and S1). The AQNO cluster was composed of several compounds showing variable distributions between the ΔwspF mutant and the PA14 wild type supernatants (Fig. 1A). Indeed, P. aeruginosa synthesizes more than 50 different alkyl-quinolones, sharing a common biosynthetic pathway with the Pseudomonas quinolone signal (PQS) (Lepine et al., 2004; Drees et al., 2018). Analysis of the AQNO sub-group identified four compounds: 2-heptyl-4-quinoline N-oxide (HQNO), 2-nonyl-4-quinoline N-oxide (NQNO), 2-decanoyl-4-quinoline N-oxide (DQNO) and 2-undecanoyl-4-quinoline N-oxide (UQNO). The metabolomic analysis detected similar amounts of HQNO and UQNO, but increased amounts of NQNO and DQNO in the ΔwspF mutant supernatants compared to those of PA14 (Fig. S2A), suggesting that these latter compounds might contribute to S. aureus killing.

A detailed analysis of the rhamnolipid cluster (cluster B in Fig. 1), almost exclusively detected in the ΔwspF mutant supernatants, revealed mainly the presence of C_{10} and C_{12} mono-rhamnolipids as well as C_{10}-C_{12} and C_{12}-C_{12} di-rhamnolipids, both being produced by the majority of P. aeruginosa isolates (Abdel-Mawgoud et al., 2010). In agreement with the metabolomic data, the orcinol assay detected 11-fold higher amounts of rhamnolipids in a ΔwspF mutant supernatant compared to those of PA14 (Fig. S2B).

Pyoverdin and pyochelin, the main siderophores produced by P. aeruginosa, were members of two clusters, which were mainly detected in the ΔwspF mutant supernatants (Fig. 1, clusters C and D). Spectrophotometric quantification of the two siderophores confirmed the metabolomics data, showing a 30-fold increase in pyoverdin (Fig. S2C) and a twofold increase in pyochelin concentrations in ΔwspF mutant supernatants compared to those of PA14 (Fig. S2D).

Altogether, the metabolomics analysis detected members of three important classes of P. aeruginosa virulence factors, namely rhamnolipids, siderophores and two AQNOs, which were present in higher amounts in a ΔwspF supernatant compared to those of PA14.

Metabolites overexpressed in the ΔwspF mutant all contribute to S. aureus killing activity

To determine whether specific compounds identified by the metabolomics analyses were essential for the S. aureus
killing activity, we created deletion mutants in their corresponding biosynthetic pathways in a ΔwspF strain background (Table S1). Deletion of the pchAD operon, abolishing pyochelin synthesis, reduced the killing activity of the ΔwspF supernatant 10-fold. Simultaneous inactivation of the pvdL gene, involved in pyoverdin synthesis, further reduced the killing activity by three orders of magnitude. Specific deletion of the rhlA gene, abolishing synthesis of all rhamnolipid species, decreased the killing activity by 3-logs, whereas deletion of the pqsL gene, encoding the N-oxide synthase responsible for AQNOs synthesis, reduced the S. aureus killing by 4-logs (Fig. 2A). We further measured HCN production, another secondary metabolite showing antimicrobial activity. HCN production

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Fig 2. *S. aureus* killing activity of ΔwspF mutant is not dependent on c-di-GMP but involves QS-regulated metabolites.

A. Killing assay with supernatants of mutants deficient in production of pyochelin (ΔwspFΔpchAD), pyochelin and pyoverdin (ΔwspFΔpchADΔpvdL), rhamnolipids (ΔwspFΔrhlA) and N-oxide quinolines (ΔwspFΔpqsL) were performed on *S. aureus* cell suspensions and scored after 24 h incubation with *P. aeruginosa* culture supernatants. Values are the average and standard deviations of three independent experiments (two sided student t-test; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001).

B. Secondary metabolites from the indicated strains were quantified by metabolomic analysis and represented in the heat map compared to the values of the ΔwspF mutant set to 100% (white).

C. Biofilm formation was determined after 12 h incubation in M14 medium at 37°C by crystal violet staining. The c-di-GMP degrading phosphodiesterase gene PA14_36990 was constitutively expressed from plasmid pPDE.

D. *S. aureus* killing activity of culture supernatants was determined by plate counts after 24 h incubation. Values are the average and standard deviations of three independent experiments (two sided student t-test, \*\*\*\*p < 0.001). [Color figure can be viewed at wileyonlinelibrary.com]
(Castric and Castric, 1983) was detectable in the ΔwspF mutant, but not in the PA14 wild type and the ΔwspΔhcn mutant (Fig. S3A). The Δhcn mutant generated in the ΔwspF background showed a 10-fold increase in S. aureus viable counts, suggesting a moderate contribution of HCN in S. aureus killing (Fig. S3B).

