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1 **Volatile hydrogen cyanide released by *Pseudomonas aeruginosa* provides a**
2 **competitive advantage over *Staphylococcus aureus* in biofilm and *in vivo* lung**
3 **environments**
4
5
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30 **Running title:** *Pseudomonas-Staphylococcus* HCN-mediated competition.
31
32
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35
36

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ABSTRACT

Diverse bacterial volatile compounds alter bacterial stress responses and physiology, but their contribution to population dynamics in polymicrobial communities is not well known. In this study, we showed that airborne volatile hydrogen cyanide (HCN) produced by a wide range of *Pseudomonas aeruginosa* clinical strains leads to at-a-distance inhibition of the growth of a wide array of *Staphylococcus aureus* strains. We determined that low oxygen level environments not only enhance *P. aeruginosa* HCN production but also increase *S. aureus* sensitivity to HCN, which impacts *P. aeruginosa*-*S. aureus* competition in microaerobic *in vitro* mixed biofilms as well as in an *in vitro* cystic fibrosis lung sputum medium. Consistently, we demonstrated that production of HCN provides a competitive advantage to *P. aeruginosa* in a mouse model of airways co-infected by *P. aeruginosa* and *S. aureus*. Our study therefore demonstrates that *P. aeruginosa* HCN contributes to local and distant airborne competition against *S. aureus* and potentially other HCN-sensitive bacteria in contexts relevant to cystic fibrosis and other polymicrobial infectious diseases.

IMPORTANCE

Airborne volatile compounds produced by bacteria are often only considered as attractive or repulsive scents, but they also directly contribute to bacterial physiology. Here we showed that volatile hydrogen cyanide (HCN) released by a wide range of *Pseudomonas aeruginosa* clinical strains inhibits *Staphylococcus aureus* growth in low oxygen *in vitro* biofilms or aggregates and *in vivo* lung environments. These results are of pathophysiological relevance, since lungs of cystic fibrosis patients are known to present microaerophilic areas and to be commonly associated with the presence of *S. aureus* and *P. aeruginosa* in polymicrobial communities. Our study therefore provides insights into how a bacterial volatile compound can contribute to the exclusion of *S. aureus* and other HCN-sensitive competitors from *P. aeruginosa* ecological niches. It opens new perspectives for the management or monitoring of *P. aeruginosa* infections in lower lung airway infections and other polymicrobial disease contexts.

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1 **INTRODUCTION**
2
3 Bacteria release a wide diversity of volatile molecules contributing to cross-kingdom
4 interactions with fungi, plants and animal (1, 2). Bacteria volatile compounds also play a
5 role in bacterial physiology by altering stress responses, antibiotic resistance, biofilm
6 formation and expression of virulence factors. Although these interactions likely
7 contribute to bacterial population dynamics, relatively little is known regarding
8 interactions mediated by volatile compounds in polymicrobial communities (2-8). Cystic
9 Fibrosis (CF) is a common genetic disease in which the patients' airways are often
10 colonized by multiple bacterial pathogens, including *Pseudomonas aeruginosa* and
11 *Staphylococcus aureus*, that are frequently found in association in the same lung lobes
12 (9-15). Whereas *S. aureus* usually colonizes the airways first during CF infection, it is
13 later outcompeted and replaced by *P. aeruginosa* (12, 16-20).
14
15 Several *P. aeruginosa* extra-cellular factors inhibiting *S. aureus* growth could contribute
16 to this colonization shift during CF infection, including siderophores, 4-hydroxy-2-
17 heptylquinoline-*N*-oxide (HQNO), proteases, RedOx and surface active compounds (19,
18 21-26). By contrast, less is known about how competitive interactions of *P. aeruginosa*
19 are mediated via the production of volatile compounds and their impact on the dynamics
20 of coinfections with *S. aureus* (27-29).
21
22 It has long been recognized that *P. aeruginosa* metabolism produces volatile hydrogen
23 cyanide (HCN) that can rapidly diffuse into the environment (30, 31). HCN is an inhibitor
24 of cytochrome c oxidases and other metallo-enzymes that bind iron, leading to the
25 inhibition of the respiratory chain (30). HCN production is restricted to *Pseudomonas,*
26 *Chromobacterium*, *Rhizobium* and several cyanobacterial species that avoid auto-
27 intoxication by expressing HCN-insensitive cytochrome oxidase (31). *P. aeruginosa*
28 HCN is produced by the oxidative decarboxylation of glycine mediated by membrane-
29 bound cyanide synthases encoded by the *hcnABC* operon (32-34). *hcnABC* expression is
30 maximal between 34 °C and 37 °C and transcriptionally up-regulated in microaerophilic
31 conditions or by high bacterial cell density conditions (31, 35). Consistently, HCN
32 production by *P. aeruginosa* is regulated by the anaerobic regulator Anr, and the LasR
33 and RhlR quorum sensing regulators (36). HCN is therefore produced in environmental

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1 conditions leading to the induction of *P. aeruginosa* virulence factors, including the
2 synthesis of alginate a constituent of *P. aeruginosa* biofilms matrix and a major virulence
3 factor in the lungs of CF patients (37-39).
4 Considering that HCN was shown to poison a wide range of eukaryotic organisms (2, 40-
5 42), it was hypothesized that cyanogenesis could also poison HCN-sensitive bacteria in a
6 range of polymicrobial niches (26, 28, 30, 31, 39, 43, 44). However, whereas *P.*
7 *aeruginosa* HCN was shown to inhibit the growth of a wide range of Staphylococci,
8 including *S. aureus* (45), the direct contribution of HCN to *P. aeruginosa* dominance over
9 *S. aureus* within polymicrobial niches such as biofilms or infected lungs is still unclear.
10

