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ORIGINAL ARTICLE

Evolution of metabolic divergence in *Pseudomonas aeruginosa* during long-term infection facilitates a proto-cooperative interspecies interaction

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The effect of polymicrobial interactions on pathogen physiology and how it can act either to limit pathogen colonization or to potentiate pathogen expansion and virulence are not well understood. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are opportunistic pathogens commonly found together in polymicrobial human infections. However, we have previously shown that the interactions between these two bacterial species are strain dependent. Whereas *P. aeruginosa* PAO1, a commonly used laboratory strain, effectively suppressed *S. aureus* growth, we observed a commensal-like interaction between the human host-adapted strain, DK2-P2M24-2003, and *S. aureus*. In this study, characterization by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) imaging mass spectrometry (IMS) and mass spectral (MS) molecular networking revealed a significant metabolic divergence between *P. aeruginosa* PAO1 and DK2-P2M24-2003, which comprised several virulence factors and signaling 4-hydroxy-2-alkylquinoline (HAQ) molecules. Strikingly, a further modulation of the HAQ profile was observed in DK2-P2M24-2003 during interaction with *S. aureus*, resulting in an area with thickened colony morphology at the *P. aeruginosa*–*S. aureus* interface. In addition, we found an HAQ-mediated protection of *S. aureus* by DK2-P2M24-2003 from the killing effect of tobramycin. Our findings suggest a model where the metabolic divergence manifested in human host-adapted *P. aeruginosa* is further modulated during interaction with *S. aureus* and facilitate a proto-cooperative *P. aeruginosa*–*S. aureus* relationship.

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Introduction

Although many bacterial infectious diseases are assumed to be monoclonal, infections most often occur in a polymicrobial context within the host (Short *et al.*, 2014). How the different microbes contribute to disease and health remains poorly understood, but it is becoming increasingly clear that interactions between bacterial pathogens and other microbial species residing in the infected host (such as co-infecting pathogens or commensal bacteria) may in some cases determine disease phenotype or the response to therapy (Peters *et al.*, 2012; Short *et al.*, 2014). It continues to be a central challenge to

identify and characterize these interactions and the biological processes they control in relation to infection. Studies of microbe–microbe interactions and their impact on the activity of bacterial pathogens have often focused on well-characterized laboratory or reference strains (Moree *et al.*, 2012; Korgaonkar *et al.*, 2013; Vega *et al.*, 2013). However, given the genetic diversity that often exists in pathogen populations and the clonal diversity that may evolve within the host during long-term infection (Lieberman *et al.*, 2011; Marvig *et al.*, 2013; Markussen *et al.*, 2014), a major issue is how well the use of any one strain as an experimental model for understanding how microbial interactions modulate pathogen activity reflects this diversity.

To begin to address this issue, we investigate chronic airway infections in patients with cystic fibrosis (CF). Complex microbial communities reside in the CF airways, where the community structure and composition may vary between patients and can

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change dramatically over time depending on disease progression and antibiotic exposure (Zhao *et al.*, 2012). Despite profound inter-individual microbial community dynamics, strains of the opportunistic human pathogens, *P. aeruginosa* and *S. aureus*, are among the dominant inhabitants in the CF airways and have been co-isolated in both early-stage as well as chronic late-stage infections (Lording *et al.*, 2006; Harrison, 2007; Fugere *et al.*, 2014). In accordance, a recent cohort study at the Danish CF Centre reported that approximately 16% of the CF patients chronically infected with *S. aureus* were also chronically infected with *P. aeruginosa* (Dalboge *et al.*, 2013). Interaction studies between these two bacterial species often use laboratory model strains of *P. aeruginosa*, which effectively kills co-cultured *S. aureus* by producing a range of anti-staphylococcal molecules such as pyocyanin, LasA, LasB, rhamnolipids and the 4-hydroxy-2-alkylquinoline (HAQ) molecule, 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) (Mashburn *et al.*, 2005; Palmer *et al.*, 2005; Hoffman *et al.*, 2006; Mitchell *et al.*, 2010; Korgaonkar and Whiteley, 2011; Korgaonkar *et al.*, 2013; DeLeon *et al.*, 2014). Therefore, limited investigations have addressed the potential effect of *S. aureus* on *P. aeruginosa*.

Important for *P. aeruginosa* pathogenesis is the hierarchical QS system, which is mediated by two chemically distinct classes of signaling molecules, the *N*-acylhomoserine lactones and the HAQ molecules (Pearson *et al.*, 1997; Heeb *et al.*, 2011). More than 50 HAQ molecules divided into five distinct series (the series A–E congeners) have been identified from *P. aeruginosa*, where especially the signaling molecule 3,4-dihydroxy-2-heptylquinoline (PQS; the most prominent Series B congener) is important for regulating the production of virulence factors (Deziel *et al.*, 2004; Heeb *et al.*, 2011). However, within bacterial populations associated with long-term infections, loci encoding the QS system have been found to accumulate mutations, and can result in extensive metabolic and phenotypic modifications of host-adapted strains compared with strains of environmental origin (Smith *et al.*, 2006; D'Argenio *et al.*, 2007; Yang *et al.*, 2011; Damkiaer *et al.*, 2013), which may subsequently affect their interaction with other microorganisms. Thus, the outcome of interaction between *P. aeruginosa* and *S. aureus* may be strain dependent.

This work expands on our previous observation with a human host-adapted *P. aeruginosa* strain, DK2-P2M24-2003, from a dominant lineage, DK2, that have evolved through decades of growth in chronically infected Danish CF patients (Yang *et al.*, 2011; Damkiaer *et al.*, 2013; Marvig *et al.*, 2013). *P. aeruginosa* DK2-P2M24-2003 showed a distinct colony morphology with centered autolysis and a metallic sheen coverage associated with the area of autolysis (Figure 1) (Michelsen *et al.*, 2014). Notably, in contrast to *P. aeruginosa* PAO1, which is a widely used laboratory strain in microbial interaction

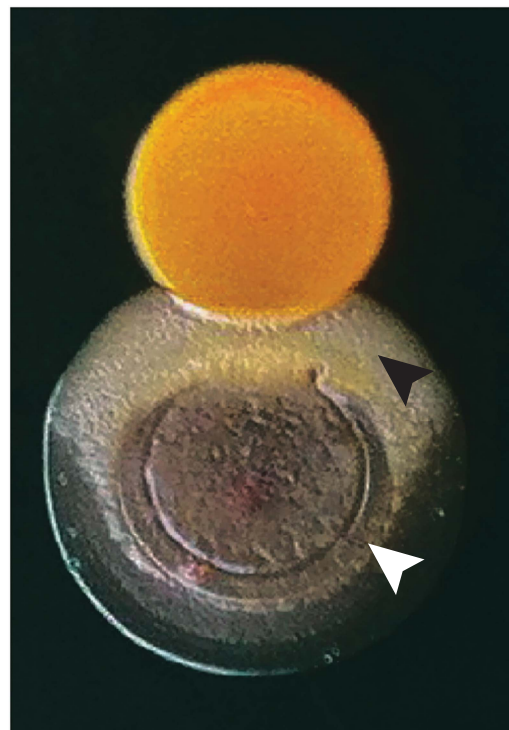


Figure 1 Co-culture of *P. aeruginosa* DK2-P24M2-2003 (lower colony) and *S. aureus* JE2 (upper colony). *P. aeruginosa* DK2-P24M2-2003 show centered autolysis and a metallic sheen coverage associated with the lysed area (indicated by a white arrowhead). In interaction with *S. aureus* JE2, a thickened (white) *P. aeruginosa* colony morphology is observed at the interface (indicated by a black arrowhead).

studies (Hoffman *et al.*, 2006; Mitchell *et al.*, 2010; DeLeon *et al.*, 2014; Phelan *et al.*, 2014), *P. aeruginosa* DK2-P2M24-2003 did not suppress the growth of *S. aureus* USA300 strain JE2. Instead a thickened colony morphology appeared at the *P. aeruginosa*–*S. aureus* interface (Figure 1). Gfp expression measured from *P. aeruginosa* DK2-P2M24-2003 tagged with a growth rate-dependent promoter:reporter gene construct, demonstrated an increased *P. aeruginosa* cell density at the area of thickened colony (Michelsen *et al.*, 2014). These results suggested a commensal-like interaction, where *P. aeruginosa* DK2-P2M24-2003 benefits from the interaction with *S. aureus*.