To assess the impact of the generated deletions in the ΔwspF mutant on the metabolome, we submitted their supernatants to metabolomics analysis. As expected, pyochelin and/or pyoverdin were undetectable in the ΔwspFΔpchAD and ΔwspFΔpchADΔpvdL mutants. Similarly, rhamnolipids and the four AQNOs were absent in supernatants of the ΔwspFΔrhlA and ΔwspFΔpqsL mutants respectively (Fig. 2B). Pyocyanin levels were unaffected in the biosynthesis mutants and were comparable to those of the wild type and the ΔwspF mutant (Fig. S2E). Surprisingly, we observed two to threefold decreased amounts of the four AQNOs and the quorum-sensing (QS) signalling molecules 3-oxo-C12-HSL and PQS in the ΔwspFΔrhlA mutant compared to the parental ΔwspF mutant. A possible explanation is that rhamnolipids promote the incorporation of these hydrophobic molecules into outer membrane vesicles (Mashburn-Warren et al., 2008). Hence, in the absence of rhamnolipids, AQNOs and 3-oxo-C12-HSL may remain attached to the bacterial membrane thereby decreasing their concentrations in the supernatants. Altogether, our combined genetic and biochemical analyses demonstrate that the four compound clusters identified by metabolomics to be overexpressed in the ΔwspF mutant, all contribute to S. aureus killing.

Expression of secreted killing factors is QS-dependent and not regulated by c-di-GMP

A hallmark of the ΔwspF mutant is the constitutive activation of the WspR cyclase through phosphorelay systems, increasing c-di-GMP levels responsible for increased biofilm formation and the characteristic wrinkly colony morphology (Hickman et al., 2005). We wondered whether increased levels of c-di-GMP would also trigger S. aureus killing activity in the ΔwspF mutant. We thus cloned the gene of the phosphodiesterase (PDE) PA14_36990, which hydrolyzes c-di-GMP (Kulasakara et al., 2006; Hickman and Harwood, 2008) and introduced the resulting plasmid pPDE in PA14 and in the ΔwspF mutant. As expected pPDE expression decreased biofilm formation of the ΔwspF mutant by 50% (Fig. 2C). In contrast, pPDE expression did not affect the S. aureus killing activity of a ΔwspF mutant (Fig. 2D), nor did it affect siderophore production (data not shown). Hence, c-di-GMP levels do not seem to play a role in the killing activity of the ΔwspF mutant.

Because rhamnolipid and AQNO synthesis is QS-controlled, we hypothesized an upregulation or advanced expression of the P. aeruginosa QS system(s) in the ΔwspF mutant. To determine the onset of the killing activity in the supernatants, we collected supernatant samples during static growth in microtitre plates. The S. aureus killing activity occurred after 6 h growth (mid-log phase) in the ΔwspF mutant supernatants, and increased until stationary phase was reached (data not shown). No killing activity was observed in the PA14 supernatants under these conditions, although PA14 reached similar cell densities. Surprisingly, we observed decreased amounts of the las-system QS molecule 3-oxo-C12-HSL (Fig. 2B and S2F) and of the PQS signalling molecule (Figs 2B and S2H) in the ΔwspF mutant compared to the wild type. In contrast, the metabolomics analysis showed a two-fold increase in signal intensity for the rhl-QS-system signalling molecule C4-HSL in the ΔwspF mutant supernatant compared to PA14 (Fig. 2B), which was corroborated by a three-fold higher expression of the rhl gene, encoding the C4-HSL synthase (Fig. S2G). We therefore suspect that increased or advanced expression of the C4-HSL dependent rhl-QS system might explain the increase in rhamnolipid production in the ΔwspF mutant.

Secreted factors display distinct killing phases

To determine whether the compounds overproduced by the ΔwspF mutant act simultaneously or sequentially, we exposed S. aureus cells to culture supernatants of P. aeruginosa mutants constructed in the ΔwspF background and followed S. aureus survival during 24 h (Fig. 3A). As our previous data showed overproduction of several secreted proteins in the ΔwspF mutant (Tognon et al., 2017), including the staphylococcal protease LasA (Kessler et al., 1997), we also tested a ΔwspFΔlasA mutant. This allowed us to distinguish three killing phases. During the initial phase (0–2 h), S. aureus viable counts declined rapidly in all supernatants with the exception of the one from the ΔwspFΔlasA mutant (Fig. 3A, green rectangle and zoomed blue box). During the second phase (2–6 h), S. aureus CFUs decreased for all supernatants by 1 to 2-logs (Fig. 3A, blue rectangle). During the third phase (6–24 h), a further 1 to 2-log decrease in CFUs occurred except for supernatants from PA14 and the ΔwspFΔpqsL and ΔwspFΔpchADΔpvdL mutants (Fig. 3A, yellow rectangle). We conclude that the first killing phase (0–2 h) is mainly dependent on the LasA protease. The second phase (2–6 h) is initially dominated (2–4 h) by the action of rhamnolipid (CFU remain stable in the ΔwspFΔrhlA mutant), whereas the third phase (6–24 h) involves AQNOs, as well as both pyochelin and pyoverdin. This complex scheme suggests an initial lysis of a subpopulation of S. aureus cells, followed by a combined and probably synergistic action among rhamnolipids, AQNOs and siderophores.
To verify that the initial drop in CFU resulted indeed from cell lysis, we examined GFP-labelled *S. aureus* cells under the microscope. Indeed the Δ*wspF* mutant supernatant lysed *S. aureus* cells within 12 min, although no lysis was observed with PA14 and Δ*wspFΔlasA* mutant supernatants, confirming that initial lysis was due to the action of the LasA protease (Fig. 3B). Rhamnolipids were not involved in cell lysis because supernatants from the Δ*wspFΔrhlA* mutant still showed *S. aureus* lysis (Fig. 3B) and addition of commercial rhamnolipids to M14 medium at concentrations of up to 400 μg ml⁻¹ did not cause *S. aureus* cell lysis (data not shown). These data support a cell-permeabilizing role of rhamnolipids during later stages of the killing process.