11 Here we showed that exposure to airborne HCN produced by *P. aeruginosa* inhibits *S.*
12 *aureus* growth and influences the dynamics of *P. aeruginosa*-*S. aureus* interactions in *in*
13 *vitro* mixed biofilms. We determined that HCN production is widespread among *P.*
14 *aeruginosa* clinical strains and particularly active in low oxygen (microaerobic)
15 conditions against a representative panel of *S. aureus* isolates. We also demonstrated that
16 *P. aeruginosa* HCN impairs *S. aureus* growth in an *in vitro* CF lung sputum model as
17 well as in a mouse model of airway co-infection by *P. aeruginosa* and *S. aureus*. Our
18 study therefore shows that volatile HCN provides *P. aeruginosa* with a competitive
19 advantage in local and at-a-distance airborne competitions against *S. aureus* and
20 potentially other HCN-sensitive bacteria in context relevant to CF and other
21 polymicrobial infectious diseases.
22

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1 **RESULTS**
2
3 **Production of volatile hydrogen cyanide by *Pseudomonas aeruginosa* leads to**
4 **airborne inhibition of *Staphylococcus aureus* growth**
5 To determine whether HCN released by *P. aeruginosa* could inhibit the growth of *S.*
6 *aureus,* we first tested its HCN production by PAO1, a commonly used strain of *P.*
7 *aeruginosa* isolated from a wound infection **(46)**. Using a semi-quantitative HCN
8 detection method based on the intensity of blue color produced upon HCN reaction with
9 copper(II) ethylacetoacetate and 4,4'-methylenebis- (N,N-dimethylaniline) (47)
10 (Supplementary Fig. S1), we detected an HCN signal emitted from WT *P. aeruginosa*
11 PAO1 grown in LB, which increased upon glycine supplementation (Fig.1A). By contrast,
12 no HCN signal could be detected from a Δ*hcnB* mutant, which lacks HCN production
13 (Fig.1A). We then exposed *S. aureus* to *P. aeruginosa* PAO1 volatile compounds in the
14 set-up described in the supplementary Fig. S1. Whereas exposure to *P. aeruginosa* PAO1
15 cultures modestly reduced *S. aureus* MW2 growth, the aerial exposure to culture
16 supplemented with glycine led to a 100-1000-fold growth inhibition dependent on *hcnB*
17 (Fig.1B). Moreover, we observed that a *S. aureus* MW2 *srrAB* mutant lacking the SrrAB
18 global regulator of the transition from aerobic to anaerobic respiration displayed an
19 increased sensitivity to HCN (Supplementary Fig. S2) (48). Finally, preventing HCN
20 release by placing a parafilm seal on the emitting plate containing *P. aeruginosa* or *P.*
21 *aeruginosa* Δ*hcnB* culture supplemented with glycine did not lead to any growth defect,
22 confirming the contribution of volatile HCN to *S. aureus* MW2 growth inhibition
23 (Fig.1B).
24
25 **Production of biogenic HCN is widespread among *Pseudomonas aeruginosa* clinical**
26 **strains and active against diverse *S. aureus* isolates**
27 To determine whether HCN production is a widespread *P. aeruginosa* property, we
28 exposed the HCN-sensitive *S. aureus srrAB* mutant to a panel of laboratory and clinical
29 *P. aeruginosa* strains, many of them isolated from airway infections (Table 1). We
30 showed that, despite variations, all tested strains aerially inhibited *S. aureus srrAB* in
31 aerobic conditions, even in the absence of glycine (Supplementary Fig. S3A). Moreover,
32 *P. aeruginosa* strains that led to a minimal reduction of *S. aureus* growth showed a strong
33 growth inhibition phenotype when grown in the presence of glycine, indicative of an

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1 increase in HCN production (Supplementary Fig. S3B). In addition, we also showed that
2 the production of HCN by *P. aeruginosa* PAO1 in the presence of glycine inhibited the
3 growth of a wide panel of distinct pathogenic *S. aureus* strains (Supplementary Fig. S4),
4 confirming HCN-mediated growth inhibition at distance over a broad range of *S. aureus*
5 strains.
6
7 **Microaerobia enhances *P. aeruginosa* HCN production while increasing *S. aureus***
8 **sensitivity to HCN**
9 *P. aeruginosa* HCN production is regulated by quorum sensing (36). Consistently, we
10 observed an increase of the HCN signal during the transition from exponential to
11 stationary phase, at culture densities OD_{600}>2 (Fig. 2A). Compared to aerobic conditions,
12 we also observed that the *P. aeruginosa* HCN signal was enhanced in microaerobic
13 conditions (0.4-0.8% O_2) (Fig. 2BD) (33). Moreover, in these microaerobic conditions, *S.*
14 *aureus* MW2 was more sensitive to *P. aeruginosa* PAO1 HCN that when grown in
15 aerobic conditions (Fig. 2CE). These results suggest that microaerobic conditions not
16 only lead to higher HCN production in *P. aeruginosa* PAO, but also increased *S. aureus*
17 MW2 sensitivity to HCN.
18
19 **Production of biogenic HCN impairs *S. aureus* growth in *in vitro* mixed biofilms**
20 Our results suggested that HCN production could contribute to the dynamics of *P.*
21 *aeruginosa*-*S. aureus* competition. Considering that microaerobic conditions prevail
22 within multi-species biofilms (49) , we hypothesized that *P. aeruginosa* HCN production
23 in biofilms could impact *S. aureus* growth dynamics in mixed *P. aeruginosa / S. aureus*
24 biofilms. To test this *in vitro*, we co-inoculated continuous-flow biofilm microfermenters
25 with *P. aeruginosa* PAO1 WT (HCN+) and or Δ*hcnB* (HCN-) mutant at a 1:1 ratio with
26 three different *S. aureus* strains, including HG001, or Xen36 and MW2. While all strains
27 displayed similar individual biofilm-forming capacities (Fig. 3A), the *P. aeruginosa* and
28 *S. aureus* proportions in the resulting two-species biofilms formed after 48h showed that
29 all tested *S. aureus* strains formed less biofilm biomass, as measured by CFU count, when
30 mixed with WT *P. aeruginosa* than with the HCN-deficient mutant (Fig. 3B). Taken
31 together, these results indicate that the production of *P. aeruginosa* biogenic HCN
32 impairs growth and outcompete *S. aureus* in mixed biofilms.

