Metabolic pathways of *Pseudomonas aeruginosa* involved in competition with respiratory bacterial pathogens

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**Background:** Chronic airway infection by *Pseudomonas aeruginosa* considerably contributes to lung tissue destruction and impairment of pulmonary function in cystic-fibrosis (CF) patients. Complex interplays between *P. aeruginosa* and other co-colonizing pathogens including *Staphylococcus aureus*, *Burkholderia* sp., and *Klebsiella pneumoniae* may be crucial for pathogenesis and disease progression.

**Methods:** We generated a library of PA14 transposon insertion mutants to identify *P. aeruginosa* genes required for exploitative and direct competitions with *S. aureus*, *Burkholderia cenocepacia*, and *K. pneumoniae*.

**Results:** Whereas wild-type PA14 inhibited *S. aureus* growth, two transposon insertions located in *pqsC* and *carB* resulted in reduced growth inhibition. *PqsC* is involved in the synthesis of 4-hydroxy-2-alkylquinolines (HAQs), a family of molecules having antibacterial properties, while *carB* is a key gene in pyrimidine biosynthesis. The *carB* mutant was also unable to grow in the presence of *B. cepacia* and *K. pneumoniae* but not *Escherichia coli* and *S. epidermidis*. We further identified a transposon insertion in *purF*, encoding a key enzyme of purine metabolism. This mutant displayed a severe growth deficiency in the presence of Gram-negative but not of Gram-positive bacteria. We identified a beneficial interaction in a *bioA* transposon mutant, unable to grow on rich medium. This growth defect could be restored either by addition of biotin or by co-culturing the mutant in the presence of *K. pneumoniae* or *E. coli*.

**Conclusion:** Complex interactions take place between the various bacterial species colonizing CF-lungs. This work identified both detrimental and beneficial interactions occurring between *P. aeruginosa* and three other respiratory pathogens involving several major metabolic pathways. Manipulating these pathways could be used to interfere with bacterial interactions and influence the colonization by respiratory pathogens.

**Keywords:** direct competition, exploitative competition, respiratory pathogens, *Pseudomonas aeruginosa*, beneficial interactions, *Staphylococcus aureus*
Introduction

The composition of the microbial respiratory flora of cystic fibrosis (CF) patients changes with age; *Haemophilus influenzae* and *Staphylococcus aureus* being present mainly in young children, while *Pseudomonas aeruginosa*, *S. aureus*, and *Burkholderia* sp. predominate in older patients (Goss and Burns, 2007; Cox et al., 2010; Lipuma, 2010). At the age of 20, 60–70% of CF-patients present intermittent colonization by *P. aeruginosa* (Folkesson et al., 2012) which progressively evolves toward a chronic colonization, that entails progressive lung tissue damage and impairment of pulmonary function. The disappearance of the early colonizing species may be explained by antibiotic treatments or by competition between co-colonizing microorganisms. Many ecological factors, including host immune responses as well as the presence of co-colonizing species interacting with *P. aeruginosa* by competition and/or cooperation, determine the dynamics of lung colonization. Bacterial interference occurs either via direct competition characterized by an active mechanism including the type VI secretion systems (Pukatzki et al., 2006; Basler et al., 2013) and production of competitor molecules (siderophores, secondary metabolites, toxins), or via exploitative competition where one organism consumes the resources of another (Cornforth and Foster, 2013; Boon et al., 2014). For example, *P. aeruginosa* can lyse *S. aureus* to obtain iron under iron-starvation conditions using LasA protease (Mashburn et al., 2005), and 4-hydroxy-2-heptylquinoline N-oxide (HQNO) allows *P. aeruginosa* to inhibit the cytochrome oxidase of *S. aureus* (Lightbown and Jackson, 1954; Machan et al., 1992; Toder et al., 1994). Furthermore, recent results demonstrated that compounds such as phenazines inhibit methicillin-resistant *S. aureus* (MRSA; Cardozo et al., 2013). Besides active competition, regulatory effects induced for instance by the resident flora can induce the expression of important virulence and metabolic genes in *P. aeruginosa* (Duan et al., 2003). In addition, positive interactions can also occur between microorganisms during polymicrobial infections. For example, *P. aeruginosa* can induce the formation of small colony variants in *S. aureus*, leading to an increased resistance of the latter to aminoglycoside antibiotics (Hoffman et al., 2006). This indicates that interspecies competition and cooperation play an important role in shaping composition and structure of polymicrobial bacterial populations, thereby potentially influencing disease progression. Increasing our understanding of these interactions is crucial (Dragoni et al., 2012) and might lead to the identification of new targets aiming at manipulating interactions inside polymicrobial communities to the disadvantage of pathogens such as *P. aeruginosa*.