We further investigated the antibacterial properties of the supernatants by measuring ROS generation. *S. aureus* cells exposed to the PA14 or Δ*wspF* supernatants showed identical hydroxyl radical production during the first 4 h. However, in the presence of PA14 supernatant, hydroxyl radicals reached a plateau level after 9 h, whereas in the presence of Δ*wspF* supernatant the hydroxyl radical production showed a steady increase.

**Fig 3.** Survival kinetics and microscopic analysis reveal three killing phases in Δ*wspF* supernatants. A. Killing activity of crude supernatants from PA14, Δ*wspF* mutant and derivatives was monitored during 24 h (left panel), a blow up is shown for the initial 2.5 h (right panel). The fast killing phase (light green background), resulting mainly from the LasA protease is followed by a second rhamnolipid dependent phase (blue background) and a third phase mainly dependent on HCN, pyochelin and HQNO (beige background). B. GFP-labelled *S. aureus* Newman cells were incubated for 12 min with filtered culture supernatants from the indicated strains and analysed under a fluorescent microscope for cell lysis. [Color figure can be viewed at wileyonlinelibrary.com]
reaching a four-fold higher level compared to PA14 supernatants after 24 h (Fig. S4). The Δ wspFΔ pvdL and Δ wspFΔ pchADΔ pvdL mutant supernatants deficient for pyoverdin and/or pyochelin production respectively showed a slower onset and reduced ROS production compared to those of the Δ wspF mutant at 24 h, suggesting the role of iron availability in the generation of hydroxyl radicals via the Fenton reaction. The signal for superoxide production was weak and did not show any significant differences between the supernatants (data not shown). The data suggest that the two siderophores pyoverdin and pyochelin, overproduced in the Δ wspF mutant, play a role in hydroxyl radical production and likely contribute to the later killing phases (Fig. 3A).

**Rhamnolipids increase staphylococcal cell permeability**

To assess the effect of rhamnolipids on *S. aureus* cell permeability, we used propidium iodide (PI) fluorescence. PI emits fluorescence when intercalating into DNA but cannot penetrate intact cell membranes. *S. aureus* strain Newman, exposed to medium or PA14 supernatant,
showed no change in PI-fluorescence during the incubation period (Fig. 4A, grey and black symbols). However, in the presence of the ΔwspF supernatant, PI-fluorescence increased rapidly during the first 60 min and reached a fourfold higher level than in the medium control (Fig. 4A, red dots), indicating fast cell permeabilization. At the end of the incubation, viable cell counts dropped by 5-logs under these conditions (Fig. 4B). Incubation with the rhamnolipid-deficient ΔwspFΔrhlA supernatant showed a linear increase in PI-fluorescence reaching twice the level of the medium control (Fig. 4A, blue dots). The ΔwspFΔrhlA supernatant showed only a 2-log decrease in viable S. aureus cell counts (Fig. 4B). S. aureus possesses a lysozyme-resistant cell wall due to O-acetylation of its peptidoglycan; hence, addition of lysozyme to the supernatants had no additional effect on PI-fluorescence or cell viability.

To assess whether the permeabilizing activity was specific to S. aureus, we repeated the experiment with S. carnosus, which has a lysozyme susceptible peptidoglycan. Although medium or PA14 supernatant alone showed no increase in PI-fluorescence (Fig. 4C, grey and black dots), addition of a sub-lethal amount of lysozyme (100 μg ml⁻¹) increased PI-fluorescence after 6–7 h of incubation (Fig. 4C, grey and black triangles), reaching a plateau level after 15 h. In the presence of the ΔwspF supernatant, PI-fluorescence increased steadily (Fig. 4C, red dots) but viable counts decreased only 10-fold compared to the medium or PA14 treatments (Fig. 4D). Interestingly, PI-fluorescence showed a rapid exponential increase when we added lysozyme to the ΔwspF supernatant (Fig. 4C, red triangles), which resulted in a drastic 5-log reduction of S. carnosus CFUs (Fig. 4D). As observed for S. aureus, no PI-fluorescence increase occurred with the ΔwspFΔrhlA mutant supernatant (Fig. 4C, blue dots), supporting the permeabilizing action of rhamnolipids also in S. carnosus. Finally, addition of lysozyme to the ΔwspFΔrhlA mutant increased PI-fluorescence (Fig. 4C, blue triangles), resulting in a concomitant 10-fold decrease in S. carnosus CFUs (Fig. 4D). Hence, rapid permeabilization by rhamnolipids, in the absence (S. aureus) or the presence (S. carnosus) of lysozyme, is crucial to cause irreversible cell damage and killing by the factors present in the ΔwspF supernatants.

