ORIGINAL ARTICLE

Co-evolution with *Staphylococcus aureus* leads to lipopolysaccharide alterations in *Pseudomonas aeruginosa*

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Detrimental and beneficial interactions between co-colonizing bacteria may influence the course of infections. In cystic fibrosis (CF) airways, *Staphylococcus aureus* prevails in childhood, whereas *Pseudomonas aeruginosa* progressively predominates thereafter. While a range of interactions has been identified, it is unclear if these represent specific adaptations or correlated responses to other aspects of the environment. Here, we investigate how *P. aeruginosa* adapts to *S. aureus* by evolving *P. aeruginosa* in the presence and absence of *S. aureus*. *P. aeruginosa* populations that evolved for 150 generations were sequenced and compared to the ancestor strain. Mutations in the Wsp signaling system were identified in both treatments and likely occurred because of low oxygen availability. Despite showing increased killing activity, *wsp* mutants were less fit in the presence of *S. aureus*. In contrast, mutations in lipopolysaccharide (LPS) biosynthesis occurred exclusively in co-cultures with *S. aureus* and conferred a fitness gain in its presence. Moreover, they increased resistance towards beta-lactam antibiotics. Strikingly, both mutations in *wsp* and LPS genes are observed in clinical isolates from CF-patients. Our results suggest that *P. aeruginosa* LPS mutations are a direct consequence of *S. aureus* imposed selection *in vitro*.
The ISME Journal (2017) **11,** 2233–2243; doi:10.1038/ismej.2017.83; published online 26 May 2017

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen responsible for acute and chronic infections in immunocompromised hosts. In cystic fibrosis (CF) patients, *P. aeruginosa* establishes chronic respiratory infections leading to progressive decrease of lung function (Folkesson *et al.*, 2012), ultimately requiring lung transplantation. *P. aeruginosa* adapts to the CF-lung and is difficult to eradicate because of its ability to resist antimicrobial treatments. Part of the adaptation to the CF-environment involves the loss or downregulation of virulence factors including lipopolysaccharide (LPS), quorum sensing (QS) regulators and siderophore receptors as well as increased antibiotic resistance (Folkesson *et al.*, 2012). A key question to address is what is driving

these evolutionary changes. While the host environment and treatment strategies likely play a fundamental role (Folkesson *et al.*, 2012), interactions with conspecifics (Köhler *et al.*, 2009) and other co-infecting species may be critical (Mashburn *et al.*, 2005).

P. aeruginosa colonizes up to 80% of adult CF-patients, while the dominant bacterial species in children and young adults is *Staphylococcus aureus* with a prevalence of up to 75% (Cystic Fibrosis Foundation Patient Registry, 2015). Crucially, *P. aeruginosa* and *S. aureus* frequently co-inhabit the CF lungs, suggesting that some of the *P. aeruginosa* adaptations may be driven by interactions with *S. aureus*. Consistent with this view, several QS-controlled strategies are used by *P. aeruginosa* to outcompete *S. aureus* *in vitro*: (i) lysis of the *S. aureus* cell wall by the secreted LasA elastase (Mashburn *et al.*, 2005), (ii) inhibition of the *S. aureus* respiratory chain by 4-hydroxy-2-heptyl-quinoline-*N*-oxide (HQNO) (Hoffman *et al.*, 2006; Kim *et al.*, 2015), and (iii) dispersal of *S. aureus* biofilms by rhamnolipids (Abdel-Mawgoud *et al.*, 2010; Diaz De Rienzo *et al.*, 2016). However, these studies contrast with others that show beneficial

interactions between the two pathogens. For instance, *P. aeruginosa* protects *S. aureus* from *Dictyostelium discoideum* phagocytosis (Yang *et al.*, 2011), and increases the survival of *S. aureus* during antibiotic treatments (Baldan *et al.*, 2014; DeLeon *et al.*, 2014). In addition, clinical strains of *P. aeruginosa* isolated from the lungs of adult CF-patients are impaired in their ability to outcompete *S. aureus*, but rather interact in a commensal-like fashion (Michelsen *et al.*, 2014; Frydenlund Michelsen *et al.*, 2016). These data illustrate a complex relationship between *P. aeruginosa* and *S. aureus* and call into question whether these phenotypic interactions are the result of selection imposed by *S. aureus* or simply correlated responses to other selection pressures.

One way to identify the potential consequences of selection imposed by *S. aureus* on *P. aeruginosa* is to conduct controlled *in vitro* evolution experiments (Harrison *et al.*, 2008), and this is the approach we took in this study. Our *in vitro* set up was designed to mimic aspects of an *in vivo* situation, where a low-density *P. aeruginosa* population encounters a pre-established high-density *S. aureus* community. We evolved *P. aeruginosa* for over as estimated 150 generations in the presence or absence of *S. aureus*, with the *S. aureus* population renewed after each cycle. Surprisingly we found that *wsp* mutants, characterized by small, wrinkly colonies of *P. aeruginosa*, which showed increased killing activity towards *S. aureus* evolved in both treatments, demonstrating this interaction to be a correlated response to selection imposed by the abiotic conditions. However, LPS-deficient mutants evolved in the presence but not the absence of *S. aureus*, and conferred a fitness advantage. These mutants were also more resistant to beta-lactam antibiotics. Lipopolysaccharide mutants with both phenotypes are frequently observed in *P. aeruginosa* CF-isolates, suggesting that exposure to *S. aureus* might play a role in the *in vivo* adaptation of *P. aeruginosa* to the CF lung-environment.

Material and methods

M14 medium composition
An M9 salt (Na$_2$HPO$_4$ 6 g l^{-1}; KH$_2$PO$_4$ 3 g l^{-1}; NaCl 0.5 g l^{-1}; NH$_4$Cl 1 g l^{-1}) base (Rudin *et al.*, 1974) was modified to obtain similar growth rates for *S. aureus* and *P. aeruginosa* under static microtiter plate growth conditions. This medium, termed M14, was supplemented with 10 g l^{-1} casamino acids (BD, Franklin Lakes, NJ, USA), magnesium sulfate (MgSO$_4$) 1 mM, 2 mg l^{-1} thiamine (vitamin B1), 2 mg l^{-1} niacin (vitamin B3), 2 mg l^{-1} calcium pantothenate (vitamin B5), 0.1 mg l^{-1} biotin (vitamin B9) and 2 g l^{-1} glucose.

Strains, plasmids and culture conditions
*P. aeruginosa* PA14 and *S. aureus* Newman strains used in this study are listed in Supplementary Table S1. Plasmids and primers are listed in Supplementary Tables S2 and S3. *P. aeruginosa* and *S. aureus* were freshly grown each day on M14 agar plates overnight at 37 °C if not stated otherwise. Bacterial suspensions were prepared in 0.9% (w/v) NaCl from overnight cultures on M14 agar plates. All experiments were performed in microtiter plates using a multimode plate reader (Synergy H1 BioTek, Franklin Lakes, NJ, USA). Run and reading parameters were as follows: incubation at 37 °C and linear shaking for 1 min before reading the optical density at 600 nm and fluorescence (excitation wavelength: 485 nm, emission wavelength: 528 nm) every hour for 24 h. All experiments were performed in technical triplicates.