In this perspective we developed a broad genetic screen to identify *P. aeruginosa* genes required for direct and exploitative competition. We searched for *P. aeruginosa* mutants affected in growth in the presence of *S. aureus*, *Klebsiella pneumoniae*, or *B. cepacia*. In addition, we examined *P. aeruginosa* mutants altered in their capacity to inhibit growth of *S. aureus*.

Materials and Methods

**Bacterial Strains, Growth Conditions and Media**

All *P. aeruginosa* mutants used in this study are derived from the clinical strain PA14 (Rahme et al., 1995; Table 1). All genetic manipulations were performed in *Escherichia coli* strain DH10B. *E. coli* strain SM10-λpir was used for conjugating plasmids into *P. aeruginosa*. Three bacterial pathogens were used for the initial screening: the *S. aureus* clinical strain COL (Dyke et al., 1966), a clinical *Burkholderia cepacia* isolate (University Hospitals of Geneva) and a non-capsulated *K. pneumoniae* strain (laboratory collection). The *S. epidermidis* and the *E. coli* strains were isolated from tracheal aspirates of two CF patients followed at the University Hospitals of Geneva (Geneva, Switzerland).

When required, plates were supplemented with 1 mM uracil, 1 mM arginine, or 2 mg/L biotin (final concentrations). For growth under hypoxic conditions, LB plates were supplemented with 5 mM KNO₃ and incubated in a jar using the GasPak Plus kit (BBL) (final O₂ concentration < 0.2%).

**Generation of the *P. aeruginosa* PA14 Transposon (Tn) Mutant Library**

The *E. coli* SM10-λpir donor strain containing pBT20 (Kulasekara et al., 2005) and the recipient *P. aeruginosa* PA14 were grown overnight at 37°C with shaking (250 rpm) in LB-broth. 200 μL of the donor and 100 μL of the recipient culture were mixed and centrifuged at 6,000 rpm for 2 min. The pellet was resuspended in 20 μL of LB and deposited on an LB agar plate. After 5 h of incubation at 37°C, cells were resuspended in 1 mL 0.9% NaCl and 100 μL of the suspension were plated on LB agar medium containing gentamicin (50 mg/L) and chloramphenicol (10 mg/L). To test for random distribution of Tn-insertions, 200 transconjugants were picked and streaked on minimal M9-salts medium supplemented with 0.2% glucose and 1 mM MgSO₄ and on an LB-agar plate. Approximately 1% of clones were auxotrophic, which is agreement with a random transposition event.

**Growth Competition Screening**

Overnight cultures of bacterial competitors were adjusted to OD₆₀₀ = 0.5. A 500 μL aliquot of fivefold (*S. aureus* and *S. epidermidis*) and 100-fold (*B. cepacia*, *K. pneumoniae*, and *E. coli*) dilutions were plated on agar plates. Bacterial lawns were incubated 2 h at 37°C. Overnight cultures of *P. aeruginosa* transposon mutants were adjusted to OD₆₀₀ = 2 and 1 μL of each culture was spotted on the competitor lawns using a 48-pin inoculator. As control, cells were spotted on a plate without competitors. Plates were incubated 18 h at 37°C. Mutants were initially screened for: (i) an alteration of the inhibition zone on the *S. aureus* lawn on Mueller Hinton agar, and (ii) an alteration of *P. aeruginosa* growth on lawns of *B. cepacia* and *K. pneumoniae* on LB-agar.

**Identification of the Transposon Insertion Site**

The transposon insertion site was identified by a two-step semi-random PCR (Friedman and Kolter, 2004; Kulasekara et al.,...
PA14 purF:: pqsCDE PCR fragment cloned into pIApX2 by using XbaI (Roche) and HindIII (Roche) This study

PA14 bioA:: pqsC complementation plasmid This study

PA14 purF:: pqsC complementation plasmid This study

PA14 purF:: pqsL complementation plasmid This study

PA14 Δ lasA LasA-deleted P. aeruginosa PA14 strain This study

PAO1 Δ pqsA pqsA::lux PqsA-deleted P. aeruginosa PAO1 strain with a copy of the pqsA promoter linked to the luxCDABE genes and inserted into a neutral site in the chromosome Fletcher et al. (2007)

PA14 mvrR− Mutated PA14 strain containing a nonsense point mutation in mvrR Cao et al. (2001)

PA14 pqsA− Mutated PA14 strain containing a non-polar deletion of pqsA Deziel et al. (2004)

PA14 pqsH− Mutated PA14 strain containing a aacC1 cassette inserted into pqsH Xiao et al. (2006a)

PA14 pqsL− Isogenic non-polar pqsL deletion mutant Lepine et al. (2004)

Escherichia coli SM10-λpir RPM-2-Tc; Mu recA, pir lysogen KmR Simon et al. (1983)

Staphylococcus aureus COL Clinical strain, MRSA Dyce et al. (1966)