The goal of this study was to provide insight into the metabolic divergence between *P. aeruginosa* DK2-P2M24-2003 and PAO1, which facilitates the commensal-like interaction between *P. aeruginosa* DK2-P2M24-2003 and *S. aureus* JE2. By combining molecular genetics with matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) imaging mass spectrometry (IMS) on agar surfaces and mass spectral (MS) molecular network analysis on microbial extracts, a significant divergence in production of antimicrobial and HAQ molecules was detected between PAO1 and DK2-P2M24-2003. Such metabolic divergence may explain the different interaction patterns with *S. aureus* observed by

PAO1 and DK2-P2M24-2003, respectively. Interestingly, the presence of *S. aureus* resulted in induction of a distinct HAQ profile in *P. aeruginosa* DK2-P2M24-2003 during interaction, which significantly differed from the otherwise autolysing HAQ profile detected in the DK2-P2M24-2003 monoculture and in interaction with the *S. aureus agrC* mutant. Furthermore, a *P. aeruginosa* HAQ-mediated protection of *S. aureus* from the antibiotic effect of tobramycin was observed. We therefore propose a model, where the HAQ molecules produced by *P. aeruginosa* DK2-P2M24-2003 provide an important factor for the proto-cooperative interaction with *S. aureus* JE2, in which both bacterial species benefit from the relationship.

Materials and methods

Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are described in Table 1. *P. aeruginosa* and *S. aureus* were routinely cultured in LB broth/agar. *P. aeruginosa*–*S. aureus* interaction assays were performed on tryptic soy broth (Sigma-Aldrich, Denmark A/S, Copenhagen, Denmark) supplemented with 1.2% (wt/vol) agar (TSA), LB with 1.2% agar (Sigma-Aldrich, Denmark A/S) or artificial sputum medium (ASM) (Kirchner *et al.*, 2012) supplemented with 1.2% agar. All liquid cultures were incubated at 37 °C with shaking (200 r.p.m.) unless other stated. Antibiotics were used in following concentrations: ampicillin, 100 µg ml⁻¹; gentamicin, 60 µg ml⁻¹ for

P. aeruginosa transposon and allelic replacement mutants; tetracycline, 50 µg ml⁻¹ for *P. aeruginosa* with inserted lux reporter fusion.

P. aeruginosa–*S. aureus* interaction assays. Stationary-phase cultures of *S. aureus* (optical density at 600 nm (OD600) of ~2) and *P. aeruginosa* (OD600 ~1.5) cultured in LB were diluted to OD600 ~0.2 and streaked or added as spots onto dry agar plates as previously described (Michelsen *et al.*, 2014). Briefly, in the cross-streak assay, one loopful of *P. aeruginosa* culture was first streaked in one direction on the plate and left to dry before the plate was cross-streaked with one loopful of the *S. aureus* culture. During spot inoculation, 5 µl of the *P. aeruginosa* culture was spotted first onto agar plates and after drying 5 µl of the *S. aureus* culture was spotted with 5 mm distance from the *P. aeruginosa* spot. The experiments were carried out in triplicate, and the plates were incubated at 37 °C under aerobic conditions or anaerobically in a Type B Vinyl Anaerobic Chamber (Coy Lab Products, Grass Lake, MI, USA) filled with an atmosphere of 95% (vol/vol) nitrogen and 5% (vol/vol) hydrogen. After 24–72 h of incubation, the bacterial colonies touched the edge of each other with < 1 mm distance between them. The plates were inspected visually, and the interaction at the *P. aeruginosa*–*S. aureus* interface was recorded.

The interaction assay with tobramycin was carried out as described by Hoffman *et al.* (2006). Briefly, the diluted *S. aureus* culture was applied to the surface of LB agar without or with addition of tobramycin (that is,

Table 1 Strains and plasmids used in this study

Strain or plasmid	Genotype	Reference
<i>Plasmids</i>		
pBT20	Gm ^r ; <i>Himar1</i> C9 transposon	Kulasekara <i>et al.</i> (2005)
prK600	Cm ^r ; <i>ori</i> ColE1 RK2-mob ⁺ RK2-mob ⁺ helper plasmid for conjugation	Kessler <i>et al.</i> (1992)
pEXG2-PAO601	Gm ^r ; <i>sacB</i> , allelic exchange vector with <i>PAO601</i> gene deletion fragment	This study
pCTX::pqsA'-lux	Tet ^r ; Promoter region and part of <i>pqsA</i> fused to <i>luxCDABE</i> inserted into the miniCTX-1 delivery plasmid	Fletcher <i>et al.</i> (2007)
pHK-pqsH::lux	Tet ^r ; Promoter region and part of <i>pqsH</i> fused to <i>luxCDABE</i> inserted into the miniCTX-1 delivery plasmid	This study
<i>E. coli</i>		
HB101	<i>recA thi pro leu hsdRM^r</i> ; Sm ^r	Kessler <i>et al.</i> (1992)
<i>P. aeruginosa</i>		
PAO1	WT, laboratory model strain	Stover <i>et al.</i> (2000)
PAO1 <i>pqsH::lux</i>	PAO1 containing a copy of the <i>pqsH</i> promoter linked to the <i>luxCDABE</i> genes in the chromosome	This study
DK2-P2M24-2003	<i>P. aeruginosa</i> DK2 isolate from 2003	Michelsen <i>et al.</i> (2014)
DK2 <i>pqsA'-lux</i>	DK2-P2M24-2003 containing a copy of the <i>pqsA</i> promoter linked to the <i>luxCDABE</i> genes in the chromosome	This study
DK2 <i>pqsH::lux</i>	DK2-P2M24-2003 containing a copy of the <i>pqsH</i> promoter linked to the <i>luxCDABE</i> genes in the chromosome	This study
DK2-Tn1	Transposon deletion of <i>pqsB</i> in DK2-P2M24-2003	This study
<i>S. aureus</i>		
JE2	Plasmid-cured derivative of USA300 (FPR3757)	Fey <i>et al.</i> (2013)
<i>agrC</i>	JE2 containing a transposon deletion of <i>agrC</i>	Fey <i>et al.</i> (2013)

0.5, 1.0, 1.25, 1.5, 1.75, 2.0 or 2.5 $\mu\text{g ml}^{-1}$) using a drigalski spatula, and 5 μl of the diluted *P. aeruginosa* cultures were added as spots to the *S. aureus* lawn. The experiments were carried out in triplicate, and the plates were incubated for 24 h at 37 °C.

Construction of reporter fusions and bioluminescence measurements

The plasmid pCTX::*pqsA'*-*lux* (Fletcher *et al.*, 2007) was used for the *pqsA* transcriptional fusion experiment. For the *pqsH*::*lux* reporter fusion, a 389-bp fragment containing the intergenic region upstream of *pqsH* was amplified from genomic DNA using Phusion polymerase (Thermo Scientific) with the primers PqsH_F-PstI (5'-GAGACTGCAGATCACGCTGCACCTCGTCGC-3') and pqsH_R-XhoI (5'-ACATCTCGAGGTCGGTGAGATGGCCGCACA-3') and ligated with T4 DNA ligase into pHK-CTX2-*lux* (Marvig *et al.*, 2014) after double digestion of the PCR fragment and vector with the restriction enzymes XhoI and PstI. The resulting plasmid pHK-*pqsH*::*lux* or pCTX::*pqsA'*-*lux* was introduced into *P. aeruginosa* strain PAO1 or DK2-P2M24-2003, respectively by triparental mating (Trieuquot *et al.*, 1993) using the helper strain *E. coli* HB101/pRK600.