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Production of biogenic HCN provides a competitive advantage to *P. aeruginosa* over *S. aureus* in CF-relevant conditions

To test whether production of HCN could provide *P. aeruginosa* with a competitive advantage over *S. aureus* in CF-relevant conditions, we first used the synthetic CF sputum (SCFM2) medium, designed to recapitulate human CF environments (50). We inoculated this medium with red fluorescent *S. aureus* LAC*dsrfp* alone or in 1:1 mix ratio with *P. aeruginosa* PAO1*gfp* WT (HCN+) or its Δ*hcnB* (HCN-) mutant. The comparison of the respective *S. aureus* and *P. aeruginosa* spatial organization revealed a strong reduction of *S. aureus* biomass development (Fig. 4A) and aggregate abundance when co-inoculated with WT *P. aeruginosa* (Fig. 4B top), in contrast to the opposite increase of *S. aureus* development in presence of the *P. aeruginosa* Δ*hcnB* mutant (Fig. 4B bottom). To further test the *in vivo* impact of HCN production on *S. aureus/P. aeruginosa* mixed community dynamics in the microaerophilic lung airways, we performed an *in vivo* competition in mice, in which lungs were intratracheally co-inoculated with *S. aureus* Xen36 strain and either *P. aeruginosa* PAO1 WT or Δ*hcnB*. Mice lungs infected individually show that *P. aeruginosa* colonizes better than *S. aureus* Xen36 (Fig. 4C left) The comparison of the number of CFUs extracted from mice lungs co-inoculated in a 2:1 mixed ratio (*P. aeruginosa : S. aureus*) showed a 2 log decrease of *S. aureus* Xen36 in presence of WT PAO1 (HCN+) as compared to *S. aureus* growth in presence of the PAO1 Δ*hcnB* mutant (HCN-) (Fig.4C). Taken together, our results demonstrate that HCN production by *P. aeruginosa* reduces *S. aureus* colonization in co-infected mouse lungs and other microaerobic biofilm-like environments.

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DISCUSSION

In this study, we showed that airborne HCN produced by a wide range of *P. aeruginosa* clinical strains is enhanced in microaerobic conditions and inhibits various *S. aureus* isolates *in vitro*, disadvantaging *S. aureus* colonization in polymicrobial biofilms. This occurs both in a CF sputum medium and in an *in vivo* mouse model of pulmonary co-infection. A number of bacterial infections are characterized by the development of polymicrobial communities in which complex interactions between bacteria can influence the outcome of diseases (12, 17, 43, 51). Colonization of the lungs during CF is one of the best examples of polymicrobial infection that is characterized by excessive mucus production in airways and decreased mucosal clearance (52). This favors lung colonization by bacterial pathogens, including *P. aeruginosa*, *S. aureus*, non-typeable *Haemophilus influenzae* and *Burkholderia cepacia*, where prevalence varies with the age and treatments of CF patients (52-55). These airway infections are difficult to eradicate despite aggressive antibiotic therapy (56, 57) and are associated with inflammation, leading to a progressive decline in lung functions and, ultimately, to respiratory failure (53, 57-59).

Interactions between microorganisms have been shown to be key determinants of their distribution and activity in most ecosystems (12, 58) with several *P. aeruginosa* secreted molecules shown to inhibit *S. aureus*'s growth (16, 19, 25, 26, 60). By contrast to local competition driven by short range diffusion (<10 µm) of most inhibitory metabolic products, volatile HCN produced by *Pseudomonas* and a number of bacterial species could play an important role in the spatial organization of microbial communities (31). HCN could indeed contribute to both local and distant, airborne competition between microorganisms in physically heterogeneous solid, liquid and gaseous environments such as the lungs and other organic tissues.