**Formulation of a lethal synthetic cocktail from P. aeruginosa metabolites**

We next tested whether the compounds identified by the metabolomic analysis would be sufficient to cause bacterial killing, when reconstituted in M14 medium at concentrations close to those identified in the ΔwspF supernatant (250 μg ml⁻¹ rhamnolipids, 4 μM HQNO, 10 μM pyochelin and 6 μM pyoverdin). Added individually to the medium at

![Fig 5. Formulation of the antibacterial cocktail and its broad-spectrum activity.](https://www.wileyonlinelibrary.com)
these concentrations only rhamnolipids showed at most a 50-fold reduction in S. aureus survival (Figs 5A and S5A). HQNO and pyocelin added either alone or in combination did not affect significantly S. aureus survival (Figs 5A and S5B and C). However, addition of rhamnolipids to HQNO and pyocelin increased S. aureus killing by 3-logs, suggesting a synergistic action of these three compounds. Poyoverdin further decreased S. aureus CFUs by 1-log. Lysozyme alone at 100 μg ml⁻¹ had no effect on S. aureus killing (Fig. 4B), whereas it further reduced CFU counts below our detection limit in the presence of the cocktail (Fig. 5A). Hence, the four metabolites identified by metabolomics all contribute to S. aureus killing and the cocktail showed a similar bactericidal activity as the one of the ΔwspF supernatant (Fig. 5A). To assess the specificity of the iron chelators, we substituted pyoverdin by the siderophores enterobactin from E. coli and protochelin from Azotobacter vinelandii or by the synthetic iron chelator 2,2’-dipyridyl. All of them enhanced the killing activity to a similar level as pyoverdin (Fig. S5D), suggesting that the Fe(III) chelating function was essential for the killing activity.

We used this cocktail, containing 2,2’-dipyridyl and lysozyme, to test its activity on other Gram-positive and Gram-negative bacteria. We found that the cocktail showed similar or even better activity than the ΔwspF supernatant against six Gram-positive and six Gram-negative bacterial species, including several respiratory isolates (Rothia mucilaginosa, Neisseria flavescens, Streptococcus parasanguinis) (Fig. 5B) as well as Gram-negative non-fermenters. All bacterial species showed at least a 2 to 3-log reduction in viable counts, suggesting that the targets of the cocktail components are conserved among distantly related bacterial species. As expected, the cocktail did not affect viability of P. aeruginosa (Fig. 5B).

Discussion

Our unbiased metabolomic comparison between a ΔwspF and PA14 wild type supernatant allowed us to identify specific P. aeruginosa metabolites, which individually showed no antibiotic activity, but when combined in a synthetic cocktail, resulted in a broad-spectrum bactericidal activity. An essential component of this cocktail were rhamnolipids, produced by Pseudomonas and Burkholderia spp (Soberon-Chavez et al., 2005). Their main function seems to be the solubilization of hydrophobic compounds such as aliphatic C-sources acquired from the environment (Noordman and Janssen, 2002) or the self-produced PQS-signalling molecule (Calfee et al., 2005). Rhamnolipids show intrinsic antimicrobial activity against both Gram-positive and Gram-negative bacteria (Haba et al., 2003; Nitschke et al., 2010; Samadi et al., 2012), as well as amoeba (Cosson et al., 2002) and fungi (Goswami et al., 2015). These glycolipids also display surfactant activity (Abdel-Mawgoud et al., 2009), required for swarming motility (Köhler et al., 2000), disrupt tight junctions in epithelial cells (Zulianello et al., 2006) and lyse polymorphonuclear neutrophils (Jensen et al., 2007). The amounts produced by the ΔwspF mutant ranged between 300 and 400 μg ml⁻¹, a concentration reported to have bacteriostatic activity against S. aureus, S. epidermidis and B. subtilis (Haba et al., 2003; Nitschke et al., 2010; Samadi et al., 2012). However, incubation with a commercial mix of rhamnolipids at concentrations of up to 500 μg ml⁻¹ caused only a modest 50-fold reduction in S. aureus and S. carnosus viable counts, which did not result from cell lysis as we confirmed by microscopic analysis. These data support the notion that at the concentrations detected in the ΔwspF supernatant, rhamnolipids alone are not sufficient to explain a 5 to 6-log decrease in S. aureus viable cell counts. We therefore conclude that rhamnolipids act mainly as a permeabilizing agent (Radlinski et al., 2017). Rhamnolipids can form micelles, which might incorporate cargo molecules, like the hydrophobic AQQNs and pyocelin (Fig. 6). Along this line, the QS signals PQS and 3-oxo-C12-HSL were shown to be integrated into outer membrane vesicles of P. aeruginosa, thereby promoting their dissemination within a bacterial community (Mashburn-Warren et al., 2008). Membrane vesicles of P. aeruginosa were reported to fuse to the membranes of other Gram-negative bacteria and even to S. aureus membranes (Kadurugamuwa and Beveridge, 1996). Whether this is the case for rhamnolipid micelles remains to be determined. The critical micellar concentration for rhamnolipids is approximately 100 μg ml⁻¹ (Klosowska-Chomiczewska et al., 2017), a concentration below the 300–400 μg ml⁻¹ measured in ΔwspF supernatants. Hence, the majority of rhamnolipid molecules in the ΔwspF supernatant should be under the form of micelles. Indeed, preliminary experiments using Nile Red to stain rhamnolipid micelles are in agreement with this hypothesis (our own unpublished observations). In the case of S. carnosus, lysozyme could further enhance the access of outer membrane vesicles or rhamnolipid micelles to the cytoplasmic membrane.