Evolution experiment
The evolution experiment was performed in 96-well microtiter plates using liquid M14 medium as illustrated in Figure 1. Based on *P. aeruginosa* growth in our experimental conditions (Supplementary Figure S1), we calculated the number of generations (*n*) by $n = (\log CFU\ t_{24} - \log CFU\ t_0)/\log 2$. Taking into account the linear growth pattern of *P. aeruginosa*, the initial bacterial concentration of 10^6 CFU ml^{-1} at t_0 and the final bacterial concentration of 10^9 CFU ml^{-1} at t_{24} we calculated $n = 9.96$ for one 24 h cycle. Evolution of *P. aeruginosa* in the presence or absence of *S. aureus* was achieved by performing 15 (24-h) cycles of incubation (15 serial transfers) within pre-incubated cultures of *S. aureus* or sterile M14 medium, respectively. On the first day, 5×10^5 CFU of *P. aeruginosa* were used to inoculate each well of the plate. Pre-established cultures of *S. aureus* were prepared each day from fresh overnight cultures using 10^7 CFU of *S. aureus*, and were incubated for 6 h at 37 °C without shaking before introduction of *P. aeruginosa*. To ensure a steady *P. aeruginosa* to *S. aureus* ratio between 1:1000 and 1:100, cultures were diluted before their transfer to the next incubation plate. In order to transfer a *P. aeruginosa* CFU range of 10^5–10^6 (bacterial concentration of 10^6–10^7 CFU ml^{-1}), 20 µl of the cultures were withdrawn and diluted 1000 × in 0.9% (w/v) NaCl. Ten microliters of the 1000 × -diluted cultures were then used to inoculate the next plate for a new 24-h round of incubation. *Staphylococcus aureus* cells could not be separated from *P. aeruginosa* cells during the transfer. Viable plate counts after each 24-h cycle allowed us to follow the survival of each species throughout the co-evolution assay (Supplementary Figure S2). Culture samples were stored at the end of each 24 h cycle at − 80 °C.

Whole genome sequencing and bioinformatics analysis
Bacterial gDNA was extracted from the whole evolved populations (triplicate samples) and sequenced on an Illumina HiSeq 2500 platform (Fasteris SA, Plan-les-Ouates, Switzerland) generating 100-bp paired end reads to obtain an average of
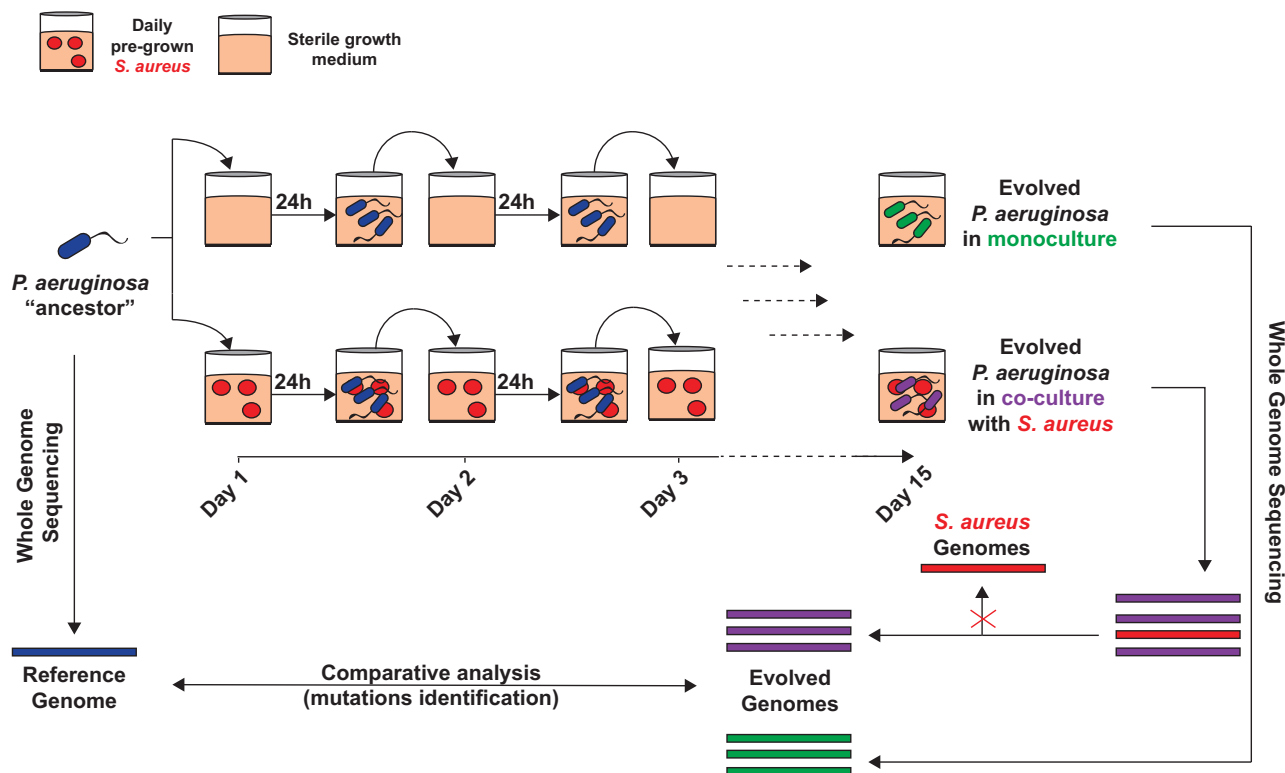
Figure 1 Evolution experiment setup. The ancestor strain of *P. aeruginosa* was evolved by performing 15 cycles of 24 h incubation each with and without the presence of pre-incubated *S. aureus* cultures. At the 15th day, the resulting evolved genomes of *P. aeruginosa* descendants were sequenced and compared to the ancestor for the identification of mutations.

9 187 682 reads (range of 70 667 874–10 707 490 reads). The software SGA (Simpson and Durbin, 2010) was used to filter the reads and to assemble genomes. Reads were processed as follows: the adapter sequence (5′-AGATCGGAAGAGC-3′) was removed at the end of the reads, and the end of the reads with a quality score below three were trimmed. Reads were discarded if the resulting length after trimming was below 49 bp. SGA was used to correct sequencing errors by searching for 27-mers with low frequency. Genomes were *de novo* assembled and specific 27-mers were mapped to the assembled contigs using SGA. The contigs were then mapped for each gene feature to the reference genome of *P. aeruginosa* PA14 RefSeq genome NC_008463.1 using Burrows-Wheeler Alignment Tool to create BAM files (Li and Durbin, 2010). The contigs were screened manually using IGV browser software (http://wwwbroadinstitute.org/igv) and blasted (NCBI Blast) to identify single nucleotide polymorphisms (SNPs), insertions/deletions (InDels) and rearrangements.

Killing assays
Killing assays were performed on *S. aureus* cells pre-grown for 6 h in M14 medium under static growth conditions in microtiter plates. 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. Supernatants were obtained after 24 h growth in M14 medium, centrifugation at 3,800 g for 5 min at 4 °C and subsequent filtration (0.22 µm Millipore filters). If required, *P. aeruginosa* supernatants were stored at − 20 °C. Growth (increase in OD_{600}) was monitored in a plate reader (Biotek Synergy H1) for 24 h. At the end of the killing assay, viable plate counts were performed to determine *S. aureus* survival.