Our results are of pathophysiological relevance, since CF lungs are known to present microaerophilic areas and to be commonly associated with the presence of *S. aureus* and *P. aeruginosa* multispecies biofilms, reaching high *P. aeruginosa* cell density, two conditions that have been shown to induce *hcnABC* gene expression and subsequent HCN

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1 production (30, 31, 34, 36, 61). HCN was indeed previously detected in the sputum and
2 bronchial-alveolar lavage fluids of CF patients infected by *P. aeruginosa* and the measure
3 of HCN levels in lungs of CF patients has been used as a non-invasive breath test to
4 diagnose *P. aeruginosa* infection (44, 62-64). This suggests that the levels of HCN
5 produced by *P. aeruginosa* in the lung environment could be sufficient to poison aerobic
6 metabolism and growth, excluding competitors from *P. aeruginosa* ecological niches (39,
7 44).
8
9 *S. aureus* had been regarded as one of the initial microbial colonizers of the CF patients'
10 airways before being displaced by *P. aeruginosa* (14, 20) and our results support the
11 hypothesis that metabolic poisoning upon HCN production could be a key determinant of
12 *Pseudomonas* distribution in the lung upon exclusion of *S. aureus* in mixed *in vitro and*
13 *in vivo* polymicrobial biofilms (19, 28, 34, 39). However, *P. aeruginosa* PAO1 was also
14 shown to reduce its toxicity towards *S. aureus* or to facilitate *S. aureus* microcolony
15 formation through alginate production, therefore promoting the coexistence of these two
16 bacteria (13, 65-67). Consistently, we observed that, although production of HCN by
17 PAO1 WT reduced the number of *S. aureus* recovered from co-inoculated lungs
18 compared to co-inoculation with PAO1Δ*hcnB*, there was a 1000-fold increase in *S. aureus*
19 abundance when comparing mono-inoculation and co-inoculation with PAO1Δ*hcnB* (Fig.
20 4C). This indicates that, in absence of HCN, *S. aureus* growth is stimulated by *P.*
21 *aeruginosa,* which further emphasizes that these two bacteria could engage in complex
22 negative and positive interactions *in vivo* (67, 68).
23
24 Our study therefore contributes to a better understanding of *P. aeruginosa* and *S. aureus*
25 competition in a context relevant to CF airway infection. Whereas further studies will be
26 required to tease out the respective ecological contribution of HCN and other *P.*
27 *aeruginosa* factors to outcompete *S. aureus*, our results further illustrate the remarkable
28 ability of *P. aeruginosa* to adapt and thrive in multispecies communities. The
29 identification of a volatile compound-based mechanism potentially underlying the
30 dynamic shift from *S. aureus* to *P. aeruginosa* dominance in polymicrobial infection
31 opens new perspectives for the management or monitoring of *P. aeruginosa* infections in
32 lower lung airway infections and other polymicrobial disease contexts.
33

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1 **MATERIALS AND METHODS**

2

3 ***Bacterial strains, plasmids and growth conditions.***

4 Bacterial strains and plasmids used in this study are listed in Table 1. All experiments
5 were performed in lysogeny broth (LB) medium, supplemented or not with 0.4% (w/v)
6 glycine and incubated at 37°C. All chemicals were purchased from Sigma-Aldrich.

7 ***Test in SCFM2 artificial sputum model.***

8 Green fluorescent *P. aeruginosa* (WT or HCN mutant) carrying pMRP9-1 and *S. aureus*
9 expressing dsRed red fluorescent protein (see Table 1) were grown overnight in Tryptic
10 Soy Broth (TSB). Cells were washed twice and resuspended in PBS. The optical density
11 at 600 nm (OD_{600}) was measured with a spectrophotometer, and washed bacterial cultures
12 were inoculated into SCFM2 at an OD_{600} = 0.05 (~10^7 CFU/mL) as individuals or in
13 combination. Cultures were vortexed for 5 to 10 s to disperse bacterial cells in SCFM2.
14 Five hundred microliters of inoculated SCFM2 was then transferred into each well of
15 four-well microchamber slides (Lab-Tek; Nunc) and incubated under static conditions at
16 37°C.

17 ***Imaging:*** All images were acquired with Zeiss LSM 700 and LSM 880 confocal laser
18 scanning microscopes utilizing Zen image capture software. Bacterial cells were
19 visualized via GFP with an excitation wavelength of 488 nm and an emission wavelength
20 of 509 nm or via dsRed with an excitation wavelength of 587 nm and an emission
21 wavelength of 610 nm or with a 63× oil immersion objective. SCFM2 images were
22 acquired by producing 512- by 512-pixel (0.26- by 0.26-µm pixels) 8-bit z-stack images
23 that were 100 µm from the base of the coverslip. The total volumes of 100-µm z-stack
24 images were 1822.5 mm^3. Control images of uninoculated SCFM2 were acquired by
25 using identical settings to determine the background fluorescence for image analysis.

26 ***Image analysis:*** All imaging was performed with identical image capture settings. To
27 determine the background fluorescence in SCFM2, a histogram of detected dsRed and
28 GFP fluorescence was produced in Imaris v 8.3.1 (Bitplane) for uninoculated SCFM2,
29 and the average of the three highest voxel values was determined as the background
30 fluorescence. Averaging across all of the control images, this value was then subtracted
31 from all experimental images with Imaris. For aggregate and biomass quantification in