Burkholderia cenocepacia Clinical strain University Hospitals of Geneva

Klebsiella pneumoniae Non-capsulated Laboratory collection

E. coli Clinical strain from CF-patient This study

S. epidermidis Clinical strain from CF-patient This study

2005). The first round PCR reaction contained dNTPs (0.25 mM final), Taq Buffer (1X final, Sigma), DMSO (5% final concentration), Taq polymerase (2.5 U/reaction, Sigma), and primers MCL195 and ARB1 (Table 2). Four microliters of bacterial lysate served as template. PCR cycling conditions were as follows: 95°C for 3 min, 36 cycles of 95°C for 20 s, 30 to 48°C for 40 s with a 0.5°C increase per cycle, 72°C for 1 min and a final extension at 72°C for 5 min. In the second round PCR reaction the nested primers ARB2 and MCL210 were used (Table 2). PCR conditions were as follows: 95°C for 3 min, 30 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 1 min and a final extension of 72°C for 4 min. PCR products were visualized on a 1.6% agarose gel. The most intense bands were extracted and purified (GeneJet Gel extraction kit, ThermoScientific). The PCR products were then submitted to DNA sequencing (Fasteris SA, Geneva, Switzerland) using the nested primers ARB2 and MCL210. Each sequence was aligned to the PA14 genome sequence in the Pseudomonas database (Winsor et al., 2011). Confirmation of the transposon insertion was done by PCR using gene and/or transposon specific primers. PCR cycling conditions were: (i) activation: 95°C for 2 min, (ii) denaturation: 95°C for 20 s, annealing: 57°C for 30 s, extension: 72°C for 1 min and 30 s, (iii) final extension: 72°C for 4 min. Step 2 was repeated 27 times. PCR products were purified and submitted to DNA sequencing.

**Exoprotein Analysis**

Rhamnolipid production was assessed following a previously described protocol (Kohler et al., 2000). Plates were incubated 18 h at 37°C, 24 h at room temperature and then 16 h at 4°C. The diameter of the rhamnolipid-containing halo formed around the bacterial colony was measured and compared with
that produced by the reference strain PA14. Determinations were done in duplicates.

Elastase activity was determined using the Elastin Congo Red assay with modifications (Pearson et al., 1997). Overnight cultures of *P. aeruginosa* strains were prepared in LB medium supplemented with 1 mM uracil to optimize the growth of the strain PA14 *carB::Tn*. Cultures were grown for 7 h at 37°C in PB medium (Essar et al., 1990). The OD600 was measured and elastase activity determined in supernatants using the Elastin Congo Red assay measuring absorption at OD495. A lld e t e r m i - nations were done in triplicate and expressed as the ratio of OD495/OD600.

### Extra-Chromosomal Complementations

The coding regions of *carB*, *pqsCDE*, *bioA*, and *purF* including 100 nucleotides upstream of the start codon were amplified by PCR from PA14 genomic DNA using the primers listed in Table 2 and following the same PCR conditions as described above. PCR products were purified and cloned into vectors pIpA2X (*pqsCDE*, *carB*) or pMMB207 (*purF*, *bioA*; Morales et al., 1991; West et al., 1994; Table 1). 1.5 μg of each vector and insert were digested with the appropriate restriction enzymes and buffers in a final volume of 25 μL (Table 1). After purification on agarose gel (GeneJet Gel extraction kit, ThermoScientific), vectors and inserts were ligated (T4 DNA ligase, Promega). *E. coli* DH10B thermo-competent cells were transformed with the ligation mixture and transformants selected on LB plates supplemented with 100 μg/mL ampicillin (*pqsCDE*, *carB*) or 15 μg/mL chloramphenicol (*purF*, *bioA*). The resulting plasmids pCarB and pPqsCDE were electroporated into the corresponding PA14 Tn-mutants and selected on LB-agar plates supplemented with 200 μg/mL carbenicillin. Plasmids pPurF and pBioA were transferred into the corresponding PA14 Tn-mutants by triparental mating using pRK2013 as a helper plasmid (Figurski and Helinski, 1979). Transconjugants were selected on LB-agar plates supplemented with 250 μg/mL chloramphenicol and 25 μg/mL gentamycin to counterselect *E. coli* donor strains.