Diluted stationary phase cultures of *P. aeruginosa* PAO1 or DK2-P2M24-2003 containing the *pqsH*::*lux* or *pqsA'*-*lux* reporter fusions were spot-inoculated together with diluted stationary phase cultures of *S. aureus* on agar plates as described above. After 24 h of incubation, luminescence was recorded continuously for 10 s with a photon camera using an Hamamatsu Argus 50 image processor (Unit One Electronics, Birkerød, Denmark) and Argus 50 Control Program vers. 4.25 (Hamamatsu Photonics K.K., Ballerup, Denmark). The experiments were carried out in triplicate and the Tukey's HSD test was used in conjunction with ANOVA in order to evaluate which means were significantly different from one another. The tests were carried out with R version 3.0.3 (R Development Core Team, 2009).

Screen of *S. aureus* or *P. aeruginosa* transposon mutant libraries

The full *S. aureus* Nebraska transposon mutant library derived from *S. aureus* JE2 (Fey *et al.*, 2013) was screened in a spot-inoculation setup with *P. aeruginosa* DK2-P2M24-2003 as illustrated in Supplementary Figure S5, to detect mutant strains which no longer induced the increased growth activity phenotype in *P. aeruginosa*. The *S. aureus* mutant strains were cultured in 200 μl LB in 96-well microtiter plates (Nunc) and *P. aeruginosa* DK2-P2M24-2003 in 50 ml LB over night (O/N) at 37 °C with shaking. Cultures of the *S. aureus* mutant strains were applied at one time onto OmniTrays (Nunc) containing LB agar by using a sterile replicator. After drying, the *P. aeruginosa* culture was applied in spots next to the *S. aureus* colonies

using a sterile replicator. After 24–72 h of incubation the plates were visually inspected and *S. aureus* mutant strains were selected for a second round of screening using cross-streak and spot-inoculated assay as described above. The screening and rescreening were repeated three times.

Transposon mutagenesis of *P. aeruginosa* DK2-P2M24-2003 was performed with the *himar1* C9 mariner transposon from pBT20 (Kulasekara *et al.*, 2005) by triparental mating using the helper strain *E. coli* HB101/pRK600. Strain DK2-P2M24-2003 transconjugants with transposons integrated into the chromosome were selected on *Pseudomonas* isolation agar plates supplemented with gentamicin. Approximately 1500 mutants were screened for an impaired interaction with *S. aureus* JE2. The *P. aeruginosa* mutant strains were cultured in 200 μl LB in 96-well microtiter plates (Nunc) and *S. aureus* JE2 in 50 ml LB O/N at 37 °C with shaking and screened in a spot-inoculation setup as described above for the *S. aureus* mutant library as illustrated in Supplementary Figure S4. For mapping transposon insertion junctions in the *P. aeruginosa* DK2-P2M24-2003 chromosome, we used arbitrary PCR (Caetano-Anolles, 1993). The PCR was performed in two rounds with two different sets of primers as described in Supplementary Table S1. The PCR products were purified using the NucleoSpin PCR clean-up kit (Macherey-Nagel GmbH & Co, Düren, Germany), as described by the manufacturer, sequenced at LGC Genomics GmbH (Berlin, Germany), and compared with the Genbank DNA sequence database using blastn (Altschul *et al.*, 1990) and the *P. aeruginosa* DK2 reference genome (Accession no.: CP003149).

Construction of PAO601 deletion by allelic exchange in *P. aeruginosa* DK2-P2M24-2003

The *PAO601* deletion in *P. aeruginosa* DK2-P2M24-2003 was performed using complete allelic exchange with a pEXG2-based construct containing a *PAO601* gene deletion fragment, pEXG2-PAO601, kindly provided by M. Whiteley (University of Texas at Austin, USA). The allelic replacement construct was transferred into *P. aeruginosa* DK2-P2M24-2003 by triparental mating using the helper strain *E. coli* HB101/pRK600. After incubation O/N, merodiploid *P. aeruginosa* was selected by plating the conjugation mixture on *Pseudomonas* isolation agar plates with gentamicin (Gm) followed by incubation until colonies appeared. Colonies were restreaked on 6% (wt/vol) sucrose-low salt (0.5% NaCl) LB agar plates. Sucrose-resistant/Gm-sensitive colonies were restreaked on 6% sucrose-low salt LB agar plates, screened for the presence of the mutated allele by PCR and verified by sequencing at LGC Genomics.

MALDI-TOF imaging mass spectrometry

P. aeruginosa was co-cultured with *S. aureus* using spot inoculation on thin TSA plates as described

above (that is, 10 ml of medium in a standard Petri dish resulting in a medium thickness of about 1.5 mm). After 24–72 h of incubation, areas of agar media containing the microbial colonies were excised from the Petri dish and transferred onto the MALDI stainless steel target plate. The subsequent matrix application was performed using a 53- μm test sieve whereby dry matrix was deposited evenly across the sample surface until the desired matrix thickness was achieved. The sample was dried O/N followed by MALDI-TOF IMS analysis using a Bruker Microflex mass spectrometer. Data were collected from 50 to 3000 m/z at 80 Hz laser frequency at a spatial resolution of 400 $\mu\text{m} \times 400 \mu\text{m}$ using a Random Walk shot pattern. Data were analyzed using the Compass 1.2 software suite (FlexImaging 2.0, FlexControl 3.0 and FlexAnalysis 3.0; Bruker Daltonics, Ballerica, MA, USA) as previously described (Gonzalez *et al.*, 2011). The resulting mass spectrum was binned at 0.5 m/z increments and manually inspected for masses of interest. Correlating the spatial distribution of signals between the bacterial monocultures and the bacterial interactions allowed identification of signal intensities of masses ascribed to a specific bacterial source. Each mass was visualized by a specific false color superimposed onto an optical image of the original sample plate. The MALDI-TOF IMS analysis was carried out in triplicate and only masses that were observed in all sample replicates were investigated further.

LC-MS/MS analysis and construction of MS molecular network

For LC-MS/MS analysis, samples were diluted eightfold. Mass spectrometry was performed by using a Bruker Daltonics Maxis qTOF mass spectrometer equipped with a standard electrospray ionization source as previously described (Phelan *et al.*, 2014). For accurate mass measurements, lock mass internal calibration used a wick saturated with hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazene ions (Synquest Laboratories, Alachua, FL, USA; m/z 922.0098) located within the source.

Ions of interest observed in MALDI-TOF IMS (*P. aeruginosa* DK2-P2M24-2003–*S. aureus* interaction at 48 h) were correlated to the collected MS/MS data on the corresponding extracts. Tandem mass spectra were converted into a MS/MS network and clustered according to the protocol described by Watrous *et al.* (2012) using the GNPS infrastructure (gnps.ucsd.edu) as MSV000078634, whereby each node is a cluster-consensus spectrum and each edge corresponds to a significant pair wise alignment. The MS molecular network was visualized in Cytoscape (Smoot *et al.*, 2011). All algorithms assumed precursor mass tolerance of 1.0 and fragment mass tolerance of 0.5 Da. MS/MS-based dereplication was performed by incorporating accumulated reference

spectra into the MS molecular network (Watrous *et al.*, 2012; Yang *et al.*, 2013).

Results

Pseudomonas aeruginosa DK2-P2M24-2003 show divergent metabolic and interspecies interaction profiles

The interactions between *P. aeruginosa* strains PAO1 or DK2-P2M24-2003 and *S. aureus* JE2 were studied by culturing the bacteria side-by-side on agar surfaces in high-cell-density spots, which previously have been used to study microbial interactions (Gonzalez *et al.*, 2011; Antonic *et al.*, 2013; Michelsen *et al.*, 2014; Phelan *et al.*, 2014). Distinct colony morphologies and interaction patterns were observed by *P. aeruginosa* strains PAO1 and DK2-P2M24-2003, respectively, during co-culturing with *S. aureus* JE2 (Figure 2), which supported our previous observations (Michelsen *et al.*, 2014). Whereas *P. aeruginosa* PAO1 developed a smooth, green-pigmented colony and inhibited the growth of *S. aureus*, DK2-P2M24-2003 instead showed a commensal-like interspecies interaction with a thickened colony morphology at the *P. aeruginosa*–*S. aureus* interface (Figure 2a), which previously has been identified as an area of increased *Pseudomonas* cell density (Michelsen *et al.*, 2014). Importantly, the distinct interaction patterns by *P. aeruginosa* DK2-P2M24-2003 and *P. aeruginosa* PAO1, respectively, were reproducible during culturing on the different agar media, LB, TSA and ASM, which mimics the nutrient conditions of natural CF sputum, as well as both under aerobic and anaerobic growth conditions (Figure 3a).