AQQNs represent another family of specialized metabolites reported to have anti-staphylococcal activity. In particular, HQNO and NQQO (Lightbow and Jackson, 1954; Machan et al., 1992; Szamosvari and Bottcher, 2017) inhibit cytochrome oxidases of the electron transport chain (CydAB in S. aureus) by blocking the access to respiratory quinones (menadione) (Voggu et al., 2006). HQNO also selects small colony variants in S. aureus, which carry mutations in heme or menadione synthesis pathways (Hoffman et al., 2006). AQQNs are produced in vitro by CF-isolates in the range of 1–10 μM (Nguyen et al., 2016), © 2020 The Authors. Environmental Microbiology published by Society for Applied Microbiology and John Wiley & Sons Ltd., Environmental Microbiology, 22, 3572–3587
but were also detected in lung biopsies from CF-patients (Garg et al., 2017). Indeed, the ΔwspF ΔpqsL mutant deficient in AQNO production, as shown by the metabolome analysis, showed strongly diminished killing activity. PqsL is responsible for the hydroxylation of the AQS to generate AQNOs (Drees et al., 2018). Addition of commercial HQNO alone had only a weak antimicrobial effect on S. aureus, suggesting that HQNO requires other components present in the ΔwspF supernatant. We cannot exclude that AQNOs with longer acyl chains (NQNO, DQNO, UQNO) or harbouring an unsaturated acyl chain (Szamosvari and Bottcher, 2017) detected in our extended metabolomics analysis, also participate in the killing activity. Interestingly, the activity of HQNO seems to increase under iron-deficient conditions (Nguyen et al., 2016), which pinpoints the potential role of siderophores in our cocktail. Surprisingly, we could demonstrate killing of S. camosus, which in contrast to S. aureus expresses a cyanide and HQNO insensitive CydB enzyme and has a lysozyme-susceptible peptidoglycan (Bera et al., 2006; Voggu et al., 2006). This would incriminate other compounds present in the ΔwspF supernatant and acting on a different S. camosus target. Alternatively, CydB might be susceptible to long-chain AQNOs, which do not gain access to its target in the absence of lysozyme treatment.

Besides the QS-controlled factors rhamnolipids and AQNOs, the ΔwspF mutant also overproduced the main siderophores pyochelin and pyoverdin. Pyochelin has been reported to have bacteriostatic effects on S. aureus and other bacterial species and was suggested to generate reactive oxygen species (Adler et al., 2012; Ong et al., 2017) in the presence of pyocyanin (Coffman et al., 1990; Britigan et al., 1992). We could show here that pyochelin participates in S. aureus killing because the pyochelin-deficient pchAD mutant showed decreased killing activity and addition of pyochelin at 200 μM resulted in a 2-log reduction in S. aureus viable counts, both in medium and in PA14 supernatant. In E. coli,
addition of catechol siderophores (enterobactin) was shown to abrogate ROS induced damage generated by pyochelin (Adler et al., 2012). However, in our study, we observed the opposite effect since (i) addition of pyoverdin (a cyclic peptide harbouring a dihydroxy quinoline chelating group) to the pyochelin containing cocktail increased S. aureus killing activity by 3-logs and (ii) deletion of pyoverdin synthesis genes in the pyochelin deficient ΔwspFΔpchAD strain decreased killing activity of the corresponding supernatants by 3-logs. We hypothesize that pyoverdin, which has a higher affinity for iron than pyochelin, chelates Fe(III) outside the cell generating apo-pyochelin (Fig. 6). In Gram-negative bacteria, TonB-dependent receptor proteins actively transport side-chains of hydroxyl radicals (Fig. 6). In Gram-negative bacteria, TonB-dependent receptor proteins actively transport side-chains of hydroxyl radicals (Fig. 6).

In summary, our data show that P. aeruginosa produces a set of metabolites, including respiratory chain inhibitors (AQNOs, HCN), siderophores (pyochelin, pyoverdin) involved in ROS production, and cell permeabilizers (rhamnolipids, LasA protease), which when combined synthetically, result in an efficient broad-spectrum bactericidal cocktail. Hence, P. aeruginosa uses a probably unique combination of effector and membrane permeabilizing compounds, targeting a broad spectrum of Gram-positive and Gram-negative bacteria. Secretion of this lethal cocktail is likely beneficial to P. aeruginosa when establishing or defending niches in the environment or in the host.