### Construction of the *P. aeruginosa lasA* Mutant

A *lasA* deletion mutant was constructed by homologous recombination using plasmid pME3087 (Voisard et al., 2007). For the 5′-region PCR fragment, we used dNTP (0.2 mM final), Taq Buffer (1X final, Sigma), DMSO (5% final), Taq polymerase (2.5 U/reaction), and 4 μL of PA14 lysate. PCR conditions were as follows: one cycle of 2 min at 95°C, 27 cycles of 20 s at 95°C, 30 s at 55°C, 1 min at 72°C followed by a final extension of 4 min at 72°C. For the 3′-region PCR fragment, we used dNTP (0.2 mM final), PFU Buffer (1X final), DMSO (5% final), PFU polymerase (2.5 U/reaction), and 4 μL of PA14 bacterial lysate. PCR conditions were as follows: one cycle of 95°C for 2 min, 30 cycles of 20 s at 95°C, 30 s at 55°C, 1 min at 72°C, followed by a final extension of 4 min at 72°C. The 5′-region and the 3′ region fragments were digested with BamHI. Both fragments were ligated and PCR re-amplified using primers LasAF-Eco and LasAR-Hind (Table 2). The generated 1.6 kbp fragment was digested using EcoRI and HindIII restriction enzymes and cloned into plasmid pME3087. The resulting plasmid plasA1 was introduced into *E. coli* strain SM10pir for subsequent conjugation into *P. aeruginosa* PA14. Transductants were selected on M9-agar plates supplemented with 25 μM citrate and 1 mM MgSO4 and 75 μg/mL tetracycline (Tc). Individual colonies were repurified on LB-agar plates containing 75 μg/mL Tc. Putative *lasR* deletion mutants generated after the second recombination event were enriched by

### Table 2: Primers used in this study

| Primers | Amplified gene(s) | Sequences (5′–3′) | Final concentration (μM) |
|---------|------------------|------------------|------------------------|
| ARB1    | *purF*           | ACAGGATGATTTTGCGGCGAC | 0.6 |
| ARB2    | *bioA*           | ACAGGATGATTGCCGAC  | 0.6 |
| BioA-R  | *lasA*           | ACAGGATGATTGCCGAC  | 0.6 |
| CarB-F  | *lasA*           | ACAGGATGATTGCCGAC  | 0.6 |
| CarB-R  | *lasA*           | ACAGGATGATTGCCGAC  | 0.6 |
| LasAF-Eco| *lasA* region   | CCGCGATGATAGCGAT      | 0.6 |
| LasAR-Bam| *lasA* region   | CCGCGATGATAGCGAT      | 0.6 |
| LasAF-Bam| *lasA* region   | CCGCGATGATAGCGAT      | 0.6 |

*Restriction enzyme recognition sites are underlined*
carbenicillin-treatment as described previously (Voisard et al., 2007). Surviving cells, which were Tc-susceptible were screened by PCR for loss of a 300 bp fragment in the lasA gene. One of three clones was selected for further analysis.

Inhibition and Lytic Activity of P. aeruginosa Supernatants on S. aureus

We measured both inhibition of the S. aureus growth as well as lysis of S. aureus cells by P. aeruginosa supernatants.

The inhibition capacity of P. aeruginosa supernatants on S. aureus growth was tested in liquid. Overnight cultures of P. aeruginosa grown in MH-broth were centrifuged and the resulted supernatants were filtered (0.22 μm, Millipore). An overnight culture of S. aureus strain COL was adjusted to an OD600 of 0.1 in MH-broth. 100 μL of this bacterial suspension was incubated with 100 μL of P. aeruginosa supernatant. All mixtures were incubated in triplicate at 37°C with intermittent shaking and OD600 was recorded for 10 h in a plate reader (BioTek Synergy H1).

The lytic activity of P. aeruginosa overnight culture supernatants on S. aureus was tested on LB medium, supplemented or not with 1 mM uracil. The overnight cultures of P. aeruginosa were adjusted to the same OD600 before pelleting cells by centrifugation (6,000 × g, 10 min). Supernatants were filtered (0.22 μm) and used immediately. An overnight culture of S. aureus strain COL was adjusted to OD600 = 1 in 0.02 M Tris-HCl pH 7.5 and cells were inactivated by heating at 95°C for 10 min. 100 μL of the S. aureus bacterial suspension was incubated with 100 μL of P. aeruginosa supernatant. Mixtures were incubated at 37°C with intermittent shaking and OD600 was recorded during 8 h in a plate reader (BioTek Synergy H1).

Quantification of PQS Production

To test whether S. aureus affects PQS production by P. aeruginosa, an overnight culture of S. aureus strain COL was adjusted to an OD600 = 0.1 in LB medium. 190 μL of this dilution was deposited in a 96 well plate and incubated for 2 h without shaking. During this incubation, P. aeruginosa overnight cultures were centrifuged (6,000 rpm, 3 min) and the pellet adjusted to an OD600 = 2 in 0.9% NaCl. 5 μL of this cell suspensions of the P. aeruginosa PA14 and PA14 carB mutant were combined with 5 μL of the cell suspension of the PQS-indicator strain PA14 ΔpqsA pqsA::lux culture (Fletcher et al., 2007) and added to the 190 μL of the pre-incubated S. aureus cultures. The microtiter plate was incubated at 37°C with intermittent shaking and optical density at 600 nm and luminescence were recorded after 6 h. Experiments were done in duplicate.