Fitness assays
The fitness assays were performed using the protocol developed by Wiser and Lenski in a long-term evolution experiment (Wiser and Lenski, 2015). Isogenic mutants constructed from the ancestor strain of *P. aeruginosa* were used to assess their fitness in the presence and absence of *S. aureus*. The fitness assay was performed using 5×10^5 CFU of *P. aeruginosa* test strain and 5×10^5 CFU of *P. aeruginosa* ancestor mixed within either sterile M14 medium or with pre-incubated *S. aureus* cultures following the same protocol as for the evolution experiment. Viable plate counts were performed as described above. All assays were done in biological duplicates.

LPS analysis
Lipopolysaccharide preparation was performed as described previously (Hao *et al.*, 2015;

Wang *et al.*, 2015). Lipopolysaccharide samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by silver staining (Hao *et al.*, 2015; Wang *et al.*, 2015).

Minimal inhibitory concentration

To determine antibiotic susceptibility, minimal inhibitory concentrations were performed in triplicates by two-fold serial dilutions in Müller-Hinton medium (Becton Dickinson, Franklin Lakes, NJ, USA) according to Clinical and Laboratory Standard Institute (CLSI) guidelines.

Results

Whole genome sequencing of evolved isolates

At the end of the evolution experiment (approximately 150 generations), the ancestor strain and the triplicate samples from *P. aeruginosa* populations, evolved with and without *S. aureus,* were submitted to whole genome sequencing. We identified two mutated genetic loci at high frequencies in all three *P. aeruginosa* monoculture replicates. The first locus was *wspA*, coding for a methyl-accepting chemotaxis-like transducer, and the second was *lasR*, coding for a QS transcriptional regulator. The mutations were either deletions (replicate A) or SNPs (replicates B and C) (Table 1). The mutations in *wspA* were all located in the predicted

methyl-accepting domain of WspA (Figures 2a and b), whereas those in *lasR* were located in the C-terminal DNA-binding domain of LasR. While the *wspA* mutation was present in 100% of the evolved genomes and therefore likely fixed within the population, the *lasR* mutation was detected in only 30–40% of genomes at the end of the experiment.

Among the *P. aeruginosa* populations evolved in the presence of *S. aureus*, we identified five to six mutations in each of the triplicates (17 in all) at the end of the experiment. The diversity of targeted genes was higher than in the monoculture, but the majority fell within two functional categories: cell signaling (5/12) and cell membrane biosynthesis (7/12). As in the mono-cultures, mutations in the cell signaling category occurred in the Wsp signaling system, but targeted the *wspE* or the *wspF* gene (Figures 2a and b). Mutations in the cell membrane biosynthesis category occurred in *orfN* and PA14_23400, two uncharacterized ORFs encoded in an LPS biosynthesis gene cluster, as well as in *dnpA* encoding a putative de-*N*-acetylase (Table 1). The majority of mutations in *orfN*, PA14_23400 and *dnpA* were either deletions or insertions, expected to result in loss of function of the corresponding proteins. These mutations occurred exclusively in clones retrieved from the co-cultures; a strong argument for a specific adaptation to the presence of *S. aureus.* The mutations in *wspE* (SNP) and *wspF* (SNP/deletion) occurred in the corresponding

Table 1 Whole genome sequencing data for evolved *P. aeruginosa* populations

| Sample name	Gene name in PA14	Functional category	Mutation type	InDel (nts)	Mutation	Mutation[a] tolerated	Relative abundance in population (%)
M15A	*wspA*	Cell signaling	Del	Δ84	S285S	No	100
	lasR	Transcription	Del	Δ17	X17	—	40
M15B	*wspA*	Cell signaling	SNP	—	A438V	No	100
	lasR	Transcription	SNP	—	T222I	No	30
M15C	*wspA*	Cell signaling	SNP	—	A418V	No	100
	lasR	Transcription	SNP	—	C188R	No	40
C15A	*wspF*	Cell signaling	SNP	—	M307V	No	92
	orfN	LPS biosynthesis	In	+1	X99	—	45
	orfN	LPS biosynthesis	In	+2	X4	—	< 1
	dnpA	LPS biosynthesis	Del	Δ20	X2	—	4
	mexI	Defense mechanisms	SNP	—	G195E	No	< 1
C15B	PA14_65570	Unknown	Del	Δ9	L173A	No	59
	wspE	Cell signaling	SNP	—	A749V	No	33
	orfN	LPS biosynthesis	Del	Δ1	X7	—	52
	pilT	Cell signaling	Del	Δ1365	Gene deleted	No	50
	PA14_05160	Unknown	Del	Δ21	Δ7aa	—	50
C15C	*wspF*	Cell signaling	Del	Δ41	X129	—	30
	PA14_23400	Unknown	Del	Δ1	X22	—	41
	orfN	LPS biosynthesis	Del	Δ1	X7	—	16
	PA14_23400	Unknown	SNP	—	Q329K	No	24
	orfN	LPS biosynthesis	SNP	—	D249G	No	25
	wspF	Cell signaling	SNP	—	A160A	Yes	24
	dnpA	LPS biosynthesis	Del	Δ20	X2	—	2

Abbreviations: Δ, deletion of nucleotides; +, insertion of nucleotides; fs, frameshift; LPS, lipopolysaccharide; SNP, single nucleotide polymorphism; X, stop codon.
Co-culture samples from the 15th day are designated as C15, monoculture samples from the 15th day as M15.
[a]as predicted by the SIFT algorithm (http://sift.jcvi.org/).