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1 SCFM2, isosurfaces were produced for all remaining voxels after background subtraction
2 with the surpass module in Imaris. To detect individual aggregates, the split objects option
3 in Imaris was enabled and aggregates were defined as objects with volumes of $>5\ \mu m^3$.
4 The total biomass (all voxels detected), average aggregate volume and number of
5 aggregates were calculated within the vantage module in Imaris. Detected aggregate
6 isosurfaces were then ordered by volume. Objects that were ≥ 0.5 and $\leq 5.0\ \mu m^3$ were
7 categorized as dispersed biomass, and objects that were $>5.0\ \mu m^3$ were categorized as
8 aggregated biomass. All image data were exported into Microsoft Excel 2016, and graphs
9 were generated with GraphPad Prism 7.
10
11 ***Screening for volatile-mediated HCN phenotypes***
12 To evaluate the activity of HCN released by bacterial liquid culture on recipient test
13 bacteria, a lidless 3.5 cm Petri dish was placed inside a 9 cm Petri dish, which external
14 ring was filled with 20 mL of 1.5% LB agar (Supplementary Fig. S1A) (4). Tested
15 recipient bacteria were spotted as 20 µL drops of 10^{-4} to 10^{-8} serial dilutions of an
16 overnight culture adjusted to OD_{600} = 1 filled and *P. aeruginosa b*acterial liquid culture
17 releasing or not volatile HCN were adjusted to OD_{600} = 3 and introduced in the middle of
18 an uncovered Petri dish. The large Petri dish was then closed and incubated for 24 h at
19 37°C, in aerobic or microaerobic conditions. Exposure under microaerobic conditions
20 (0.4 - 0.8% O_2) was performed in a C400M Ruskinn anaerobic-microaerophilic station.
21
22 ***Detection of HCN production***
23 Semi quantitative determination of the levels of HCN production used a method adapted
24 from previous studies (47). Briefly, using the set-up described in Supplementary Fig.
25 S1A , Whatman chromatography paper soaked into HCN detection reagent containing
26 copper(II) ethyl acetoacetate (100mg) and 4,4'-methylenebis-(N,N-dimethylaniline)
27 (100mg) solubilized in 20 mL chloroform was laid on the surface of the central,
28 uncovered 3.5cm Petri dish of containing bacterial liquid culture releasing or not volatile
29 HCN. The large Petri dish was then closed and incubated for 24 h at 37°C in aerobic or
30 microaerobic conditions. Exposure under microaerobic conditions (0.4 – 0.8% O_2) was
31 performed in a C400M Ruskinn anaerobic-microaerophilic station. The level of HCN was
32 evaluated based on the intensity of blue color resulting from exposure to bacterial HCN.

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1 ***In vivo experiments***

2 Specific opportunistic pathogen free (SOPF) Balb/c mice (male, 7 weeks, in particular
3 free of detectable *S. aureus* and *P. aeruginosa* strain) were ordered in Janvier Labs
4 (France) and housed in the Institut Pasteur animal facilities. All experiments were
5 approved by the Ethics Committee of Institut Pasteur (reference 2014-0014). Mice were
6 infected intratracheally as described previously (19). In brief, mice were anesthetized by
7 intraperitoneal injection of ketamine (Imalgene 1000®, 90mg/kg)/xylazine (Rompun®,
8 10mg/kg) suspended in PBS. The anesthetized animals were subjected to non-invasive
9 intratracheal catheterization through which *P. aeruginosa* (1×10^6 CFU) and/or *S. aureus*
10 (5×10^5 CFU) suspended in 50 µL of PBS was/were introduced to initiate the infection.
11 Twenty-four hours post infection, the animals were sacrificed by intraperitoneal injection
12 of a lethal dose of pentobarbital. The lungs were harvested and homogenized as described
13 previously (19). The lung homogenates were serially diluted, and the number of bacterial
14 CFU in the lung was determined by plating and counting bacteria on LB agar (all bacteria)
15 and/or on *P. aeruginosa* selective PIA plates and *S. aureus* selective MSA plates.

16

17 ***Biofilm competition experiments in microfermenters***

18 Continuous-flow biofilm microfermenters containing a removal glass spatula were used
19 as described in (69) (see also https://research.pasteur.fr/en/tool/biofilm-
20 microfermenters/**)**. Medium flow was adjusted to 60 mL/h with internal bubbling
21 agitation with filter-sterilized compressed air to minimize planktonic growth over biofilm
22 development. Inoculation was performed by dipping the glass spatula for 10 min in
23 overnight OD_{600}=1 LB cultures of *S. aureus* and *P. aeruginosa* strains mixed at a 1:1
24 ratio. The spatula was then reintroduced into the microfermenter and the resulting 48 h
25 mixed biofilms grown on the fermenter spatula were recovered and corresponding serial
26 dilutions were plated on LB agar (all bacteria) and/or on *P. aeruginosa* selective PIA
27 plates and *S. aureus* selective MSA plates.

28

29 ***Statistical analysis***

30 Two-tailed unpaired *t-test* with Welch correction analyses were performed using Prism
31 9.0 for Mac OS X (GraphPad Software). Each experiment was performed at least three
32 times.

33

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AUTHORS CONTRIBUTION

S.L., Y.W., S.E. D., J.-M.G and C.B. performed the experiments; J.-M.G., S.L., L.T., C.B., M.W and S.E.D designed the experiments. S.L., C.B., L.T. S.E.D, M.W and J.-M.G., analyzed the data. J.-M.G. provided resources and funding. J.-M.G. wrote the manuscript with significant contribution from all co-authors.

COMPETING INTEREST

We declare no competing financial interests.