Growth Competition Assay with Supernatants of Competitor Cultures

Pseudomonas aeruginosa cultures were also spotted on MH-plates seeded with 500 μL of filtered supernatants from overnight competitor cultures. Overnight (18 h) cultures of competitor bacteria were centrifuged 10 min at 6,000 rpm. Supernatants were filtered (0.22 μm) and 500 μL of the filtrate was spread on agar plates. P. aeruginosa cultures were prepared as described above.

Results

Generation of the P. aeruginosa PA14 Transposon Insertion Library and Selection of Mutants Affected in their Capacity to Compete with other Respiratory Pathogens

We generated a library of 2,288 transposon insertion mutants in P. aeruginosa strain PA14. Three bacterial pathogens also commonly found in the lungs of CF patients were selected for the initial competition screening: S. aureus, B. cepacia, and K. pneumoniae. The PA14 mutant library was screened for: (i) an alteration of the inhibition zone produced by P. aeruginosa on a lawn of S. aureus (Figure 1A), and (ii) an alteration of P. aeruginosa growth in the presence of B. cepacia and K. pneumoniae (Figure 1B). Based on this screening, 64 mutants were retained displaying an altered inhibition zone (35 mutants) or growth (25 mutants) or both (4) in the presence of competitors when compared to wild-type PA14. Each of the selected mutants was re-tested in quadruplicates and four yielded reproducible phenotypes. Two mutants showed a reduction of the inhibition zone on a lawn of S. aureus, while the two others were affected in growth on lawns of K. pneumoniae or B. cepacia. Growth of these mutants was not affected on agar plates in the absence of competitors (data not shown).

Role of the P. aeruginosa pqs Pathway During Competition with S. aureus

We determined the transposon insertion site in the P. aeruginosa clone most affected in inhibition of S. aureus growth, by a two-step semi-random PCR. The transposon was inserted at nucleotide position 136 of the pqsC gene, which belongs to the pqsABCDE operon (Xiao et al., 2006b). A pqsC mutant is deficient for the production of 4-hydroxy-2-alkylquinolines (HAQs; Dulcey et al., 2013). We complemented our pqsC transposon mutant by introducing a plasmid-encoded copy of the pqsCDE genes from PA14. The complemented mutant showed a partial restoration of the wild-type inhibition zone (Figure 2A). Because pqsC is part of a multigene operon, we tested the phenotype of strains mutated in other genes belonging to the PQS biosynthesis pathway. As expected, PA14 derivatives mutated in pqsA or mvfR (pqsR), both deficient in HAQ biosynthesis, did not inhibit S. aureus growth (Figure 2A). The same phenotype was observed for the pqsH mutant.

To test whether these phenotypes were limited to growth on solid medium, supernatants of P. aeruginosa overnight cultures were incubated together with a cell suspension of the S. aureus strain COL. Whereas supernatant of wild-type PA14 inhibited growth of S. aureus, supernatants from pqsA, pqsC, mvfR, and pqsH1 mutants showed no inhibitory activity. In agreement with the data obtained on solid medium, the pqsL mutant showed a partial inhibition of S. aureus growth in liquid medium (Figure 2B).

The LasA staphylolytic protease of P. aeruginosa cleaves the pentaglycine cross-links in the peptidoglycan of S. aureus leading to cell lysis (Kessler et al., 1993). We therefore tested the supernatant of a lasA mutant in competition with S. aureus, which did
not alter the wild-type inhibition profile comparable to the one of the wild-type PA14 in our model (Figure 2B). These results demonstrate that the observed inhibition profile is not the consequence of the expression of LasA, but can be associated to the pqs pathway activity in wild-type PA14. To further distinguish between inhibition and lytic activity, we performed the same experiment on heat-inactivated staphylococcal cells (Figure 2C). Optical density related to S. aureus cells rapidly decreased after addition of PA14 supernatants. We observed the same profile with the pqsC mutant, indicating that HAQs do not contribute to S. aureus cell lysis but to growth inhibition. In contrast, PA14 Δ lasA was affected in its capacity to lyse S. aureus as expected from previous reports.

Because products of the PQS pathway are known to suppress growth in Gram-positive bacteria by inhibiting the respiratory chain in aerobic condition, we hypothesized that this phenotype may be abolished under hypoxic conditions. As expected, we observed no inhibition zone even for PA14 under hypoxic conditions (Figure 2D). Because nitrate is a key compound for the growth of P. aeruginosa under hypoxic conditions, we performed the experiment without and with addition of KNO₃ as terminal electron acceptor. However, addition of KNO₃ had no effect on the phenotype. This suggests that HAQs are unlikely to play a role in S. aureus growth inhibition under hypoxic conditions.

Taken together our experiments suggest that the lasA gene product is responsible for the lytic activity of P. aeruginosa, while HAQs are responsible for growth inhibition of S. aureus under aerobic conditions.