protein domains involved in signal reception (WspE) and methylesterase activity (WspF). Phenotypes associated with these mutations will be described below. Additional mutations occurred in the *mexI* gene belonging to the *mexGHI-opmD* efflux operon (<1% in replicate A) and in the *pilT* gene involved
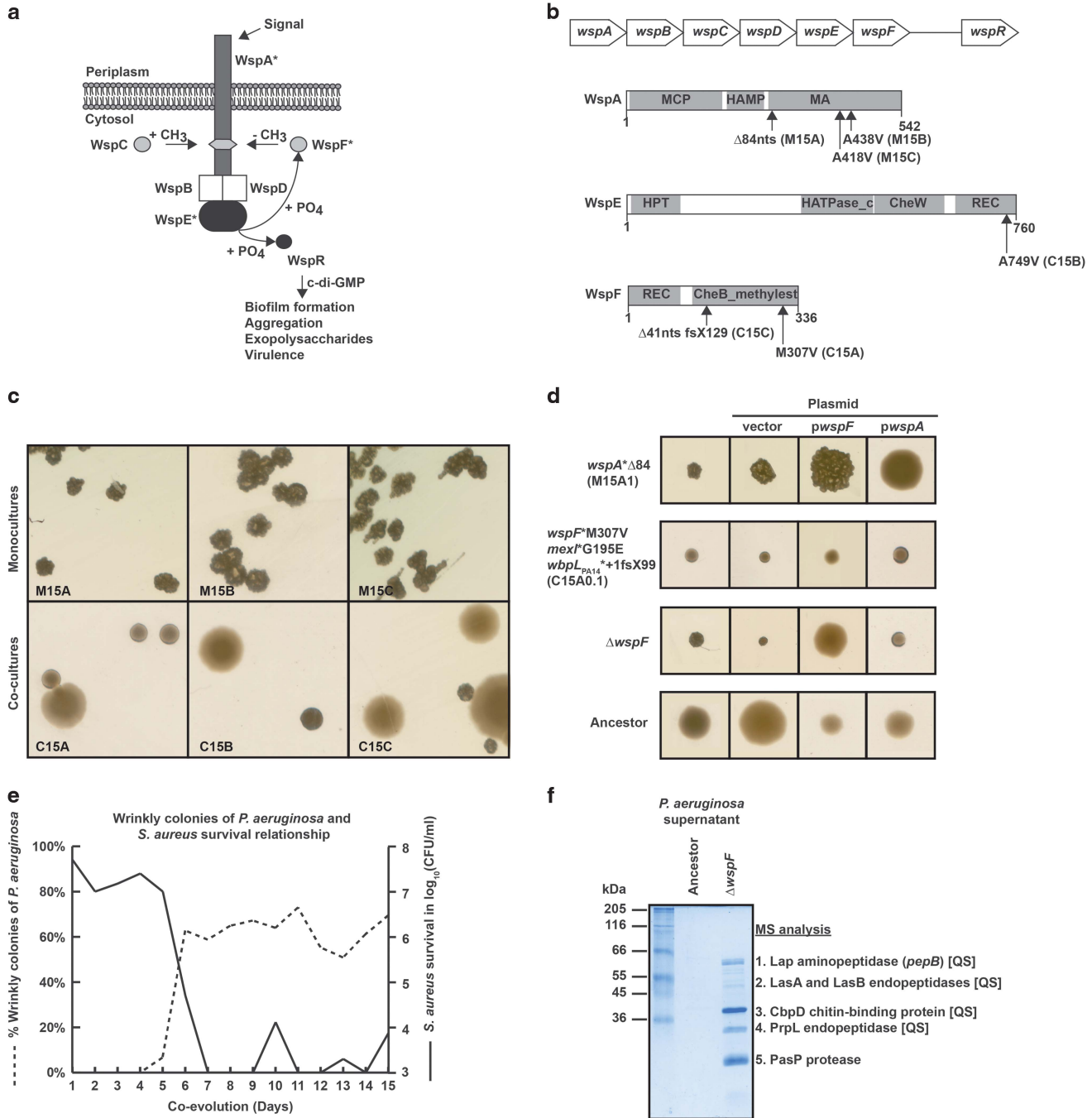
Figure 2 Scheme of the Wsp signaling system adapted from Hickman *et al.* (2005). Mutations identified in the evolution experiment are marked by asterisks (**a**). The *wsp* operon organization is shown at the top. Mutations identified are located in the methyl-accepting domain (MA) of WspA, in the receiver domain (REC) of WspE and the methylesterase domain (CheBmethylest) of WspF. Sample replicates are indicated in parenthesis, fs: frameshift, X: stop codon, Δ: deletion of nucleotides, +: insertion of nucleotides (**b**). Small wrinkly colonies (SWC) of *P. aeruginosa* from mono- (top-panels) and co-culture (lower panels) replicates at the end (15th day) of the evolution experiment (**c**). Mutations in the Wsp system were associated with wrinkly colony phenotype, which was restored by genetic complementation with the corresponding *wsp* gene (**d**). Relative abundance of small wrinkly colonies (SWC) of *P. aeruginosa* (dotted line) and survival of *S. aureus* during the evolution experiment (solid line), data shown represent the mean of three replicates (standard error (s.e.)<10%) (**e**). Twenty microliters of filtered culture supernatants collected from the ancestor and a Δ*wspF* mutant were run on an SDS-PAGE gel and stained with Coomassie blue. The five most prominent protein bands were excised and analyzed by mass spectrometry (MS). Four of the five proteases are known to be regulated by quorum sensing (QS) (**f**). SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

in pili synthesis regulation (50% in replicate B). We also noticed the absence of *lasR* mutations in *P. aeruginosa* isolated from the co-cultures, which might have been counter-selected by the presence of *S. aureus*. In contrast to the mono-cultures, the frequency of the identified mutations was highly variable in the populations (between 2 and 92%) and none of them reached fixation, which could reflect the dynamic interactions between *P. aeruginosa* and *S. aureus* in the co-culture.

Wsp-deficient mutants appear as small wrinkly colonies and their abundance is negatively correlated with S. aureus survival

When compared to the ancestor morphotype, smaller colonies with wrinkled appearance emerged in all replicates of both mono- and co-cultures (Figure 2c). This change in morphotype was linked to mutations in *wspA* for the mono-culture and to *wspE* and *wspF* for the co-cultures (Figures 2c and d). The wrinkled phenotype reached 100% at the end of the experiment in the mono-cultures (100% *wspA* mutants), while the proportion of small, wrinkled colonies was variable in the co-cultures (Figure 2c), and appeared to correspond to the observed variable proportions of *wspF* (30 and 92%) and *wspE* (33%) mutant genomes (Table 1). A similar wrinkled colony phenotype was reported for a *wspF* deletion mutant of *P. aeruginosa* strain PAO1 (Hickman *et al.*, 2005). To confirm that the mutations in the Wsp system were responsible for this phenotype, we cloned the wild-type PA14 *wspA* and *wspF* alleles on a plasmid (p*wspA* and p*wspF*) and expressed them in *wspA* and *wspF* mutants from the last cycle (Supplementary Materials). As expected, the wild-type morphology was restored in the *wspA* mutant in the presence of p*wspA,* but not p*wspF*. The *wspF* mutant, which also harbors mutations in *mexI* and *orfN*, showed a wrinkly phenotype after 72 h, and complementation with p*wspF* did not completely restore wild-type morphology, possibly due to the presence of the additional mutations (Figure 2d). We therefore constructed a deletion in the *wspF* gene in the ancestor strain. This Δ*wspF* mutant displayed the wrinkled phenotype and could be complemented by p*wspF* (Figure 2d). Thus the wrinkled phenotype is associated with mutations in the *wsp* genes, which appeared both in the mono- and co-cultures.

To follow the survival of *P. aeruginosa* and *S. aureus* during the co-evolution assay, we monitored viable counts throughout the 15 cycles of co-culture. We observed a significant decrease in *S. aureus* survival after 5 days of co-evolution. This decrease in viable *S. aureus* counts coincided with the appearance of *wsp* mutants and their relative abundance within the *P. aeruginosa* populations (Figure 2e). No effect on *P. aeruginosa* survival was observed at the same time point. We therefore hypothesized that the acquisition of *wsp* mutation increased the killing activity of *P. aeruginosa*

towards *S. aureus*. To assess whether this killing activity was caused by secreted factors, we collected supernatants of the ancestor and the constructed Δ*wspF* mutant and compared their protein contents by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figure 2f). The Δ*wspF* supernatants displayed a pattern of overexpression in several proteins that were identified by mass spectrometry as proteases (LasA, LasB, Lap, PrpL, PasP) and a chitin-binding protein CbpD (Figure 2f). Interestingly, the identified proteins, with the exception of PasP, are all QS regulated in *P. aeruginosa* (Arevalo-Ferro *et al.*, 2003; Marquart *et al.*, 2005).