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1 **Table 1: Plasmids and strains used in the study**
2

	Relevant characteristics or origin	**Reference / source**
Plasmids		
pMRP9-1	*gfp*-expressing plasmid	(70)
***Pseudomonas aeruginosa* strains**		
PAO1	Isolate from wound infection, Melbourne	(46)
PAO1*gfp*	Green fluorescent PAO1 containing pMRP9-1	This study
PAO1 Δ*hcnB*	HCN deficient Δ*hcnB* strain	University of Washington Genome Center, Gift from O. Lesouhaitier
PAO1 Δ*hcnB gfp*	Green fluorescent PAO1Δ*hcnB* containing pMRP9-1	This study
PA14	Clinical isolates from burn patients	(71)
PAK	Virulent strain sensitive to Pf phage	(72)
7508	Bronchial secretion	(73)
8931	Lung transplant	(73)
9854	Nasal swab	(73)
11989	Tonsil swab	(73)
12269	Sputum	(73)
13305	Bronchial secretion	(73)
Psae1152	Drainage catheter	(73)
Psae1471	Respiratory tract	(73)
Psae1659	Respiratory tract	(73)
Psae1716	Blood	(73)
Psae1928	Respiratory tract	(73)
Psae2328	Urine	(73)
BJN8	Catheter related blood stream infection related BSI from Beaujon Hospital. Patient#8.	(74)
BJN33	Catheter related blood stream infection related BSI from Beaujon Hospital. Patient#33.	(74)
BJN53	Catheter related blood stream infection related BSI from Beaujon Hospital. Patient#53.	(74)
BJN66	Catheter related blood stream infection related BSI from Beaujon Hospital. Patient#66.	(74)
Staphylococcus aureus		
MW2	Community-acquired methicillin-resistant	(75)
MW2*ΔsrrAB*	Deletion of *srrAB* genes	Gift from I. Lasa (76)
15981	Biofilm-forming strain isolated at the Microbiology Department of the University Clinics of Navarra	(77)
COL	Initially isolated from the operating theatre in a hospital in Colindale, England in the early 1960s.	(78)
Newman	*S. aureus* strain Newman was isolated in 1952 from a human infection	(79)
Xen36	Xen 36	Caliper Life Sciences,
HG001	Highly virulent strain derivative of NCTC 8325 originally used to propagate bacteriophage 47	(80)
N315	hospital-acquired methicillin-resistant isolated in 1982 from a pharyngeal smear of a patient in Japan.	(81)
USA300LAC	Epidemic community-associated methicillin-resistant isolated from the Los Angeles County jail	(82)
USA300LAC*dsred*		(83)
V329	Biofilm forming bovine mastitis isolate	(84)

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FIGURES

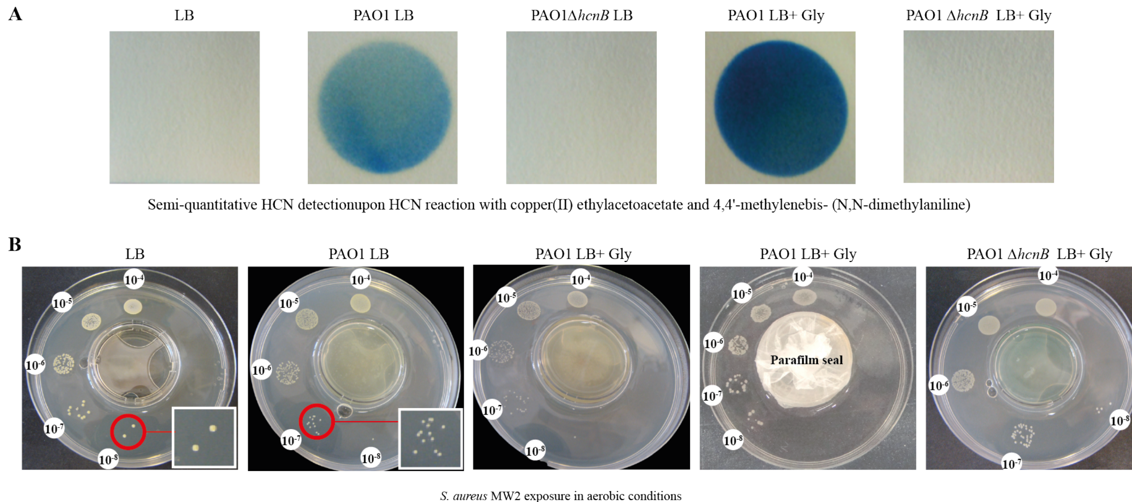

Figure 1. ***P. aeruginosa* production of hydrogen cyanide leads to airborne inhibition of *S. aureus* growth. A:** Semi-quantitative detection of volatile HCN emitted from *P. aeruginosa* WT and Δ*hcnB,* in LB supplemented or not with 0.4% (w/v) glycine, after 24h incubation at 37°C in aerobic conditions. HCN detection is based on HCN reaction with copper (II) ethylacetoacetate and 4,4'-methylenebis-(N,N-dimethylaniline). **B:** Serial dilution of *S. aureus* MW2 upon exposure to *P. aeruginosa* WT or Δ*hcnB* cultures in LB supplemented or not with 0.4% (w/v) glycine in the 2-Petri-dish assay (see Supplementary Fig. S1). No inhibition of *S. aureus* MW2 growth is observed when the middle plate containing *P. aeruginosa* culture is covered and sealed with parafilm. Pictures were taken after 24h of incubation at 37°C in aerobic conditions. Each experiment was performed at least three times.