Experimental procedures

Bacterial strains, growth conditions and supernatant preparation

Bacterial strains, plasmids and primers used in this study are listed in Table S1. M14 medium was adapted from the literature (Rudin et al., 1974) and is based on M9 salts (Na2HPO₄ 6 g L⁻¹; KH₂PO₄ 3 g L⁻¹; NaCl 0.5 g L⁻¹; NH₄Cl 1 g L⁻¹) supplemented with casamino acids (BD™) 10 g L⁻¹, magnesium sulfate (MgSO₄·7H₂O) 1 mM, thiamine (vitamin B1) 2 mg L⁻¹, niacin (vitamin B3) 2 mg L⁻¹, calcium pantothenate (vitamin B5) 2 mg L⁻¹, biotin (vitamin B9) 0.1 mg L⁻¹ and glucose 2 g L⁻¹. Casamino acids, vitamins and glucose solutions were sterilized by filtration and stored separately at 4°C. M9 salts and magnesium sulfate were sterilized by autoclaving at 121°C for 15 min (Tognon et al., 2017). Supernatants of bacterial cultures were recovered after 24 h of static growth at 37°C in microtitre plates (TPP, Switzerland). The cultures were pooled and centrifuged at 8000 rpm for 5 min. Supernatants were sterilized by filtration (0.22 μm filters, Millipore, Switzerland) and stored at −20°C.

Mass spectrometry analysis

Metabolite profiling was performed on an Acquity UPLC system (Waters, Milford, MA) interfaced to a high-resolution Q-Exactive Focus mass spectrometer (Thermo Scientific, Bremen, Germany), using a heated electrospray ionization (HESI-II) source. The LC conditions were as follows: column: Waters BEH C18 100 × 2.1 mm, 1.7 μm; mobile phase: (A) water with 0.1% formic acid; (B) acetonitrile with 0.1% formic acid; flow rate: 500 μl min⁻¹; injection volume: 1 μl; gradient: isocratic at 2% B for 0.2 min followed by a linear gradient of 2–100% B over 11 min and isocratic at 100% B for 3 min, return to initial condition in 0.2 min and equilibration step for 2.4 min. In positive ion mode, diisooctyl phthalate C₂₄H₄₀O₄ (M + H)⁺ ion (m/z 391.28429) was used as internal lock mass. The optimized HESI-II parameters were the following: source voltage: 3.5 kV (pos), sheath gas flow rate (N2): 48 units; auxiliary gas flow rate: 11 units; spare gas flow rate: 2.0; capillary temperature: 256.2°C (pos), S-Lens RF Level: 45. The mass analyser was calibrated using a mixture of caffeine, methionine-arginine-phenylalanine-alanine-acetate (MRFA), sodium dodecyl sulfate, sodium taurocholate and Ultramark 1621 in an acetonitrile/methanol/water solution containing 1% formic acid by direct injection. The data-dependent MS/MS events were performed on the three most intense ions detected in full scan MS (Top3 experiment). The MS/MS isolation window width was 1 Da, and the normalized collision energy (NCE) was set to 15, 30 and 45 units. In data-dependent MS/MS experiments, full scans were acquired at a resolution of 35,000 FWHM (at m/z 200) and MS/MS scans at 17500 FWHM both with an automatic maximum injection time. After being acquired in the MS/MS scans, parent ions were placed in a dynamic exclusion list for 2.0 s.

Metabolomics and mass spectrometry data treatment

PA14 and ΔwspF mutant supernatants were profiled by UHPLC coupled to a Q-Exactive Focus Mass-spectrometer with automated acquisition of MS/MS spectra. The MS data were converted from .RAW (Thermo) standard data format to .mzXML format using the MSConvert software, part of the ProteoWizard package (Chambers et al., 2012). The converted files were treated using the MZMine software suite v. 2.39 (Pluskal et al., 2010). The dereplication
Molecular networks generation

The molecular network was created using the online workflow at GNPS (http://gnps.ucsd.edu). A network was then created where edges were filtered to have a cosine score above 0.7 and more than six matched peaks. Further edges between two nodes were kept in the network if and only if each of the nodes appeared in each other’s respective top 10 most similar nodes. The spectra in the network were then searched against GNPS spectral libraries. All matches kept between network spectra and library spectra were required to have a score above 0.6 and at least six matched peaks. To separate isomers and consider semi-quantitative information, a Feature-Based Molecular Networking workflow was followed (Nothias et al., 2019). The molecular networking parameters and the complete results are accessible on the GNPS platform at the following address: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=a85e2cb7e0c84ab3a739e0b46e86ef73. The molecular network and the associated ISDB-DNP annotations are provided as a .cys file in Supplementary Material. This network file can be opened using Cytoscape (https://cytoscape.org/download.html).