The spatial and temporal distribution of metabolites was analyzed in the side-by-side *P. aeruginosa*–*S. aureus* interactions on agar surfaces by MALDI-TOF IMS (Yang *et al.*, 2009). Differences in distribution of m/z signals between *P. aeruginosa* PAO1 and DK2-P2M24-2003 were examined and revealed very divergent metabolic profiles (Figure 2b). Several m/z signals were detected from *P. aeruginosa* PAO1, which were not present in *P. aeruginosa* DK2-P2M24-2003. These m/z signals corresponded to the antimicrobials pyocyanin (m/z 211, 1) and different rhamnolipids (for example, represented by m/z 673, 2) (Figure 2b) that previously have been identified from *P. aeruginosa* PAO1 using MALDI-TOF IMS (Phelan *et al.*, 2014). In addition, some unidentified *S. aureus* m/z signals of m/z 231 (3) and m/z 256 (4) were observed with increased intensity at the interface with *P. aeruginosa* DK2-P2M24-2003 but not with *P. aeruginosa* PAO1 (Figure 2b), which could be due to the suppression of *S. aureus* by PAO1. Notable, the signaling HAQ molecule, PQS, and the antimicrobial HAQ molecule, HQNO, (m/z 260, 6), both of which were present in *P. aeruginosa* PAO1, were not detected in *P. aeruginosa* DK2-P2M24-2003 (Figure 2b).

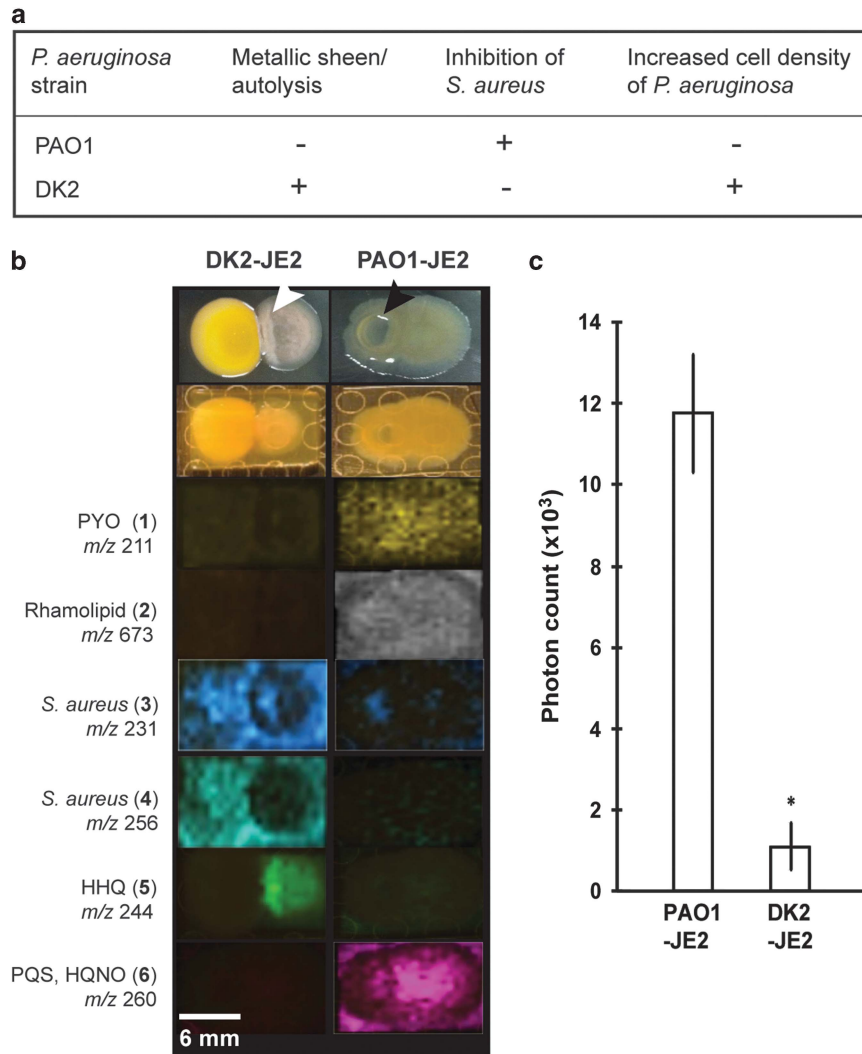


Figure 2 (a) Recorded phenotypes of *P. aeruginosa* PAO1 and DK2-P2M24-2003 in interaction with *S. aureus* JE2. Phenotypes were scored as: +: Yes or -: No. (b) *P. aeruginosa* (right colony)–*S. aureus* JE2 (left colony) interactions on agar surfaces with *P. aeruginosa* DK2-P2M24-2003 (DK2-JE2) or PAO1 (PAO1-JE2) co-cultured for 72 h on TSA. Black arrowhead indicates suppression of *S. aureus* and white arrowhead indicates the zone of thickened DK2-P2M24-2003 colony morphology. The *P. aeruginosa*–*S. aureus* interactions were transferred to a MALDI plate before MALDI-TOF IMS analysis (second row of pictures). Strain-specific *m/z* signals in *P. aeruginosa*–*S. aureus* interactions were identified by MALDI-TOF IMS analysis and visualized by different colors. The *m/z* distributions of the *P. aeruginosa* antimicrobial molecules, (1) pyocyanin (PYO) (yellow, *m/z* 211) and (2) a representative example for the rhamnolipid family (gray, *m/z* 673), (3, 4) unidentified *S. aureus* molecules of *m/z* 231 (blue) and *m/z* 256 (cyan), respectively, and the *P. aeruginosa* HAQ molecules, (5) HHQ (green, *m/z* 244) and (6) PQS and HQNO (purple, *m/z* 260) are shown. The MALDI-TOF IMS spatial resolution was 500 μm , with a detection range of 50–3000 Da. (c) Bioluminescence measured by photon counting from *P. aeruginosa* PAO1 or DK2-P2M24-2003 (DK2) with a *pqsH::lux* reporter fusion in interaction *S. aureus* JE2. * $P < 0.05$ by Tukey's HSD test compared with PAO1. Error bars represent s.d., $n = 3$.

DK2-P2M24-2003 instead showed an increased production of 2-heptyl-4-hydroxyquinoline, HHQ (*m/z* 244, 5) (Figure 2b), the biosynthetic precursor of PQS (Deziel *et al.*, 2004). PqsH catalyzes the conversion of HHQ to PQS in *P. aeruginosa* (Deziel *et al.*, 2004). Thus, to investigate whether the lack of PQS production in *P. aeruginosa* DK2-P2M24-2003 was due to an altered expression of the *pqsH* gene, we constructed a *luxCDABE*-based *pqsH* promoter fusion (Becher and Schweizer, 2000), which was incorporated into the PAO1 and DK2-P2M24-2003 chromosome, respectively. Indeed, a significant decrease in *pqsH* expression, determined by emitted bioluminescence, was observed by *P. aeruginosa*

DK2-P2M24-2003 compared with *P. aeruginosa* PAO1 (Figure 2c). Thus, given what is known about PQS and virulence factor production in *P. aeruginosa* (Deziel *et al.*, 2004; Heeb *et al.*, 2011), its loss could predict to facilitate co-existence of *P. aeruginosa* and *S. aureus*.