**Impact of the carB Mutation During Direct Competition between P. aeruginosa and S. aureus**

The second mutant affected in its capacity to inhibit growth, and therefore to compete with S. aureus, harbored the transposon inserted at position 1,632 of the carB gene (PA14_62910), encoding the large subunit of the carbamoyl phosphate synthase (Winsor et al., 2011). CarB is involved in the pyrimidine pathway leading to uracil synthesis. Indeed, supplementation with uracil restored growth of the carB mutant, but not the S. aureus growth inhibition (Figure 3A). Recently, pyrF, another gene required for uracil and pyrimidine synthesis was shown to affect the quorum-sensing (QS)-circuit in strain PA14 (Ueda et al., 2009). We therefore tested the QS-dependent virulence factor production in the carB mutant. However, we observed no difference in rhamnolipid (Figure 3B) and elastase production (Figure 3C) between the carB mutant and the wild-type PA14. To determine if the effect observed with the carB mutant during competition with S. aureus was due to an alteration of the pqs pathway we measured the production of PQS by quantifying luminescence produced by the PAO1ΔpqsA pqsA::lux reporter strain. The quantity of luminescence produced was identical between the carB mutant and the wild-type PA14 strain, indicating that carB does not affect the PQS production (Figure 3D) during competition with S. aureus. These results suggest that carB is required for inhibition of S. aureus growth, through a QS-independent mechanism.

**Role of the carB Gene During Competition between P. aeruginosa, B. cepacia, and K. pneumoniae**

Two transposon mutants of P. aeruginosa were affected in their growth in the presence of B. cepacia and K. pneumoniae as competitors. One of these mutants was the carB mutant described above. This growth defect was complemented by the addition of a plasmid-encoded copy of carB, as well as by the addition of uracil (Figure 4A). Remarkably, growth of the carB mutant was not affected when competing with E. coli and S. epidermidis (data not shown). In contrast the addition of arginine alone, the second final product of this metabolic pathway (Figure 5), had no impact on the growth of the carB mutant (Figure 4B). To determine whether soluble factors secreted by the competitors could be involved, we plated supernatants of bacterial competitors on
Role of the \textit{pqs} pathway of \textit{P. aeruginosa} in direct competition with \textit{S. aureus}. (A) Effect of \textit{pqsC} disruption on the inhibition of \textit{S. aureus} growth. Spots of \textit{P. aeruginosa} cultures were deposited on LB agar plates seeded with a lawn of \textit{S. aureus} strain COL. Inhibition zone diameters were measured, normalized against the diameter of the corresponding spots and expressed as a percentage against the wild-type PA14 values. Error bars are standard deviations calculated on at least four replicates. Statistical significance was determined by using a \textit{t}-test with unequal variances (\(p < 0.01\)). (B) Inhibition activity of \textit{P. aeruginosa} supernatants on \textit{S. aureus} growth. Supernatants of \textit{P. aeruginosa} overnight cultures were incorporated on \textit{S. aureus} cells. \textit{S. aureus} growth (OD600) was monitored during 10 h at 37\(^\circ\)C in presence to these supernatants. (C) Lytic activity of \textit{P. aeruginosa} supernatants on inactivated \textit{S. aureus} cells. Supernatants of \textit{P. aeruginosa} overnight cultures were incorporated on heat-inactivated \textit{S. aureus} cells. OD600 was monitored during 8 h at 37\(^\circ\)C in presence to these supernatants to evaluate \textit{S. aureus} lysis. (D) Role of the \textit{pqs} pathway in hypoxic conditions. Spots of \textit{P. aeruginosa} cultures were deposited on LB agar plates covered by a \textit{S. aureus} lawn. Plates were incubated during 48 h in hypoxic conditions.

LB-agar plates prior to \textit{P. aeruginosa} spotting. Culture supernatants of \textit{B. cepacia} and \textit{K. pneumoniae} did not affect the growth of the \textit{carB} mutant, suggesting that the presence of metabolically active competing bacteria is required to repress growth of this mutant (Figure 4A). Taken together, these data demonstrate that the \textit{carB} gene, and by analogy the uracil/pyrimidine biosynthesis, is an essential metabolic pathway for the competition of \textit{P. aeruginosa} against \textit{B. cepacia}, \textit{K. pneumoniae}, and \textit{S. aureus}.

Effect of the \textit{purF} Mutation on the \textit{P. aeruginosa} Growth in Presence of \textit{B. cepacia}, \textit{K. pneumoniae}, and \textit{E. coli}

The second mutant with a growth defect in the presence of competitors had the transposon inserted at position 264 of the \textit{purF} (PA14_23920) gene, encoding an amidophosphoribosyl transferase. This protein is a key enzyme of purine metabolism, supplying nucleotides for DNA and RNA synthesis (Sampei and Mizobuchi, 1988). The \textit{purF}:\textit{Tn} mutant did not grow when cells were spotted on plates containing lawns of \textit{B. cepacia}, \textit{K. pneumoniae}, and \textit{E. coli} (Figure 4C). This alteration was complemented by the addition of a plasmid-encoded copy of the \textit{purF} gene. In contrast, the \textit{purF} mutant showed normal growth in the presence of \textit{S. epidermidis} and \textit{S. aureus} (data not shown). As observed above for the \textit{carB} mutant, culture supernatants of competing bacteria did not affect growth of the \textit{purF}-disrupted strain (Figure 4C). This suggests that the \textit{purF} gene is essential for \textit{P. aeruginosa} growth in presence of the \textit{B. cepacia}, \textit{K. pneumoniae}, and \textit{E. coli}.