Supernatants of wsp mutants display QS-dependent killing towards S. aureus and other Gram-positive and Gram-negative bacterial species

To verify that the *S. aureus* killing activity was present in the culture supernatants of the *P. aeruginosa wsp* mutants, we incubated stationary phase *S. aureus* cultures with filter-sterilized *P. aeruginosa* supernatants (50% vol/vol). Supernatants from *wspA*, *wspF* and *wspE* mutants were able to decrease *S. aureus* viability by at least six orders of magnitude after 24 h incubation (Figure 3a). The culture supernatant from the constructed Δ*wspF* mutant showed a similar level of killing activity, suggesting that the *wsp* mutations in the evolved *P. aeruginosa* isolates are responsible for this activity. There were no significant differences in *S. aureus* viability using a 0.9% (w/v) NaCl solution, sterile M14 medium, and sterilized spent M14 medium from 24-h *S. aureus* or *P. aeruginosa* ancestor cultures (Supplementary Figure S3). Since the LasA protease has been identified as a staphylolytic protease (Kessler *et al.*, 1993), we wondered whether increased LasA secretion by the *wsp* mutants could be responsible for the observed *S. aureus* killing. We therefore constructed a *lasA* deletion in the Δ*wspF* mutant. Supernatants of this Δ*wspF*Δ*lasA* double mutant still showed *S. aureus* killing activity (Figure 3a). However, when the QS-regulatory gene *lasR* was deleted in the Δ*wspF* strain, or altered by selection in the *wspA* mutant (*wspA**Δ84*lasR**Δ17fsX17), the level of killing activity was dramatically decreased (Figure 3a). Since the three QS-systems show intricate interdependencies and overlaps in their target genes (Jimenez *et al.*, 2012), we further investigated whether this killing activity was also affected by the Rhl and MvfR QS systems. We thus constructed deletions in *rhlR* and *mvfR* in the Δ*wspF* strain background to obtain the double mutants Δ*wspF*Δ*rhlR* and Δ*wspF*Δ*mvfR*. As shown in Figure 3a, deletion of *mvfR* strongly reduced the killing activity, similar to a *lasR* mutant, while the *rhlR* mutation completely abolished killing activity. This suggests that factor(s) controlled by either of these three QS-systems are involved in *S. aureus* killing. To assess whether the killing activity was
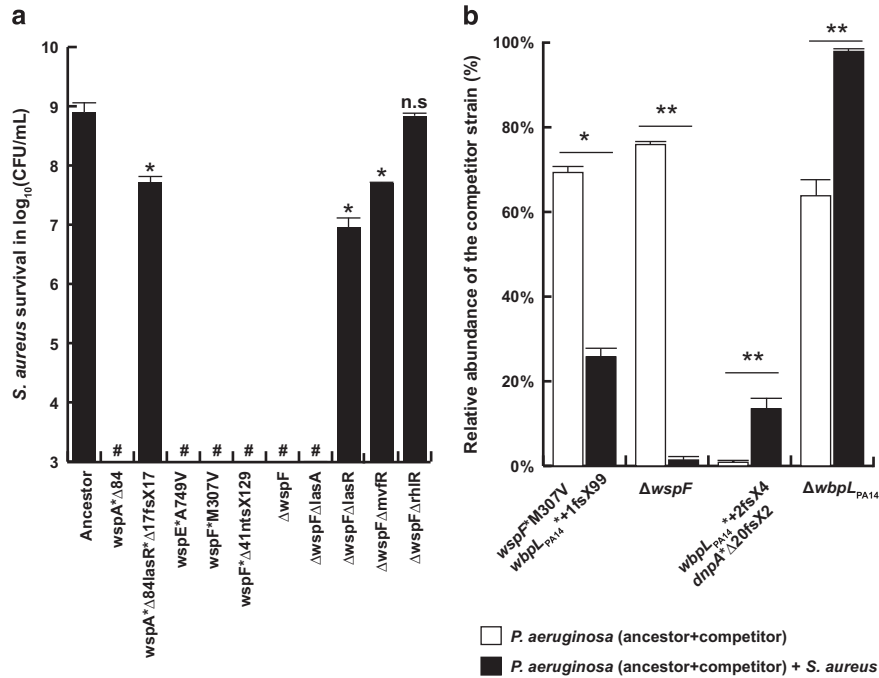
Figure 3 Killing activity of filter-sterilized *P. aeruginosa* supernatants on *S. aureus* cells. #: below detection level (10^3 CFU ml^{-1}) (**a**). Fitness assay comparing the ability of evolved and constructed *P. aeruginosa* mutants to invade the ancestor strain, starting from a 1:100 ratio, in the presence or absence of a pre-established *S. aureus* population (**b**). Statistical significance was determined using paired Student *t*-tests (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$; ns, not significant).

specific to the *S. aureus* Newman strain, we performed similar survival assays with different Gram-positive and Gram-negative bacterial species. A similar 6-log reduction in viable counts was observed with two different *S. aureus* strains (RN6390 (MSSA) and USA300 (MRSA)), whereas Δ*wspF* supernatant reduced viable counts of lung commensal strains *Actinomyces israelli* and *Neisseria flavescens* by 3–4 logs. In contrast, growth inhibition, but only weak reduction in viable counts (<50 fold) was observed with *Klebsiella pneumonia*, *Rothia mucilaginosa* and *Staphylococcus epidermidis* (Supplementary Figure S4).

Mutants in the Wsp system are adapted to the experimental growth conditions

Since mutations in the *wsp* system occurred in both mono- and co-cultures, we hypothesized that mutations in the Wsp system reflect an adaptation to our experimental set-up rather than to the presence of *S. aureus*. We therefore tested the fitness of *wspF* mutants, in the presence and absence of pre-established *S. aureus* populations, against the ancestor strain (Figure 3b). The proportion of *wspF* mutants increased from initially 1% to >70% in the absence of *S. aureus.* In contrast, the proportion increased only to 30% in the *wspF* M307V mutant and did not increase in the constructed Δ*wspF* knockout mutant in presence of *S. aureus* (Figure 3b and Supplementary Figure S5), suggesting that a *wspF* mutant is less fit in the presence than in the absence of *S. aureus*.

During the co-evolution assay, we observed a pellicle at the liquid surface of the *wspF* mutants. This is reminiscent of the wrinkly spreader phenotype of *P. fluorescens* SBW25, which appears as an adaptation to oxygen limitation by forming cellular mats at the air–liquid interface, when grown under static conditions (Spiers *et al.*, 2002, 2003; Goymer *et al.*, 2006; Bantinaki *et al.*, 2007). We wondered whether the *wsp* mutants resulted from a similar adaptation to our static microtiter plate assay conditions. We therefore repeated the co-evolution experiment over 5 days under both static and shaking growth conditions. Wrinkly colonies of *P. aeruginosa* appeared exclusively in static monocultures (data not shown). Two selected clones each harbored a different mutation in the methylesterase domain of WspF. Hence, we conclude that *wsp* mutants apparently occurred in response to oxygen limitation in our experimental set-up, and not as a selection imposed by *S. aureus*.

Mutations abolishing O-specific antigen LPS synthesis are selected in response to S. aureus

Mutations in *P. aeruginosa* that appeared specifically in the co-cultures were mainly classified within the cell wall/membrane synthesis category. Only the *orfN* locus was mutated in all three replicates, representing 45%, 52% and 41% of the genomes in replicates A, B and C, respectively (Table 1). Closer analysis of the OrfN amino acid sequence revealed that it shares 60% identity to one of the glycosyltransferase WbpL of PAO1 involved in the initiation

of the biosynthesis of the common polysaccharide antigen and the O-specific antigens (OSA) (Rocchetta *et al.*, 1998). Furthermore, *orfN* is located within the LPS biosynthesis gene cluster involved in O19 serotype synthesis of PA14. Based on these and the following data we will refer to *orfN* as *wbpL*~PA14~. To confirm the role of WbpL~PA14~, we compared the LPS profiles of the ancestor and a constructed Δ*wbpL*~PA14~ mutant by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining (Figure 4). As expected, Δ*wbpL*~PA14~ showed impaired production of OSA. However, this defect could be restored by complementation with the cloned wild-type allele from PA14. Thus, loss of WbpL~PA14~ prevents the biosynthesis of the OSA side chain; hence only the fast migrating core LPS band was observed in the Δ*wbpL*~PA14~ mutant (Figure 4).