The presence of S. aureus JE2 induces a distinct HAQ profile and altered colony morphology in P. aeruginosa DK2-P2M24-2003

Compared with the metabolic profile of *P. aeruginosa* DK2-P2M24-2003 spotted in monoculture, we noticed by MALDI-TOF IMS an increased intensity

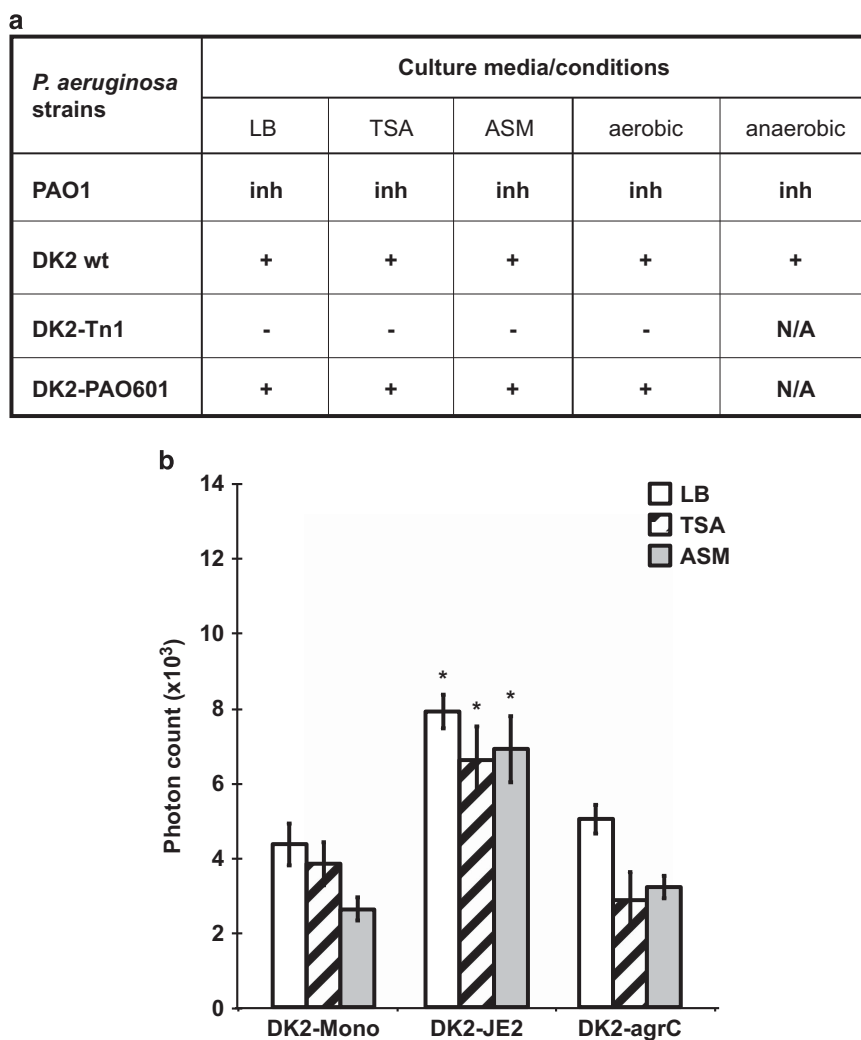


Figure 3 (a) Interactions between the different *P. aeruginosa* strains, PAO1, DK2-P2M24-2003 (*DK2 wt*), the DK2-P2M24-2003 *pqsB* mutant (*DK2-Tn1*) or the *PAO601* mutant (*DK2-PAO601*) and *S. aureus* JE2 when cultured on different agar media, LB, TSA, ASM and under aerobic or anaerobic conditions, respectively. 'inh': inhibition of *S. aureus* growth by *P. aeruginosa*; '+': thickened *P. aeruginosa* colony morphology in interaction with *S. aureus*; '-': no thickened *P. aeruginosa* colony morphology; 'N/A': not analyzed. (b) Bioluminescence measured by photon counting from *P. aeruginosa* DK2-P2M24-2003 with a *pqsA*'-lux reporter fusion in monoculture (*DK2-Mono*), in interaction with *S. aureus* JE2 (*DK2-JE2*) or the *agrC* mutant (*DK2-agrC*) when cultured on LB, TSA or ASM agar surfaces, respectively. * $P < 0.05$ by Tukey's HSD test compared with *DK2-mono*. Error bars represent s.d., $n = 3$.

of at least five *Pseudomonas*-derived molecules with the signals m/z 244, 258, 270, 272 and 298, respectively, at the *P. aeruginosa* DK2-P2M24-2003–*S. aureus* JE2 interface (Figure 4a). The increased intensity of the different molecules was most pronounced after 48 and 72 h of interaction with *S. aureus* JE2, and correlated with the area of thickened *P. aeruginosa* DK2-P2M24-2003 colony morphology (that is, increased cell density) (Figure 4a and Supplementary Figure S1).

To facilitate the identification of the molecules observed in MALDI-TOF IMS, organic extracts were prepared of a duplicate plate of the interacting *P. aeruginosa* DK2-P2M24-2003 and *S. aureus* colonies and were analyzed using LC-MS/MS. This enabled us to construct a MS/MS-based molecular network (Watrous *et al.*, 2012) of the extracted *P. aeruginosa* and *S. aureus* molecules, which were

visualized in Cytoscape (Smoot *et al.*, 2011) (Supplementary Figure S2). In this network, the parent ions (m/z) are displayed as nodes and nodes with high MS/MS spectral similarity are clustered together representing the same chemical class of molecules. The MS molecular network analysis showed a majority of parent ions derived from the *P. aeruginosa* DK2-P2M24-2003 extracts, and by incorporating MS/MS data of known molecules (Watrous *et al.*, 2012; Yang *et al.*, 2013), a cluster with members of the quinolone family (HAQ molecules) was identified (Supplementary Figure S2). Five different series A and D congeners, HHQ, C8 HHQ, C9:db NHQ, NHQ and C11:db UHQ, respectively were annotated using spectral comparison and fragmentation analysis (Figure 4b and Supplementary Figure S3), whereas the rest of the molecules within the cluster remained uncharacterized