Quantification of specialized metabolites

Rhamnolipid quantification was performed as described (Wittgens et al., 2011) with slight modifications. Samples containing 2 ml of filtered supernatant were treated with equal volumes of ethyl acetate. A standard curve was established by dissolving a mix of mono and di-rhamnolipids (R90-10G, Sigma Aldrich, Switzerland) in 2 ml ddH2O and subsequent extraction with equal volumes of ethyl acetate. After vortexing for 1 min, samples were centrifuged for 1 min at 5000 rpm to separate the phases. The organic solvent was evaporated in a SpeedVac (Thermo Scientific), and residues containing rhamnolipids were resuspended in 100 μl of ddH2O and mixed with an equal volume of 1.6% orcinol (Sigma Aldrich, Switzerland) in ddH2O. Of note, 80 μl of 60% sulphuric acid (vol/vol) was added, and samples were incubated for 45 min at 80°C. Then, 100 μl of the reaction mix were transferred to a microtitre plate, and OD420 was measured in a Synergy H1 Multi-Mode plate reader (BioTek®). For graphic representation, the background level of medium absorbance was subtracted.

Measurement of pyoverdin in supernatants was performed as described (Hoey, 2014). Of note, 100 μl of filtered supernatants were transferred to a microtitre plate, and fluorescence was measured in a Synergy H1 Multi-Mode plate reader (BioTek®) with excitation at 400 nm and emission at 460 nm. For graphic representations, fluorescence values were divided by OD600 of culture from which supernatants were obtained.

Measurement of relative levels of pyocyanin produced by P. aeruginosa was performed as described with some modifications (Hoey et al., 2014). Five millilitres of sterile supernatant was adjusted to pH = 3.0 by adding solid citric acid. Ten millilitres CH3Cl2 was added to the acidified supernatant, and pyocyanin was extracted by vortexing the 50 ml falcon tube for 1 min. The organic phase was collected and dried under a nitrogen flow overnight. The residue was resuspended in 1 ml 100 mM Tris–HCl buffer (pH 8) and used for pyocyanin detection by spectrophotometry or stored at −20°C. Of note, 200 μl of the suspension was transferred to a microtitre plate, and pyocyanin fluorescence (ex 355 nm/em 430 nm) was determined in duplicates. Relative levels of pyocyanin were expressed as RFU/OD600.

HCN production by growing P. aeruginosa cultures was performed as described (Castic and Castic, 1983), except that the filter paper was placed above a microtitre plate well during growth in M14 medium.

Pyocyanin in P. aeruginosa supernatants was quantified as previously described (Frank and Demoss, 1959; Köhler et al., 2014). Of note, 0.5 ml of CHCl3 was added to 0.75 ml of supernatant and vortexed for 30 s. After centrifugation (2 min at 13,000 rpm), the lower organic phase containing pyocyanin was transferred to a new tube. Then, 0.5 ml of 0.2 N HCl was added and tube was vortexed for 30 s. Two hundred microlitres were transferred to a microtitre plate. Relative levels of pyocyanin were expressed as ratio OD520/OD600.

Measurement of 3-oxo-C12-HSL was performed using E. coli JM109 strain, harbouring the bioluminescence reporter plasmid pSB1075 (plasmid: luxCDABE, lasR). Briefly, JM109 (pSB1075) (Winson et al., 1998) was grown overnight in LB medium supplemented with 50 μg ml−1 ampicillin. Overnight culture was diluted 1:10 in LB medium. Of note, 190 μl of diluted cell suspension was distributed in a microtitre plate, and 10 μl of supernatant was added. Bioluminescence was measured using the kinetic program in a BioTek Synergy H1 plate reader.

Measurement of PQS was performed using the PAO1 biosensor strain (Δpqsa mutant harbouring a chromosomal CTX-pqsa::luxCDAB fusion) (Fletcher et al., 2007).
Briefly, the PQS biosensor strain was grown overnight in LB medium. Next day the biosensor strain culture was adjusted to OD_{600} = 1.0 and diluted 1:50. Then, 100 μl of the test bacterial supernatant was distributed into a 96 well plate and mixed with 100 μl of the diluted biosensor strain. Bioluminescence was measured using the kinetic program in a BioTek plate reader and expressed as relative light units (RLU) per OD_{600}.

Killing assays

Killing assays were performed on S. aureus cells grown for 6 h in M14 medium under static growth conditions in microtitre plates (Tognon et al., 2017). After the 6 h incubation, 100 μl of S. aureus culture was removed and replaced with either 100 μl M14 medium or P. aeruginosa supernatant. Growth (OD_{600}) was monitored in a plate reader (BioTek®) for 24 h. Viable plate counts were performed to determine survival S. aureus cells. Other bacterial strains were grown on LB-plates or specific growth media. Cells were scraped form the plate, and a suspension was prepared and adjusted to obtain 10^8 CFU ml^{-1}. After 24 h incubation in the presence of culture supernatants, surviving cells were determined by plate counts.