We hypothesized that if *wbpL*~PA14~ inactivation, and hence loss of OSA LPS, is a specific response to the
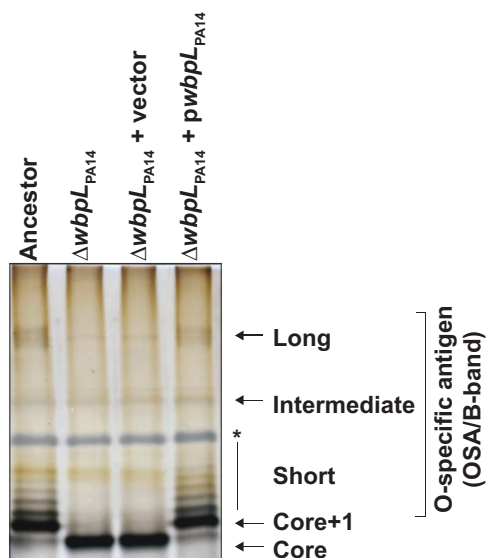
Figure 4 Lipopolysaccharide (LPS) profiles of *P. aeruginosa* ancestor strain and *wbpL*~PA14~ mutant. The O-specific antigen LPS synthesis is absent in the *wbpL*~PA14~ mutant. Asterisk indicates band corresponding to proteinase K.

presence of *S. aureus*, we would observe an increased fitness of *wbpL*~PA14~ mutants in the presence of the competitor. We therefore performed the same fitness assays as described above with the *wspF* mutant. Indeed, while the *wbpL*~PA14~-*dnpA* mutant remained at the initial 1% ratio to the ancestor in the absence of *S. aureus*, the ratio increased to 12% in the presence of *S. aureus* (Figure 3b). Interestingly, the *wbpL*~PA14~ mutations were detected between the third and fourth day of co-evolution (Supplementary Table S1), before the appearance of *wsp* mutations (Days 5–6). We therefore conclude that LPS loss is the main response of the adaptation of *P. aeruginosa* when co-cultured with *S. aureus* in our experimental setting.

The loss of *wbpL*~PA14~ increases the resistance of *P. aeruginosa* to beta-lactams

The loss of WbpL~PA14~ caused *P. aeruginosa* to produce a truncated version of LPS with only core oligosaccharide and devoid of OSA (Figure 4). We wondered whether this would affect the antibiotic susceptibility of the *wbpL*~PA14~ mutants. We performed antibiotic susceptibility tests by measuring minimal inhibitory concentrations on the ancestor, the evolved *wbpL*~PA14~*D249G single-mutant (C15C3) and a defined Δ*wbpL*~PA14~ mutant. Both the evolved and the constructed *wbpL*~PA14~ mutants showed increased antibiotic resistance to beta-lactam antibiotics, but not to ciprofloxacin or polymyxin (Table 2). The antibiotic-resistance phenotype could be reversed by complementation with the cloned wild-type allele of *wbpL*~PA14~. These results showed that adaptation to *S. aureus* has led to selection of *wbpL*~PA14~ mutants, which have improved fitness in the presence of *S. aureus* and displayed increased resistance to beta-lactam antibiotics.

Discussion

Evolution of *P. aeruginosa* in the presence and absence of *S. aureus* identified the Wsp signal transducing system (mono- and co-culture) and LPS

Table 2 Minimal inhibitory concentrations (µg ml^{-1}) for *P. aeruginosa* strains

Antibiotic	Strain						
	Ancestor	C15C3	C15C3 vector	C15C3 pwbpL	AncestorΔwbpL	AncestorΔwbpL vector	AncestorΔwbpL pwbpL
Aztreonam	4	16 (R)	8	4	8	8	4
Carbenicillin	32	128	NA	NA	128	NA	NA
Cefepime	2	8 (R)	4	2	4	4	2
Ceftazidime	1	4 (S)	2	1	2	4	1
Cefotaxime	16	64 (R)	32	16	32	32	16
Meropenem	0.125	0.125 (S)	0.125	0.125	0.5	0.5	0.125
Piperacillin-Tazobactam	2	8 (S)	8	2	8	8	2
Ciprofloxacin	0.0625	0.0625 (S)	0.0625	0.0625	0.0625	0.0625	0.0625
Polymyxin	2	2 (S)	2	4	—	2	4

Abbreviations: NA, not applicable; R, resistant; S, susceptible according to the EUCAST breakpoints (2014).

biosynthesis (co-culture) as the main targets. Parallel evolution in both mono- and co-culture samples suggests a strong selective pressure in our experimental setup. Mutations in the Wsp system resulted in a wrinkly colony morphology (Kirisits and Parsek, 2006; Haussler and Fuqua, 2013) and secretion of extracellular factor(s) able to kill *S. aureus* cells. The *wbpL* mutations that occurred only in the co-culture resulted in loss of OSA LPS-biosynthesis and conferred a fitness gain when co-cultured with *S. aureus.* These mutants were also more resistant towards beta-lactam antibiotics.

The Wsp system of *P. aeruginosa* transduces a signal through its inner membrane sensor WspA to WspE, which in turn phosphorylates both WspF, a negative regulator of the system, and the diguanylate cyclase WspR (Hickman *et al.*, 2005). This results in increased amounts of the second messenger cyclic-di-GMP, which regulates the expression of several virulence factors (Guvener and Harwood, 2007) (Figure 1a). The mutations identified in our study in the methyl-accepting domain of WspA, the receiving domain of WspE and the methylesterase domain of WspF are expected to cause auto-induction of the Wsp signaling cascade. These mutations prevent the resetting of the system to the default state mediated by methylation of WspF, and phenotypically translates into the formation of wrinkled colonies. These findings are consistent with studies in *Burkholderia cenocepacia*, where evolution in static monocultures led to the selection of *wsp* mutants (Cooper *et al.*, 2014; O'Rourke *et al.*, 2015). Interestingly, a A407V substitution in the WspA protein of *B. cenocepacia*, corresponding to our A418V mutation, resulted in Wsp auto-induction. In our study, we presume that *wsp* mutants were selected because of a fitness gain imposed by limiting oxygen availability under static growth conditions. In agreement with wrinkly spreaders in *Pseudomonas fluorescens*, we propose that the increased production of Pel polysaccharide in *wsp* mutants of *P. aeruginosa* (Hickman *et al.*, 2005) allows the attached cells to reach the air–liquid interface. Since our *wsp* mutants were non-motile the cell surface could not be reached by swimming motility (Supplementary Figure S6). The attached cells form a pellicle at the air–liquid interface where oxygen availability is optimal (Koza *et al.*, 2011). Unexpectedly, the *wsp* mutants, whether selected in the mono- or the co-culture, displayed an impressive killing activity (> 6-log reduction), which was not limited to *S. aureus*. The killing activity was LasR-dependent, but independent of LasA protease activity. Since both RhlR and MvfR (Pqs) mutations also abolished the killing activity it seems that factor(s) co-regulated by these systems are involved in the *S. aureus* killing. These could include other proteases, HCN, pyocyanin or rhamnolipids. Interestingly, *wsp* mutants that show the same or similar mutational loci have been identified in *P. aeruginosa* isolates from chronically infected CF-patients (Smith *et al.*, 2006; Marvig *et al.*, 2013, 2015) and were associated with a poor prognosis (Starkey *et al.*, 2009; Malone, 2015). Although selection of these mutations could happen for different reasons, similarities might exist between the growth conditions within the CF-lung and our experimental conditions.