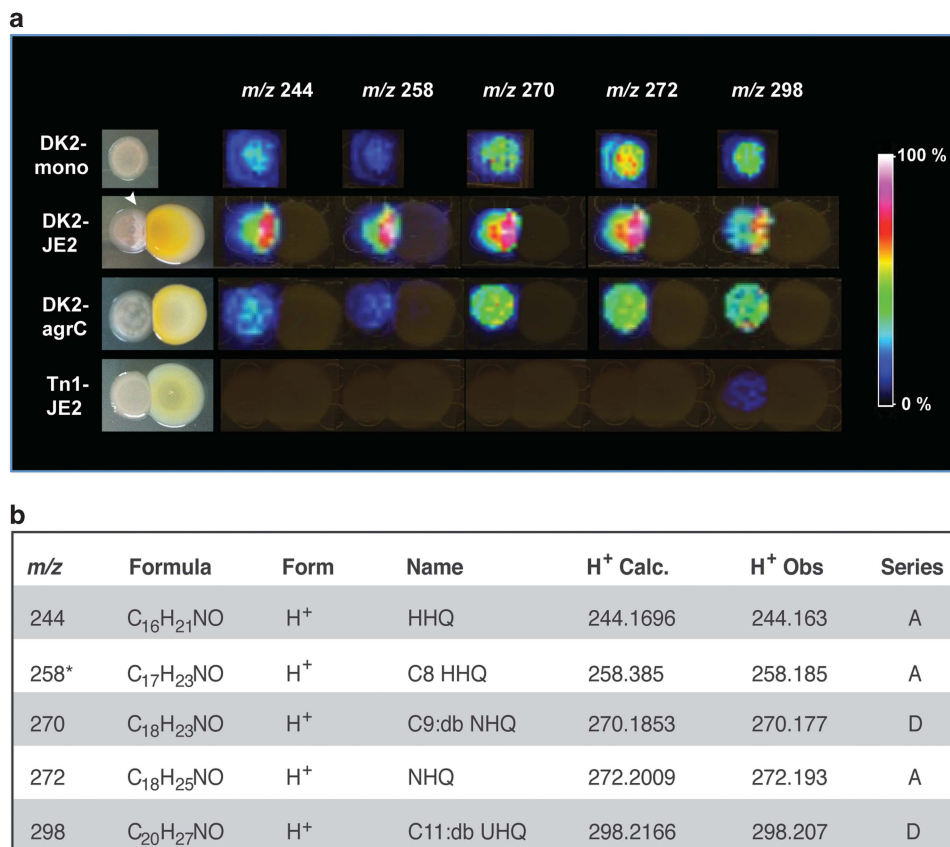


Figure 4 (a) MALDI-TOF IMS analysis of *P. aeruginosa* DK2-P2M24-2003 in monoculture (*DK2-mono*), in interaction with *S. aureus* JE2 (*DK2-JE2*) or in interaction with the *S. aureus agrC* mutant (*DK2-agrC*) as well as the *P. aeruginosa* DK2-P2M24-2003 *pqsB* mutant, Tn1, in interaction with *S. aureus* JE2 (*Tn1-JE2*) after 48 h of co-culturing. The *m/z* distributions of *P. aeruginosa* molecules are displayed as false color overlays of an optical image. Ion-intensity color scaling indicates a highest production of molecules with *m/z* 244, 258, 270, 272 and 298, respectively, at the interface between *P. aeruginosa* DK2-P2M24-2003 and *S. aureus* JE2. White arrowhead indicates the zone of thickened *P. aeruginosa* colony morphology. (b) HAQ molecules were annotated from the MS molecular network using MS/MS-based dereplication or fragmentation analysis (indicated by *). Nomenclature, calculated and observed [M+H]⁺ masses, as well as the HAQ series are shown for the different HAQ molecules according to previous studies (Deziel *et al.*, 2004; Heeb *et al.*, 2011).

(Supplementary Figure S2). The annotated HAQ molecules corresponded to the molecules detected with increased intensity in the MALDI-TOF IMS analysis of the *P. aeruginosa* DK2-P2M24-2003–*S. aureus* JE2 interaction (Figure 4). Similar to the observation from MALDI-TOF IMS, the signaling and antimicrobial HAQ molecules, PQS and HQNO (that is, *m/z* 260), respectively were not detected nor identified using MS molecular networking on the *P. aeruginosa* DK2-P2M24-2003 extracts (Supplementary Figure S2).

The *pqsABCDE* operon is involved in the biosynthesis of HAQ molecules in *P. aeruginosa* (Deziel *et al.*, 2004). Thus, in order to confirm an increased production of HAQ molecules by *P. aeruginosa* DK2-P2M24-2003 during interaction with *S. aureus* JE2, we constructed a *luxCDABE*-based reporter gene fusion (Becher and Schweizer, 2000) with the promoter of *pqsA*. This construct was incorporated into the DK2-P2M24-2003 chromosome. By measuring the bioluminescence from *P. aeruginosa* DK2-P2M24-2003, we observed an up to threefold increase in *pqsA* expression in interaction with

S. aureus JE2 compared with DK2-P2M24-2003 in monoculture when analyzed on the same media (LB, TSA and ASM, respectively) on which the thickened *Pseudomonas* colony morphology was observed (Figure 3b). Taking together, these results indicated that the induced HAQ profile is important for the thickened *P. aeruginosa* DK2-P2M24-2003 colony morphology observed during interaction with *S. aureus* JE2.

Screen for mechanisms involved in the interaction between *P. aeruginosa* DK2-P2M24-2003 and *S. aureus* JE2

A knockout mutation in the gene, *PAO601*, which previously has been identified to be important for the sensing of *S. aureus* by *P. aeruginosa* (Korgaonkar *et al.*, 2013), was constructed in *P. aeruginosa* DK2-P2M24-2003, however, did not affect the interaction between DK2-P2M24-2003 and *S. aureus* JE2 (Figure 3a)

Therefore, we established a DK2-P2M24-2003 *mariner*-based transposon mutant library of 1500

mutant strains and performed a genetic screen in a spot-interaction setup with *S. aureus* JE2 (Supplementary Figure S4), to further identify genes involved in the *P. aeruginosa* DK2-P2M24-2003 response to *S. aureus* JE2. Four distinct *P. aeruginosa* mutant strains, designated Tn1, Tn2, Tn3 and Tn4, respectively were found, showing no thickened colony morphology in interaction with *S. aureus* JE2 (Figure 4a and Supplementary Figure S4). These mutant strains had the transposon inserted into the *pqsB* gene (Tn1), which is part of the PQS biosynthetic operon, *pqsA-E* (Heeb et al., 2011); a gene encoding a tryptophan permease (Tn2); a gene encoding a putative permease (Tn3); and a gene encoding a UvrD/REP helicase (Tn4), respectively (Supplementary Figure S4). In addition, the *P. aeruginosa* mutant Tn1 did not show the autolysing phenotype of the DK2-P2M24-2003 wild-type strain (Figure 4a and Supplementary Figure S4). MALDI-TOF IMS analysis of *P. aeruginosa* Tn1 revealed a defective production of HAQ molecules, likely because of the *pqsB* gene knockout in this mutant (Figure 4a), which further supported an importance of the induced HAQ profile in DK2-P2M24-2003 for the thickened colony morphology (that is, increased cell density) detected at the interface with *S. aureus* JE2.

In addition to the genetic screen of *P. aeruginosa* DK2-P2M24-2003, the 1920 mutants included in the *S. aureus* JE2 Nebraska Transposon Mutant Library (Fey et al., 2013) were screened in a similar spot-interaction setup with *P. aeruginosa* DK2-P2M24-2003 (Supplementary Figure S5). We identified nine distinct *S. aureus* mutants from this mutant screen, which did not induce the thickened colony morphology in *P. aeruginosa* DK2-P2M24-2003 (Supplementary Figure S5). These mutants were the previously identified strains with the transposon inserted into the *agrC*, *agrB*, *clpP* or *sarA* genes, respectively, (Michelsen et al., 2014) as well as *S. aureus* strains with transposons inserted in *agrA* (NE1532) or in genes encoding different membrane transporter proteins (mutants NE496, NE969 and NE1509, respectively) (Supplementary Figure S5). The molecular interaction between the *S. aureus agrC* mutant and *P. aeruginosa* DK2-P2M24-2003 was further investigated by MALDI-TOF IMS analysis. In contrast to the interaction with the *S. aureus* JE2 wild-type strain, no induction of HAQ molecules was detected in DK2-P2M24-2003 by the presence of the *S. aureus agrC* mutant, which instead resembled the autolysing HAQ profile of the DK2-P2M24-2003 monoculture (Figure 4a). In addition, the *pqsA* expression profile in *P. aeruginosa* DK2-P2M24-2003 during interaction with *S. aureus agrC* was similar to that of DK2-P2M24-2003 in monoculture (Figure 3b), which suggests that a functional *agr* system in *S. aureus* is required for inducing the distinct HAQ profile and thickened colony morphology in *P. aeruginosa* DK2-P2M24-2003.