Reactive oxygen species detection

Reactive oxygen species (ROS) were measured using the total ROS/Superoxide Detection Kit (Enzo Life Science, Farmingdale, NY) according to the manufacturer’s instructions with slight modifications. Briefly, ROS and oxidative stress of cell suspensions were detected by staining with the two fluorescent dyes from the ROS detection kit. S. aureus cells were grown for 6 h in M14 medium under static growth conditions in microtitre plates as described (Tognon et al., 2017). At that point, 100 μl of S. aureus culture was removed and replaced with either 100 μl M14 medium, P. aeruginosa supernatant. A 50 μl aliquot of M14 medium containing the two fluorescent dyes was added to each well (8.75 μM final conc.). Fluorescence of the fluorescein (ROS) (ex 488 nm/em 520 nm) and rhodamine-based (superoxide) dyes (ex 550 nm/em 610 nm) was monitored in a plate reader (Synergy 1, BioTek®) every 15 min for 24 h.

Cell permeabilization measurement

S. aureus and S. carnosus cells were grown for 6 h in M14 medium under static growth conditions in microtitre plates. At that point, 100 μl of bacterial culture was removed and replaced with either 100 μl M14 medium or crude, filtered P. aeruginosa supernatants, supplemented with PI at a final concentration of 1 μM. Where indicated, lysozyme (Serva, Germany) was added at a final concentration of 100 μg ml^{-1}. Fluorescence (ex 535 nm/em 617 nm) was monitored in a plate reader (BioTek®) every 20 min at 37°C. Kinetic curves represent background-deduced values (supernatant with PI but without bacterial cells) and are the mean of technical triplicates. At the end of the assay, viable plate counts were performed to determine bacterial survival.

Biofilm formation

Staining and quantification of biofilm was performed as described with some modifications (Coffey and Anderson, 2014). P. aeruginosa strains were grown in static condition in microtitre plates in M14 medium. After 20 h, planktonic cells were removed by inverting the 96-well plate. The plate was then rinsed two times with ddH_2O. Wells were stained with crystal violet (225 μl of a 0.1% solution) and incubated for 10 min. The plate was rinsed twice with ddH_2O and allowed to dry. For quantification, 150 μl 30% acetic acid was added to each well and incubated for 10 min to solubilize the biofilm. Of note, 100 μl of each sample was transferred to a new 96-well optically clear flat-bottom plate. Optical density of all samples was measured in a plate reader at 550 nm.

Construction of cyclic-guanylate-specific phosphodiesterase (PDE) expression plasmid

The coding region, including 62 bp upstream of the ATG initiation codon and 120 bp downstream of the STOP codon, was amplified with primers PA14_36990 BamHI-F and PA14_36990-BamHI-R by PCR from genomic DNA of P. aeruginosa PA14. The amplified 1050 bp fragment was digested with BamHI-HindIII restriction enzymes and cloned into the expression vector pIApX2 yielding plasmid pPDE. The Q5 high-fidelity DNA polymerase (NEB) was used for all amplifications. PCRs were performed in a Biometra PCR thermal cycler (Analytik Jena AG, Germany), using the following conditions: denaturation at 98°C for 2 min, followed by 27 cycles of 98°C for 20 s, 57°C for 30 s, 72°C for 2 min and a final extension at 72°C for 4 min. Plasmids were transferred into P. aeruginosa by electroporation and cells were spread on LB-agar supplemented with carbenicillin at 200 mg L^{-1}. All constructs were verified by Sanger sequencing.

Generation of P. aeruginosa knockout mutants

The generation of unmarked knockout mutants was based on the protocol described by Hoang et al. (Hoang et al., 1998). Briefly, DNA fragments of 500–700 bps flanking the gene of interest were PCR-amplified using primer pairs F1/R1 and F2/R2 respectively (Table S1). After amplification, the obtained fragments were gel-
purified and used in a PCR fusion amplification with primers F1 and R2. For deletion of the hcnABC operon, a streptomycin cassette was introduced between the two flanking DNA fragments using the BamHI restriction site. The resulting fusion products were gel-purified and further cloned into the suicide vector pEXG2 via HindIII/Sacl (hcnABC operon) or EcoRI/HindIII restriction sites (rhaI and wspF). The gene replacement vectors were mobilized into P. aeruginosa via bi-parental conjugation, and the generation of the unmarked mutants was carried out as previously described (Pletzer et al., 2014). The constructed gene knockout strains were verified by PCR amplification of the flanking region using the external primers followed by Sanger sequencing.

Quantitative real-time PCR

For RNA extraction, strains were grown for 24 h at 37°C in microtitre plates. Three wells were used to combine the RNA. RNA was extracted using the RNeasy kit (Qiagen, Germany) followed by a DNase treatment (RQ1 DNase, Promega). A 500 ng aliquot of RNA was reverse transcribed using the ImProm-II reverse transcriptase DNAse, Promega). A 500 ng aliquot of RNA was reverse transcribed using the ImProm-II reverse transcriptase (Promega). PCR reactions were performed using the SYBR Green Quantitite Kit (Qiagen, Germany) in duplicates. Primers are shown in Table S1. qPCRs were performed in a RotorGene 3000 (Corbett Research) using the 96-well microtiter plate. Three wells were combined to form a PCR reaction product, melt curves were run after the amplification reaction. The ppsL gene was used as a reference housekeeping gene (Dumas et al., 2006).

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 Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Figure S1. Supporting information.

Table S1. Strains, plasmids and primers used in this study.