Interestingly, *lasR* mutants emerged in the mono-cultures but not in the co-cultures. Under QS-requiring growth conditions, mutants in *lasR* emerge within a cooperator population. These are considered as cheaters, since they do not pay their share for the production of public goods. Since *lasR* is not required for growth in the M14 medium (data not shown), *lasR* mutations might have emerged simply to compensate the energy burden imposed by the *wsp* mutations, resulting in overexpression of QS-regulated extracellular products (proteases, exopolysaccharides). Why the *lasR* mutants did not emerge in the co-culture is unclear. Since *lasR* deficiency reduced the *S. aureus* killing activity of the *wspF* mutant, it would probably prevent *P. aeruginosa* from taking advantage of nutrients released by lysed *S. aureus* cells.

Lipopolysaccharide represents the main component of the outer cell membrane of Gram-negative bacteria and constitutes an important virulence factor. Most *P. aeruginosa* strains produce two chemically distinct forms of LPS, capped with common polysaccharide antigen and OSA, O-antigen respectively. In *P. aeruginosa* PAO1, the WbpL glycosyltransferase is involved in the synthesis of both the common polysaccharide antigen and OSA LPS (Murphy *et al.*, 2014). Interestingly, PA14 does not produce the common polysaccharide antigen (Hao *et al.*, 2015); thus, the loss of OSA leaves only uncapped LPS cores, a characteristic found in host-adapted clinical isolates of *P. aeruginosa* that chronically infect CF-patients (Folkesson *et al.*, 2012). Loss of OSA was associated with fitness gain in the presence of *S. aureus*. Furthermore, this particular feature could explain the commensal-like interactions between clinical isolates of *P. aeruginosa* and *S. aureus* (Michelsen *et al.*, 2014). However, it does not provide an explanation for the selection of these mutants. At physiological pH, bacterial cells of *P. aeruginosa* that express only the OSA LPS display a hydrophilic surface, whereas those expressing only the core LPS are more hydrophobic (Makin and Beveridge, 1996). This hydrophobicity of the bacterial cell surface could represent a defense mechanism to a substance produced by *S. aureus* targeting *P. aeruginosa* either directly, or indirectly by enhancing biofilm-formation or cell aggregation. Both the co-culture evolved strain (C15C3) harboring the *wbpL*$_{PA14}$*D249G mutation, and a $\Delta wbpL_{PA14}$ mutant showed increased resistance to beta-lactams, which could be restored by extrachromosomal complementation with the *wbpL*$_{PA14}$ wild-type allele. Loss of LPS has been previously associated with an increased

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resistance to antibiotics and more specifically to small charged molecules (Alvarez-Ortega *et al.*, 2010). Effects on the AmpC cephalosporinase expression or exopolysaccharide overexpression, occurring in the *wspF* mutant, could be possible alternative explanations. Taken together, LPS seems to play a role in the interactions between *P. aeruginosa* and *S. aureus*, although the precise nature of these interactions remains to be determined.

In conclusion, we showed that *wsp* mutants occurred in both mono- and co-cultures, and likely represent an adaptation to the oxygen-limiting conditions in our static *in vitro* evolution assay. Interestingly, all tested *wsp* mutations showed increased production of secreted proteins and probably other soluble factors, which have bactericidal activity on other Gram-negative and Gram-positive bacterial species. Thus, selection of *wsp* mutants does not represent adaptation to the *S. aureus* population. In contrast, mutations in the LPS biosynthesis pathway emerged exclusively within the co-cultures. Mutations in $wbpL_{PA14}$ were present in all replicates, causing the loss of OSA-LPS leading to increased resistance towards beta-lactams. Whether LPS loss is a defense mechanism against *S. aureus* or a stealth-mode strategy requires further investigations. Strikingly, both *wspF* and LPS-deficient mutants are found among CF-isolates, suggesting that interspecies adaptation drives evolution in the CF-lung, which can also contribute to antibiotic resistance. Our study emphasizes that evolved interactions between bacterial species can be the result of both direct selection imposed by interacting species and a correlated response to selection imposed by other aspects of the environment.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

References

Abdel-Mawgoud AM, Lepine F, Deziel E. (2010). Rhamnolipids: diversity of structures, microbial origins and roles. *Appl Microbiol Biotechnol* **86**: 1323–1336.

Alvarez-Ortega C, Wiegand I, Olivares J, Hancock RE, Martinez JL. (2010). Genetic determinants involved in the susceptibility of Pseudomonas aeruginosa to beta-lactam antibiotics. *Antimicrob agents chemother* **54**: 4159–4167.

Arevalo-Ferro C, Hentzer M, Reil G, Gorg A, Kjelleberg S, Givskov M *et al.* (2003). Identification of quorum-sensing regulated proteins in the opportunistic pathogen Pseudomonas aeruginosa by proteomics. *Environ Microbiol* **5**: 1350–1369.

Baldan R, Cigana C, Testa F, Bianconi I, De Simone M, Pellin D *et al.* (2014). Adaptation of *Pseudomonas aeruginosa* in cystic fibrosis airways influences virulence of *Staphylococcus aureus* in vitro and murine models of co-infection. *PLoS One* **9**: e89614.

Bantinaki E, Kassen R, Knight CG, Robinson Z, Spiers AJ, Rainey PB. (2007). Adaptive divergence in experimental populations of Pseudomonas fluorescens. III. Mutational origins of wrinkly spreader diversity. *Genetics* **176**: 441–453.

Cooper VS, Staples RK, Traverse CC, Ellis CN. (2014). Parallel evolution of small colony variants in Burkholderia cenocepacia biofilms. *Genomics* **104**: 447–452.

Cystic Fibrosis Foundation Patient Registry. (2015). Annual data report. Bethesda, MD, USA.

DeLeon S, Clinton A, Fowler H, Everett J, Horswill AR, Rumbaugh KP. (2014). Synergistic interactions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an in vitro wound model. *Infect Immun* **82**: 4718–4728.

Diaz De Rienzo MA, Stevenson PS, Marchant R, Banat IM. (2016). Effect of biosurfactants on *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms in a BioFlux channel. *Appl Microbiol Biotechnol* **100**: 5773–5779.

Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Hoiby N *et al.* (2012). Adaptation of Pseudomonas aeruginosa to the cystic fibrosis airway: an evolutionary perspective. *Nature rev Microbiol* **10**: 841–851.

Frydenlund Michelsen C, Hossein Khademi SM, Krogh Johansen H, Ingmer H, Dorrestein PC, Jelsbak L. (2016). Evolution of metabolic divergence in Pseudomonas aeruginosa during long-term infection facilitates a proto-cooperative interspecies interaction. *ISME j* **10**: 1323–1336.