HAQ molecules produced by *P. aeruginosa*

DK2-P2M24-2003 protect *S. aureus* from the antibiotic effect of tobramycin

HQNO produced by *P. aeruginosa* have previously been attributed to protect *S. aureus* from killing by aminoglycosides such as tobramycin (Hoffman et al., 2006). While MALDI-TOF IMS and the MS molecular network analysis did not detect HQNO in *P. aeruginosa* DK2-P2M24-2003, we were curious whether DK2-P2M24-2003 was still able to protect *S. aureus* against tobramycin during co-culture. The effect of DK2-P2M24-2003 was compared with that of the *P. aeruginosa* DK2-P2M24-2003 *pqsB* mutant, Tn1 or *P. aeruginosa* PAO1, respectively. In the absence of tobramycin *P. aeruginosa* PAO1 suppressed the growth of *S. aureus* JE2, whereas no *S. aureus* inhibition was observed by the *P. aeruginosa* DK2-P2M24-2003 wild-type or Tn1 strain (Figure 5). In the presence of tobramycin levels above the minimal inhibitory concentration of *S. aureus* JE2 (MIC; 1.5 µg ml⁻¹), however, we observed growth of *S. aureus* in a zone surrounding the HQNO producing *P. aeruginosa* PAO1 (Figure 5), indicating a protective effect of *S. aureus* by HQNO as previous described (Hoffman et al., 2006). Surprisingly, *S. aureus* growth was also detected in a zone surrounding *P. aeruginosa* DK2-P2M24-2003, which was not able to produce HQNO, whereas no growth was observed in the presence of *P. aeruginosa* Tn1, deficient in HAQ biosynthesis (Figure 5). These results suggested a more complex HAQ-mediated protection of *S. aureus* against the effect of tobramycin. The HAQ-mediated protection from *P. aeruginosa* DK2-P2M24-2003 was not induced exclusively by the presence of *S. aureus* JE2, as a similar protective effect was observed of the *S. aureus agrC* mutant (Supplementary Figure S6).

Discussion

The importance of understanding interspecies interactions in complex polymicrobial infections is manifested by enhanced antibiotic resistance or persistency of bacterial pathogens at the infection site. One example is the interaction between the opportunistic human pathogens *P. aeruginosa* and *S. aureus* that may cause more severe infections when found together compared with when present alone (Peters et al., 2012; Hubert et al., 2013; Pastar et al., 2013). However, the nature of long-term interactions during co-existence of these two species (as occurs in chronic polymicrobial infection) has proven difficult to study *in vitro*, due to *S. aureus* growth suppression by *P. aeruginosa* exoproducts (Duan et al., 2003; Hoffman et al., 2006; Mitchell et al., 2010; DeLeon et al., 2014). Here, we postulate differences in strains that form polymicrobial infections contribute to this. Accordingly, we observed an effective killing of *S. aureus* JE2 by the laboratory reference strain *P. aeruginosa*

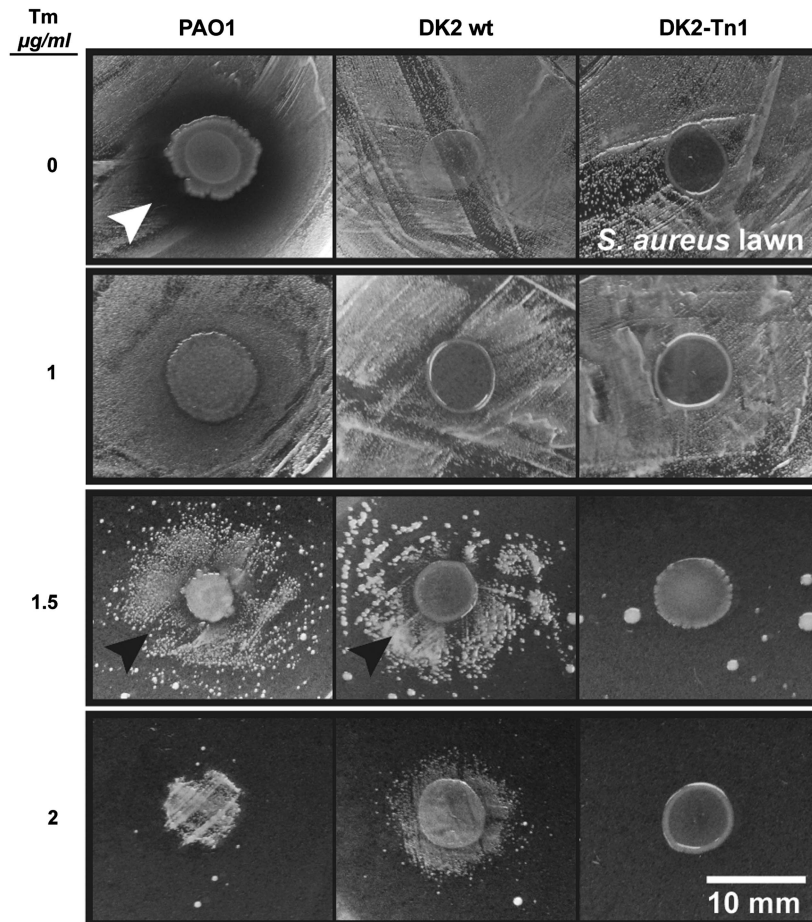


Figure 5 Colonies of *P. aeruginosa* PAO1, the DK2-P2M24-2003 wild type (*DK2 wt*) or the *pqsB* mutant, Tn1 (*DK2-Tn1*) grown on a lawn of *S. aureus* JE2 on LB agar plates with the addition of tobramycin (Tm) as indicated. *P. aeruginosa* PAO1 produced HQNO, whereas DK2-P2M24-2003 and Tn1 were defective for HQNO or HAQ production, respectively. On LB agar without tobramycin *P. aeruginosa* PAO1, but not DK2-P2M24-2003 or Tn1, suppressed the growth of *S. aureus* (indicated by a white arrowhead). By tobramycin concentrations $> 1.5 \mu\text{g ml}^{-1}$ *S. aureus* JE2 growth was observed in a zone surrounding the *P. aeruginosa* PAO1 and DK2-P2M24-2003 colonies (indicated by black arrowheads), but not the Tn1 colony.

PAO1 when co-cultured on agar surfaces. However, in contrast to PAO1, suppression of *S. aureus* JE2 growth was not observed by the human host-adapted *P. aeruginosa* strain, DK2-P2M24-2003. This co-existence of DK2-P2M24-2003 and *S. aureus* JE2 was reproducible on different agar media, including ASM, and under aerobic and anaerobic growth conditions, which thereby allowed us to explore the molecular-genetic basis of co-culture interactions between these strains.

By combining molecular genetics with MALDI-TOF IMS and MS molecular networking, we observed a significant divergent metabolic profile between *P. aeruginosa* PAO1 and DK2-P2M24-2003. Although *P. aeruginosa* PAO1 produced pyocyanin, rhamnolipids and HQNO, which are all part of the antimicrobial circuitry of *P. aeruginosa* (Hoffman et al., 2006; Strateva and Mitov, 2011), these antimicrobials were not detected in *P. aeruginosa* DK2-P2M24-2003, and most likely explain why DK2-P2M24-2003 and *S. aureus* JE2 are able to co-exist. It has been well established that *P. aeruginosa* evolves

during long-term infections in human hosts (Smith et al., 2006; Marvig et al., 2013). Loss-of-function mutations in the *lasR* gene are commonly detected during *P. aeruginosa* adaptation to the CF airways (Smith et al., 2006; D'Argenio et al., 2007), which encodes a central quorum sensing-dependent transcriptional activator of multiple virulence genes (Gallagher et al., 2002; D'Argenio et al., 2007). In addition, LasR regulates the expression of *pqsH*, which is involved in the biosynthesis of HAQ molecules (that is, responsible for the conversion of the precursor HHQ to the signaling molecule, PQS; Deziel et al., 2004). Together with other regulatory mutations, the *lasR* loss-of-function mutation has been detected in the human host-adapted *P. aeruginosa* DK2 lineage (Yang et al., 2011; Damkiaer et al., 2013) and is also present in *P. aeruginosa* DK2-P2M24-2003 (Michelsen et al., 2014). Therefore, this mutation could serve as a contributing factor for the divergent metabolic profile observed in DK2-P2M24-2003 compared to PAO1. Besides LasR our previous study with a *lasR*