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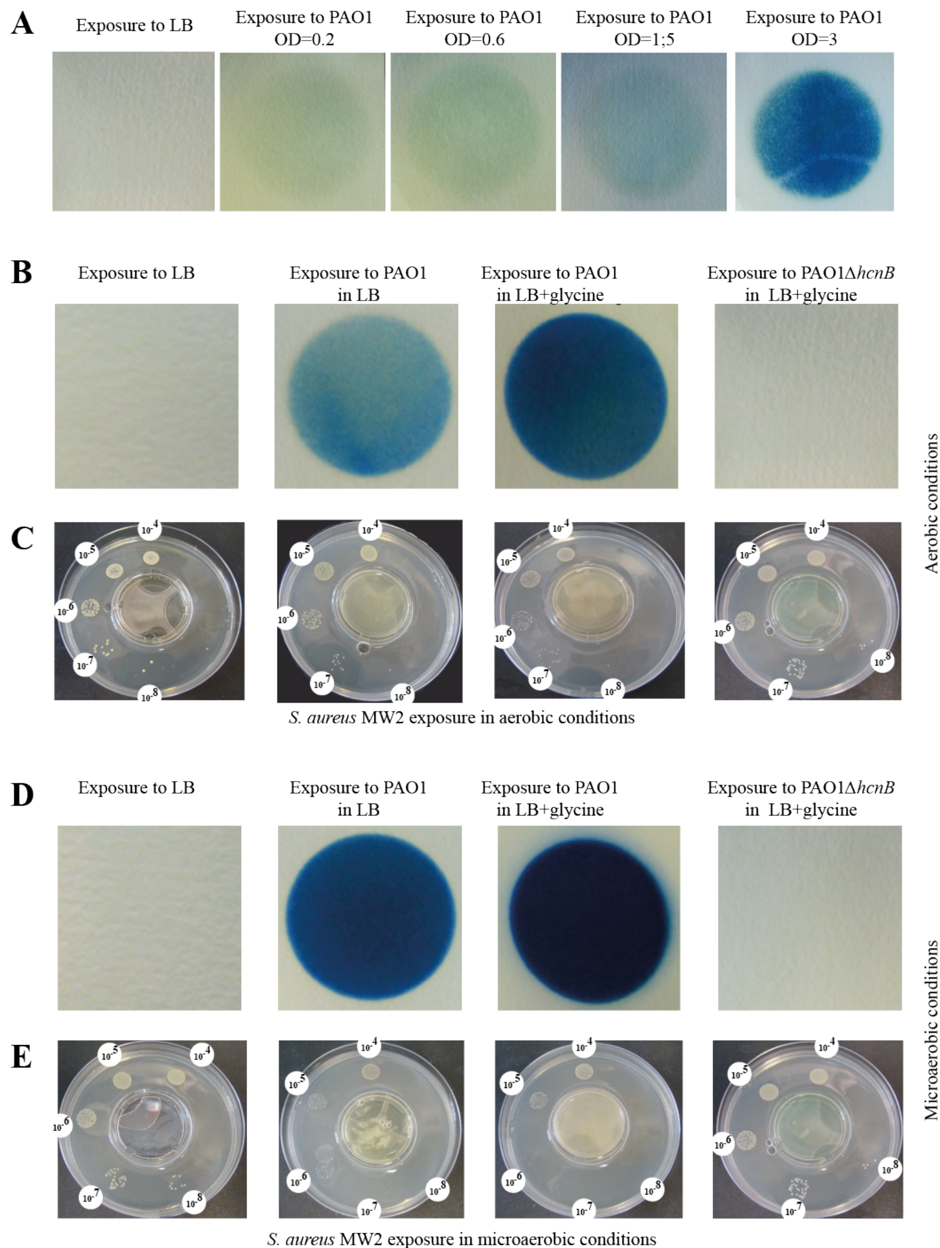

Figure 2. **Microaerobia stimulates *P. aeruginosa* HCN production and increase *S. aureus* sensitivity to HCN. A:** Semi-quantitative detection of volatile HCN showed an increased production of HCN by *P. aeruginosa* PAO1 at different stages of growth stages in LB medium. Each experiment was performed at least three times. **B, D:** Semi-quantitative detection of volatile HCN from *P. aeruginosa* WT and Δ*hcnB,* in LB supplemented or not with 0.4% (w/v) glycine, after 24h incubation at 37°C in aerobic (B) or microaerobic (D) conditions. Each experiment was performed at least three times. **C,E:** Growth of serial dilution of *S. aureus* MW2 WT upon exposure to *P. aeruginosa* WT or Δ*hcnB* cultures in LB supplemented or not with 0.4% (w/v) glycine, after 24h incubation at 37°C in aerobic (C) or microaerobic (E) conditions, using the 2-petri-dish assay described in Fig S1. Each experiment was performed at least three times.