Goymer P, Kahn SG, Malone JG, Gehrig SM, Spiers AJ, Rainey PB. (2006). Adaptive divergence in experimental populations of Pseudomonas fluorescens. II. Role of the GGDEF regulator WspR in evolution and development of the wrinkly spreader phenotype. *Genetics* **173**: 515–526.

Guvener ZT, Harwood CS. (2007). Subcellular location characteristics of the *Pseudomonas aeruginosa* GGDEF protein, WspR, indicate that it produces cyclic-di-GMP in response to growth on surfaces. *Mol Microbiol* **66**: 1459–1473.

Hao Y, Murphy K, Lo RY, Khursigara CM, Lam JS. (2015). Single-nucleotide polymorphisms found in the migA and wbpX glycosyltransferase genes account for the intrinsic lipopolysaccharide defects exhibited by *Pseudomonas aeruginosa* PA14. *J bacteriol* **197**: 2780–2791.

Harrison F, Paul J, Massey RC, Buckling A. (2008). Interspecific competition and siderophore-mediated cooperation in Pseudomonas aeruginosa. *ISME j* **2**: 49–55.

Haussler S, Fuqua C. (2013). Biofilms 2012: new discoveries and significant wrinkles in a dynamic field. *J Bacteriol* **195**: 2947–2958.

Hickman JW, Tifrea DF, Harwood CS. (2005). A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proc Natl Acad Sci USA* **102**: 14422–14427.

Hoffman LR, Deziel E, D'Argenio DA, Lepine F, Emerson J, McNamara S *et al.* (2006). Selection for Staphylococcus aureus small-colony variants due to growth in the presence of Pseudomonas aeruginosa. *Proc Natl Acad Sci USA* **103**: 19890–19895.

Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ. (2012). The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. *Microbiol Mol Biol Rev* **76**: 46–65.

Kessler E, Safrin M, Olson JC, Ohman DE. (1993). Secreted LasA of Pseudomonas aeruginosa is a staphylolytic protease. *J Biol Chem* **268**: 7503–7508.

Kim S, Yoon Y, Choi KH. (2015). Pseudomonas aeruginosa DesB promotes *Staphylococcus aureus* growth inhibition in coculture by controlling the synthesis of HAQs. *PLoS One* **10**: e0134624.

Kirisits MJ, Parsek MR. (2006). Does Pseudomonas aeruginosa use intercellular signalling to build biofilm communities? *Cell Microbiol* **8**: 1841–1849.

Köhler T, Buckling A, van Delden C. (2009). Cooperation and virulence of clinical Pseudomonas aeruginosa populations. *Proc Natl Acad Sci USA* **106**: 6339–6344.

Koza A, Moshynets O, Otten W, Spiers AJ. (2011). Environmental modification and niche construction: developing O2 gradients drive the evolution of the Wrinkly Spreader. *ISME J* **5**: 665–673.

Li H, Durbin R. (2010). Fast and accurate long-read alignment with Burrows–Wheeler transform. *Bioinformatics* **26**: 589–595.

Makin SA, Beveridge TJ. (1996). The influence of A-band and B-band lipopolysaccharide on the surface characteristics and adhesion of Pseudomonas aeruginosa to surfaces. *Microbiology* **142**(Pt 2): 299–307.

Malone JG. (2015). Role of small colony variants in persistence of Pseudomonas aeruginosa infections in cystic fibrosis lungs. *Infect Drug Resist* **8**: 237–247.

Marquart ME, Caballero AR, Chomnawang M, Thibodeaux BA, Twining SS, O'Callaghan RJ. (2005). Identification of a novel secreted protease from *Pseudomonas aeruginosa* that causes corneal erosions. *Invest Ophthalmol Vis Sci* **46**: 3761–3768.

Marvig RL, Johansen HK, Molin S, Jelsbak L. (2013). Genome analysis of a transmissible lineage of pseudomonas aeruginosa reveals pathoadaptive mutations and distinct evolutionary paths of hypermutators. *PLoS genet* **9**: e1003741.

Marvig RL, Sommer LM, Molin S, Johansen HK. (2015). Convergent evolution and adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. *Nat Genet* **47**: 57–64.

Mashburn LM, Jett AM, Akins DR, Whiteley M. (2005). *Staphylococcus aureus* serves as an iron source for *Pseudomonas aeruginosa* during in vivo coculture. *J bacteriol* **187**: 554–566.

Michelsen CF, Christensen AM, Bojer MS, Hoiby N, Ingmer H, Jelsbak L. (2014). *Staphylococcus aureus* alters growth activity, autolysis, and antibiotic tolerance in a human host-adapted *Pseudomonas aeruginosa* lineage. *J bacteriol* **196**: 3903–3911.

Murphy K, Park AJ, Hao Y, Brewer D, Lam JS, Khursigara CM. (2014). Influence of O polysaccharides on biofilm development and outer membrane vesicle biogenesis in *Pseudomonas aeruginosa* PAO1. *J bacteriol* **196**: 1306–1317.

O'Rourke D, FitzGerald CE, Traverse CC, Cooper VS. (2015). There and back again: consequences of biofilm specialization under selection for dispersal. *Front Genet* **6**: 18.

Rocchetta HL, Burrows LL, Pacan JC, Lam JS. (1998). Three rhamnosyltransferases responsible for assembly of the A-band D-rhamnan polysaccharide in *Pseudomonas aeruginosa*: a fourth transferase, WbpL, is required for the initiation of both A-band and B-band lipopolysaccharide synthesis. *Mol Microbiol* **28**: 1103–1119.

Rudin L, Sjostrom JE, Lindberg M, Philipson L. (1974). Factors affecting competence for transformation in Staphylococcus aureus. *J bacteriol* **118**: 155–164.

Simpson JT, Durbin R. (2010). Efficient construction of an assembly string graph using the FM-index. *Bioinformatics* **26**: i367–i373.

Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA *et al.* (2006). Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. *Proc Natl Acad Sci USA* **103**: 8487–8492.

Spiers AJ, Kahn SG, Bohannon J, Travisano M, Rainey PB. (2002). Adaptive divergence in experimental populations of Pseudomonas fluorescens. I. Genetic and phenotypic bases of wrinkly spreader fitness. *Genetics* **161**: 33–46.

Spiers AJ, Bohannon J, Gehrig SM, Rainey PB. (2003). Biofilm formation at the air-liquid interface by the Pseudomonas fluorescens SBW25 wrinkly spreader requires an acetylated form of cellulose. *Mol Microbiol* **50**: 15–27.

Starkey M, Hickman JH, Ma L, Zhang N, De Long S, Hinz A *et al.* (2009). Pseudomonas aeruginosa rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. *J bacteriol* **191**: 3492–3503.

Wang S, Hao Y, Lam JS, Vlahakis JZ, Szarek WA, Vinnikova A *et al.* (2015). Biosynthesis of the common polysaccharide antigen of *Pseudomonas aeruginosa* PAO1: characterization and role of GDP-D-Rhamnose: GlcNAc/GalNAc-diphosphate-lipid alpha1,3-D-rhamnosyltransferase WbpZ. *J bacteriol* **197**: 2012–2019.

Wiser MJ, Lenski RE. (2015). A comparison of methods to measure fitness in *Escherichia coli*. *PLoS One* **10**: e0126210.

Yang L, Liu Y, Markussen T, Hoiby N, Tolker-Nielsen T, Molin S. (2011). Pattern differentiation in co-culture biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *FEMS Immunol Med Microbiol* **62**: 339–347.

Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)