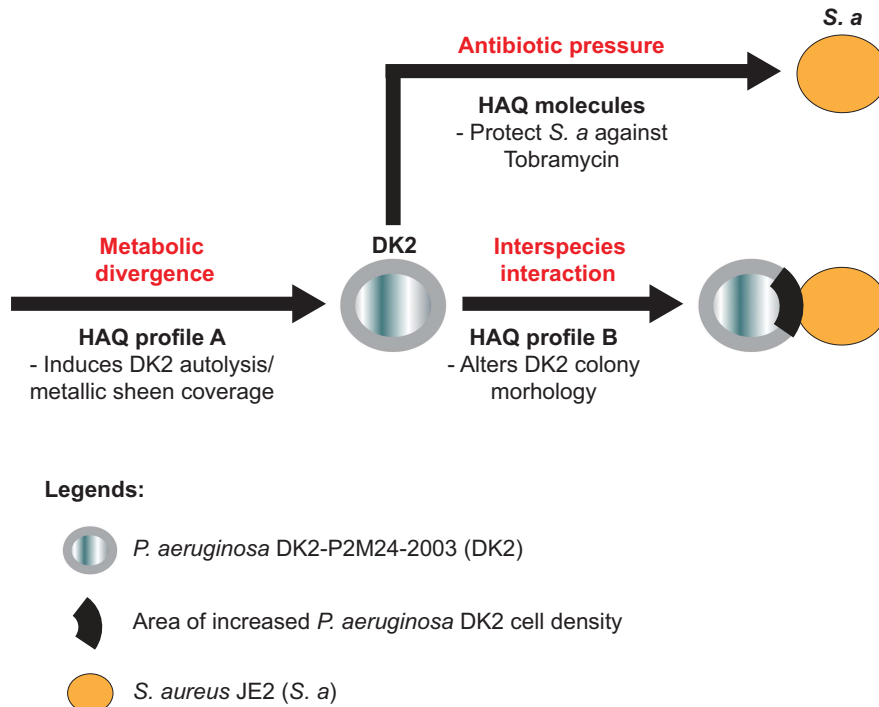


Figure 6 Model for *P. aeruginosa* DK2-P2M24-2003–*S. aureus* JE2 proto-cooperation and the importance of the *P. aeruginosa* produced HAQ molecules for this interaction. According to the model, genetic adaptation during long-term infection is manifested by metabolic divergence in DK2-P2M24-2003 (DK2) resulting in an autolysing HAQ profile (HAQ profile A) and distinct colony morphology as well as the ability to co-exist with *S. aureus* (*S. a.*). In interaction with *S. aureus*, a thickened colony morphology (i.e., increased cell density) is observed in DK2-P2M24-2003 explained by an induction of a second HAQ profile (HAQ profile B). In addition, HAQ molecules produced by DK2-P2M24-2003 mediate a protection of *S. aureus* from the killing effect by tobramycin frequently used in CF therapy.

mutation in a PAO1 background clearly stated that additional mutations could contribute as well (Michelsen *et al.*, 2014). A characteristic phenotype of *P. aeruginosa lasR* mutants is visible autolysis and an iridescent metallic sheen coverage of colonies (D'Argenio *et al.*, 2007). This phenotype has been attributed to an increased ratio of 'Series A congeners' (for example, HHQ) to 'Series B congeners' (for example, PQS) of HAQ molecules (D'Argenio *et al.*, 2007; Gupta and Schuster, 2012). Autolysis and metallic sheen coverage was also observed of the DK2-P2M24-2003 colony. However, besides an increased HHQ:PQS ratio, additional Series A and D congeners (NHQ, UHQ and derivatives, respectively) were detected by MALDI-TOF IMS and MS molecular network analysis, which suggest a more complex autolysing HAQ profile in this strain.

Previous work has reported that *P. aeruginosa* responds to the *S. aureus* cell wall components, peptidoglycan and GlcNAc, with an increased production of virulence factors, and thereby further suppressing *S. aureus* during interaction (Korgaonkar and Whiteley, 2011; Korgaonkar *et al.*, 2013). A strikingly different response to *S. aureus* was observed by *P. aeruginosa* DK2-P2M24-2003 in this study. Instead of an increased production of virulence factors, the presence of *S. aureus* JE2 induced a distinct HAQ profile of accumulated series A and D congeners in DK2-P2M24-2003, thereby substituting the autolysing HAQ profile at the

P. aeruginosa–*S. aureus* interface. Thus, the distinct HAQ profile in DK2-P2M24-2003 at the interface with *S. aureus* JE2 could explain the observed thickened colony morphology, which we have previously identified as a zone with increased *Pseudomonas* cell density (Michelsen *et al.*, 2014). This model is summarized in Figure 6. Our model was further supported by a genetic screen of 1500 *P. aeruginosa* DK2-P2M24-2003 transposon mutants in interaction with *S. aureus* JE2. Here we identified two mutations, in a gene encoding a tryptophan permease and the *pqsB* gene, respectively that no longer responded to *S. aureus*. Tryptophan provides a precursor for HAQ molecules (Farrow and Pesci, 2007; Heeb *et al.*, 2011), whereas *pqsB* is directly involved in the biosynthesis of HAQs (Deziel *et al.*, 2004; Heeb *et al.*, 2011). The DK2-P2M24-2003 *pqsB* mutant (Tn1) showed a distinct smooth colony morphology with no autolysis, which could be explained by the abolished HAQ production and thereby no autolysing HAQ profile in this strain as revealed by MALDI-TOF IMS. In addition to the effect on *P. aeruginosa* itself, the HAQ molecules produced by DK2-P2M24-2003 were associated with a protection of *S. aureus* from killing by tobramycin, an antibiotic commonly used in CF treatments (Gibson *et al.*, 2003). These results of reciprocal change in growth phenotype of both strains supported a model of proto-cooperation between *P. aeruginosa* DK2-P2M24-2003 and *S. aureus* JE2.

It became clear that peptidoglycan and GlcNAc were not involved in the induced HAQ profile in *P. aeruginosa* DK2-P2M24-2003 by *S. aureus* JE2, since a knockout of the sensing mechanism, PAO601 (Korgaonkar *et al.*, 2013), in *P. aeruginosa* DK2-P2M24-2003 did not eliminate the thickened colony morphology. The genetic background of *S. aureus* JE2 has been used as a host for creating a well-characterized sequence-defined transposon mutant library, that is the Nebraska Transposon Mutant Library (NTML), to investigate *S. aureus* biology and pathogenesis (Fey *et al.*, 2013). We have previously identified the genes, *agrC*, *agrB*, *clpP* and *sarA*, in *S. aureus* JE2 to be involved in the induction of the DK2-P2M24-2003 thickened colony morphology (Michelsen *et al.*, 2014). The involvement of these genes was also confirmed in this study by a genetic screen with the full *S. aureus* JE2 NTML consisting of 1952 mutant strains. MALDI-TOF IMS analysis of the interaction between DK2-P2M24-2003 and the *S. aureus agrC* mutant compared with *S. aureus* JE2 revealed no induction of the distinct HAQ profile in DK2-P2M24-2003, which could explain why the thickened *Pseudomonas* colony morphology was not observed in this interaction. Instead an autolysing HAQ profile resembling the profile in DK2-P2M24-2003 monoculture was found in the *S. aureus agrC* interaction. Our genetic screen identified additional membrane transporter proteins, suggesting further the involvement of *S. aureus* extracellular molecules as agents for HAQ profile modifications in *P. aeruginosa*.

In summary, by combining molecular genetics and MALDI-TOF IMS analysis we demonstrate here a difference in the production of antimicrobial and signaling (HAQ) molecules between the laboratory model strain, *P. aeruginosa* PAO1, and the human host-adapted strain, *P. aeruginosa* DK2-P2M24-2003. The metabolic divergence explains their distinct interaction patterns, that is, antagonistic versus commensal-like, respectively with *S. aureus* JE2. The HAQ profile in *P. aeruginosa* DK2-P2M24-2003 was further modulated during interaction with *S. aureus* JE2, which facilitated a proto-cooperative relationship between the two bacteria. Thus, our results emphasize the importance of HAQ molecules in this interspecies interaction (as summarized in Figure 6). In the context of long-term infections, our finding of strain diversity cautions against generalizing conclusions of interactions between bacterial pathogens, which are based on experimental models with one or a few selected strains.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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