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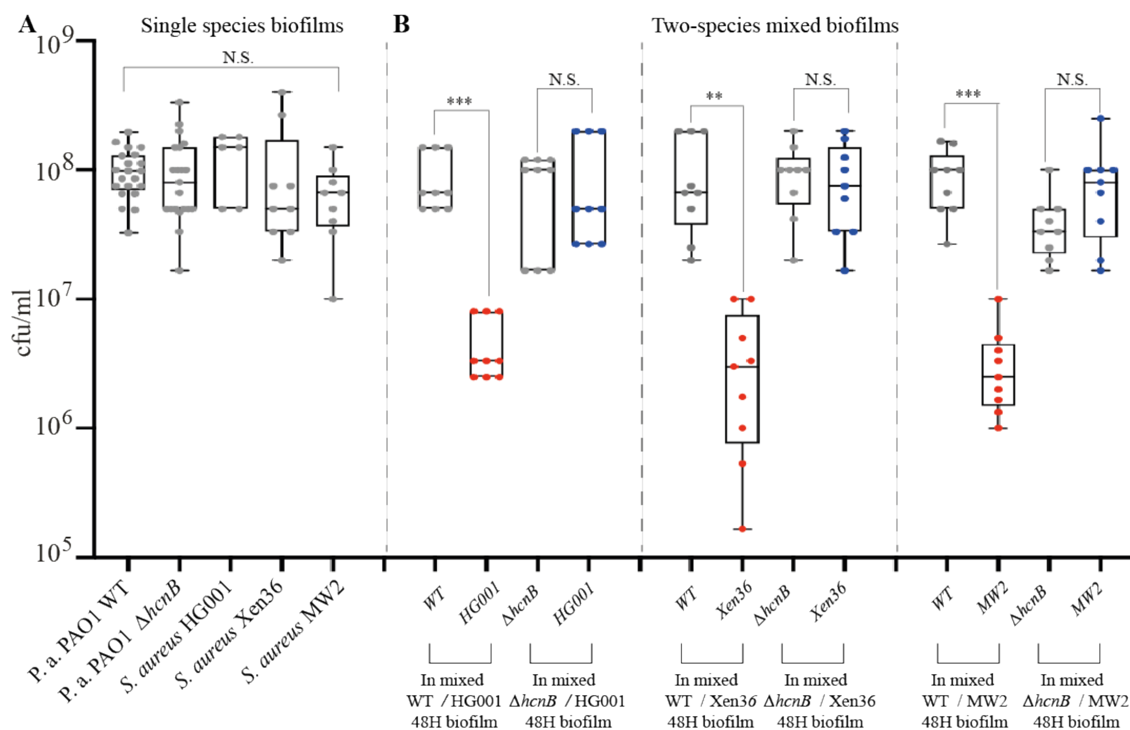

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3 Figure 3. **Production of biogenic HCN impairs *S. aureus* growth in *in vitro* mixed biofilms.**
4 **A:** Number of CFU in indicated single species biofilms grown in continuous-flow
5 microfermenters, in LB medium for 48h at 37°C. **B:** Number of CFU in indicated two-species
6 mixed biofilms: each *S. aureus* strain was mixed with either WT *P. aeruginosa* PAO1 or its Δ*hcnB*
7 mutant at a 1:1 ratio. The biofilms were grown in continuous flow microfermenters in LB medium
8 for 48h at 37°C. Statistics correspond to two-tailed unpaired *t-test* with Welch correction.
9 N.S.: not significant, ** $p \leq 0.01$ and *** $p \leq 0.001$.
10

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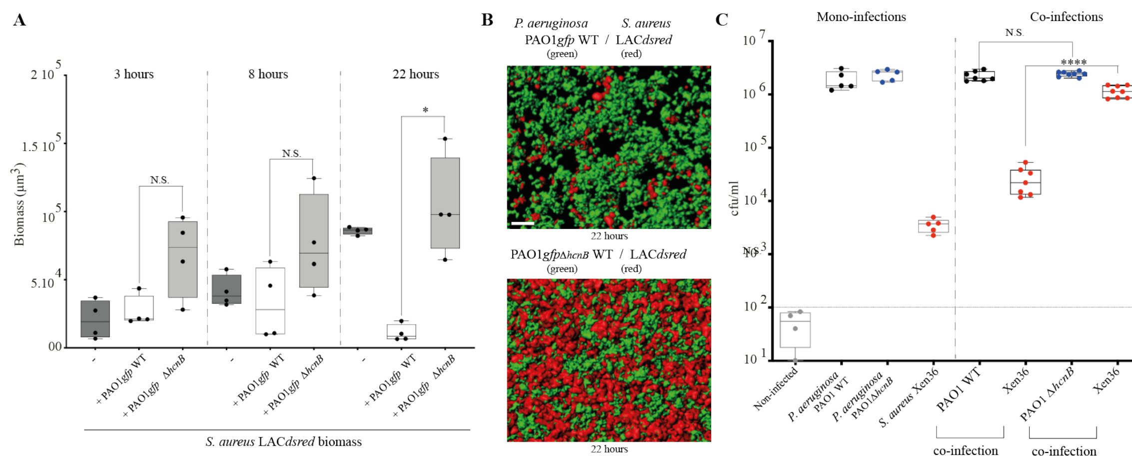

Figure 4. ***P. aeruginosa* production of biogenic HCN inhibits *S. aureus* growth in in CF-relevant conditions. A:** Total biomass of *S. aureus* aggregates as monoculture or in co-infection with PAO1 wildtype and/or *hcnB* mutant. Isolates were cultured in SCFM2 and imaged using confocal microscopy at 3, 8 and 22 hours. Statistics correspond to two-tailed unpaired *t-test* with Welch correction. N.S.: not significant, * p≤0.05. **B:** Representative rendered confocal micrograph of *S. aureus* and *P. aeruginosa* coinfection in SCFM2. Top – wild type *P. aeruginosa* in green, *S. aureus* in red. Bottom – *P. aeruginosa hcnB* mutant in green, *S. aureus* in red. **C**: *In vivo* competition experiments in mice lungs. Mono and 1:2 mixed ratio co-inoculation of *S. aureus* Xen36, *P. aeruginosa* PAO1 WT or Δ*hcnB* mutant. Number of bacteria CFU was counted in the lung homogenates of mice 24 h after infection. Non infected SOPF mice showed minimal lung bacterial contamination with CFY<100 (horizontal dotted line). Statistics correspond to two-tailed unpaired *t-test* with Welch correction. N.S.: not significant, *** p≤0.0001.