Role of the Biotin Synthesis Pathway on Beneficial Interactions between \textit{P. aeruginosa} and other Respiratory Pathogens

To determine if growth of \textit{P. aeruginosa} can be promoted by the presence of other bacterial pathogens, we screened the transposon library for mutants that grew better in the presence of
a competitor than in its absence on rich medium. We identified one transposon mutant that grew poorly on MH-agar, but showed wild-type growth in the presence of *K. pneumoniae* and *E. coli* (Figure 4D). This growth "enhancement" was not seen in the presence of *B. cepacia*, *S. epidermidis*, and *S. aureus* bacterial lawns (data not shown). We identified the transposon insertion in this mutant at position 474 of the *bioA* (PA14_05460) gene, encoding an adenosylmethionine-8-aminooxononanoate aminotransferase, involved in the biotin synthesis pathway (Stoner and Eisenberg, 1975). Indeed the addition of biotin, as well as extra-chromosomic complementation with the *bioA* gene, restored growth of the *bioA*-disrupted mutant (Figure 4D). Finally, growth of the *bioA* transposon mutant was not restored by supernatants of *K. pneumoniae* and *E. coli*, suggesting that the presence of metabolically active cells of these organisms is required to complement the growth deficit (Figure 4D).

**Discussion**

Our study identified metabolic pathways involved in either detrimental or beneficial interactions between *P. aeruginosa* and four bacterial respiratory pathogens. By screening a transposon library of 2,288 mutants in PA14, we identified mutants displaying altered growth inhibition, or growth phenotypes in the presence of co-colonizing bacterial species. We found that both *pqsC* and *carB*, responsible, respectively, for HAQ and uracil/pyrimidine synthesis, play an essential role in the direct competition of *P. aeruginosa* with *S. aureus*. Secondly, we identified *carB* and *purF*, the latter involved in purine synthesis, as essential for exploitative competition against *K. pneumoniae* and *B. cepacia* (Figure 5). Finally, we found that co-colonizing strains may also have beneficial effects, as illustrated by growth restoration of the *P. aeruginosa* *bioA* mutant by *K. pneumoniae* and *E. coli* (Figure 5).

Studies on microbial interactions have already revealed the role of the *Pseudomonas* quinolone signal (PQS) biosynthesis pathway in the inhibition of *S. aureus* growth. It has been demonstrated that 2-heptyl-4-quinolone N-oxide (HQNO), one of the final products of the *pqs* pathway, suppresses growth of Gram-positive bacteria, but not of Gram-negative bacteria (Machan et al., 1992). In aerobic conditions, HQNO binds to quinone-reacting cytochromes and inhibits respiratory electron transfer from quinone to cytochromes (Magalon et al., 1998; Toyofuku et al., 2008). The present study confirms the role of the PQS-biosynthesis pathway in the direct competition with *S. aureus*. Indeed, *pqsC* acts at the beginning of the metabolic PQS-pathway and its inactivation leads to the absence of all HAQs including PQS, 2-heptyl-4-quinolone (HHQ), as well as their N-oxide derivatives. The *pqsC* mutant identified in our screening was unable to inhibit growth of *S. aureus* in rich medium. The wild-type phenotype was partially restored in the *pqsC* mutant by complementation with plasmid-encoded copies of the *pqsCDE* genes. The partial complementation can be explained by a gene dosage effect due to overexpression from the constitutive promoter present on the vector plasmid pIApX2. In addition, we
demonstrated that the PQS-pathway does not play a role under hypoxic conditions. This result was not unexpected since under hypoxic conditions, the aerobic respiratory chain is not functional and cannot be inhibited by HQNO (Machan et al., 1992). Furthermore, HQNO and PQS seem to be produced only under aerobic conditions due to the oxygen requirement of the PqsH mono-oxygenase to catalyze the final conversion of HHQ to PQS (Schertzer et al., 2010). This suggests that in the thickened mucous layers in the airways of CF patients, where low oxygen concentrations are prevailing (Worlitzsch et al., 2002), HQNO may play only a limited role in interference between P. aeruginosa and S. aureus. We can also hypothesize that the role of the PQS-system on S. aureus is limited in mixed biofilms, representing microaerophilic growth conditions. Nevertheless, HHQ and PQS were identified in sputum samples from CF patients (Collier et al., 2002; Palmer et al., 2005) indicating that maybe, in the more oxygenated upper respiratory tract, HQNO might play a more prominent role in growth inhibition of Gram-positive bacteria.

Our results further suggest that the N-oxide derivatives of HAQs are responsible primarily for the S. aureus growth inhibition. As described above, the pqsH mutant was affected in the conversion of HHQ to PQS. PQS contributes to the positive feedback regulation of the pqsA-D genes via PqsR. Thus, a defect in PQS production should lead to the absence (or major decrease) of HHQ and HQNO in a pqsH mutant. In addition our results showed a small residual activity of the pqsL mutant, which displayed only a weak inhibition of S. aureus growth both on agar and in liquid. PqsL is a putative mono-oxygenase converting HHQ to HQNO, and PQS to its N-oxide derivative (Lepine et al., 2004). A pqsL mutant was shown to produce increased amounts of PQS (D’Argenio et al., 2002), and maybe also of other HAQs, suggesting that the non-N-oxide derivatives may also display a weak growth inhibitory effect on S. aureus.

In addition to the role of PQS, we demonstrated that the P. aeruginosa carB gene is required for direct competition with S. aureus. Without uracil, the carB-disrupted strain exhibited an alteration in its capacity to inhibit S. aureus growth. However, the growth of this mutant appeared to be slightly affected. Nevertheless, when we added uracil in the medium, the growth of the carB-disrupted strain was totally restored, but the inhibition of S. aureus growth was still diminished. Previous reports suggested that uracil influences all three QS-systems in P. aeruginosa (i.e., las, rhl, pqs; Ueda et al., 2009). Since uracil is the final product of the biosynthesis pathway containing the carB gene, we tested if the phenotype exhibited by the carB-disrupted strain can be linked to QS. However, phenotypic analysis of QS-dependent traits showed that none of the three QS-systems was affected in the carB-disrupted strain. This
indicates that a QS-unrelated mechanism was responsible for reduced inhibition of \textit{S. aureus} growth in the \textit{carB}-disrupted strain.

Interestingly, the \textit{carB} gene also appeared essential for the exploitative competition between \textit{P. aeruginosa} and \textit{S. aureus}, as well as with \textit{B. cepacia} and \textit{K. pneumoniae}. These results suggest that \textit{carB} is involved in the competition for resources between \textit{P. aeruginosa} and these three competitors. \textit{CarB} is an enzyme catalyzing the transformation of L-glutamine into carbamoyl-phosphate, an intermediate in the pyrimidine, arginine, and proline metabolisms (Figure 5). We show that the phenotype of the \textit{carB}-disrupted strain can be restored by the addition of uracil, but not of arginine, demonstrating that the role of the \textit{carB} gene in the exploitative competition can be associated to the pyrimidine biosynthesis pathway.

In addition to \textit{carB}, the \textit{purF} gene also appeared to be an essential gene for exploitative competition. Interestingly, growth of the \textit{purF} mutant was not affected in the presence of Gram-positive bacteria (\textit{S. aureus} and \textit{S. epidermidis}) but was severely affected in grow in the presence of the Gram-negative strains tested (\textit{B. cepacia}, \textit{K. pneumoniae}, \textit{E. coli}). The \textit{purF} gene encodes an amidophosphoribosyltransferase involved in the conversion of L-Glutamine into 5-Phospho-ribosylamine, a primary product of the purine metabolism (Figure 5). Taken together, these results highlight the essential role of nucleic acid biosynthesis pathways for exploitative competition. Interestingly, the purine and pyrimidine synthesis pathways were also shown to be important for colonization of the mouse intestine by \textit{E. coli} (Vogel-Scheel et al., 2010), an environment where invading organisms have to compete with resident microbial flora.

Finally, we identified beneficial interactions between \textit{P. aeruginosa} and other lung co-colonizing species via the biotin synthesis pathway. Whereas a \textit{P. aeruginosa} \textit{bioA}-disrupted mutant was unable to grow on rich medium, the presence of \textit{K. pneumoniae} and \textit{E. coli} complemented the growth defect of this strain (Figure 5). In other words, the presence of \textit{K. pneumoniae} and \textit{E. coli} can be beneficial to \textit{P. aeruginosa} when biotin supply is limited. However, the molecular basis of this beneficial interaction remains to be elucidated.
Conclusion

Understanding the interactions between the various bacterial communities colonizing the CF-airways is of considerable importance as interactions can potentially affect the metabolism of pathogens, alter the population structure and eventually influence disease progression. It is clear from our experiments that complex interactions take place between CF-lung colonizing species which may lead to profound changes in bacterial community structures in CF-patients. Detrimental and beneficial interactions not only modulate the richness and the diversity of species but also create a complex environment in which bacterial species need to adapt to co-colonizing species aiming to increase their relative fitness. The metabolic pathways involved in these complex interactions could potentially be exploited to manipulate microbial population structure to improve the clinical outcome of chronic infections.

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