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Small-Colony Variant Selection as a Survival Strategy for *Staphylococcus aureus* in the Presence of *Pseudomonas aeruginosa*▽

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Previously it has been demonstrated that *Staphylococcus aureus* is sensitive toward *Pseudomonas*-secreted exotoxins, which preferentially target the electron transport chain in staphylococci. Here it is shown that a subpopulation of *S. aureus* survives these respiratory toxins of *Pseudomonas aeruginosa* by selection of the small-colony variant (SCV) phenotype. Purified pyocyanin alone causes the same effect. A *hemB* mutant of *S. aureus* survives cocultivation with *P. aeruginosa* without a decrease in CFU.

Pseudomonas aeruginosa and *Staphylococcus aureus* are opportunistic pathogens and frequently coinfect the lungs of patients with cystic fibrosis (CF). *P. aeruginosa* excretes an arsenal of small respiratory inhibitors, like pyocyanin (5), hydrogen cyanide (2), or quinoline *N*-oxides (9), that may act against the commensal microbiota as well as host cells. Previously it has been demonstrated that *S. aureus* is sensitive toward the toxic products generated by *P. aeruginosa* and that these exotoxins preferentially target the electron transport chain (17).

Staphylococcal species can be divided into two groups: the sensitive group, comprising pathogenic species such as *S. aureus* and *S. epidermidis*, and the resistant group, represented by nonpathogenic species such as *S. carnosus*, *S. piscifermentans*, and *S. gallinarum*. The resistance in the latter group was due to *cydAB* genes, which encode a pyocyanin- and cyanide-resistant cytochrome *bd* quinol oxidase (17). It has also been shown that the resistant or sensitive phenotype is determined by the CydB subunit, which is part of the cytochrome *bd* quinol oxidase of *S. aureus.* Despite its sensitivity to these exotoxins, *S. aureus* has frequently been coisolated with *P. aeruginosa* from the skin, eyes, and catheter infections and from the lungs of patients with CF. The aim of this study is to elucidate the escape mechanism of *S. aureus* by cocultivating *S. aureus* and *P. aeruginosa*. The findings indicate that a subpopulation of the staphylococcal community can survive in the presence of *P. aeruginosa* by the selection of small-colony variants (SCVs), which usually are defective in the electron transport chain. SCVs grow as tiny, nonpigmented colonies and are auxotrophic to hemin, menadione, or thymidine (14). Here we show that both a culture supernatant of *P. aeruginosa* and purified pyocyanin select for the SCV phenotype in *S. aureus*.

Cocultivation of *S. aureus* and *P. aeruginosa* can select for *S. aureus* SCVs. *S. aureus* was grown in monoculture or in coculture with *P. aeruginosa* (1:1, optical density at 578 nm) in tryptic soy broth (TSB) medium under biofilm or planktonic conditions. Biofilm studies using *S. aureus*(pCtuf-*gfp*) and *P. aeruginosa*::pUT-tell-*rfp* grown in TSB medium supplemented with 0.5% glucose under static conditions for 36 h showed that both *S. aureus* and *P. aeruginosa* form thicker biofilm in monocultures, while in a mixed biofilm with *P. aeruginosa* only few *S. aureus* cells were visible (Fig. 1A).

Titers of *S. aureus* grown under planktonic conditions in monoculture and in coculture were determined by plating 0.1-ml samples on Chapman agar (selection medium for staphylococci) after diluting appropriately with TSB medium. The samples grew almost parallel up to 10^9 CFU for approximately 8 h. Then, the CFU of the *S. aureus* cells in coculture declined steadily for the next 24 h (Fig. 1B). This correlates exactly with the onset of pyocyanin production in *P. aeruginosa*, as it has been shown that *P. aeruginosa* produces pyocyanin in an aerobic medium when cells enter the stationary phase, at approximately 1.2×10^9 cells/ml in batch culture (15). In contrast to the growth of *S. aureus*, the growth of *S. carnosus* did not decline in cocultivation with *P. aeruginosa*, because of its pyocyanin- and cyanide-resistant cytochrome *bd* quinol oxidase (Fig. 1C). *P. aeruginosa* titers were determined by plating 0.1-ml samples on Cetrimid agar (selection medium for *P. aeruginosa*) after diluting appropriately. No difference in the CFU of *P. aeruginosa* grown in monoculture and coculture was observed (data not shown).

S. aureus colonies had the appearance of SCVs after 24 h of cocultivation (Fig. 2A and B). Typically, this phenotype disappeared upon subculture, indicating that the growth limitation is reversible. Equivalent results were obtained with *P. aeruginosa* PAO1 and SH1 strains and with different clinical isolates of *S. aureus*. The stationary-phase culture supernatant of *P. aeruginosa* can select for SCVs (Fig. 2D).

Pyocyanin is secreted into the medium by *P. aeruginosa* in copious amounts. Previously it has been shown that pyocyanin blocks the respiratory chain of *S. aureus* (17). To determine whether pyocyanin can induce electron transport-defective SCV selection, pyocyanin was isolated and purified and its purity was verified with high-performance liquid chromatography as described earlier (4, 9, 17). To see the effect of pyocyanin on *S. aureus*, we used a disc diffusion assay in which sterile filter discs were spotted with various concentrations of pyocyanin and

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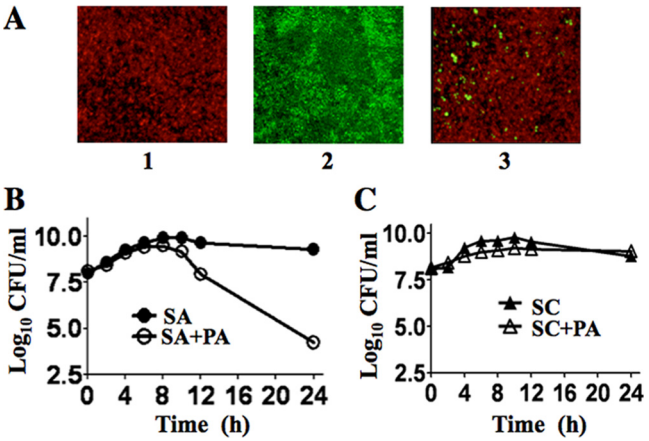

FIG. 1. (A) Confocal laser scanning microscopy analysis of biofilm formation by *S. aureus*(pCtuf-*gfp*) and *P. aeruginosa*::pUT-tell-*rfp*, expressing red fluorescent protein (RFP) and green fluorescent protein (GFP), respectively, after 24 h. (1) *P. aeruginosa*. (2) *S. aureus*. (3) *S. aureus* and *P. aeruginosa*. (B) Planktonic growth of *S. aureus* in monoculture (SA) and in coculture with *P. aeruginosa* (SA+PA). (C) Growth of *S. carnosus* in monoculture (SC) and in coculture with *P. aeruginosa* (SC+PA).

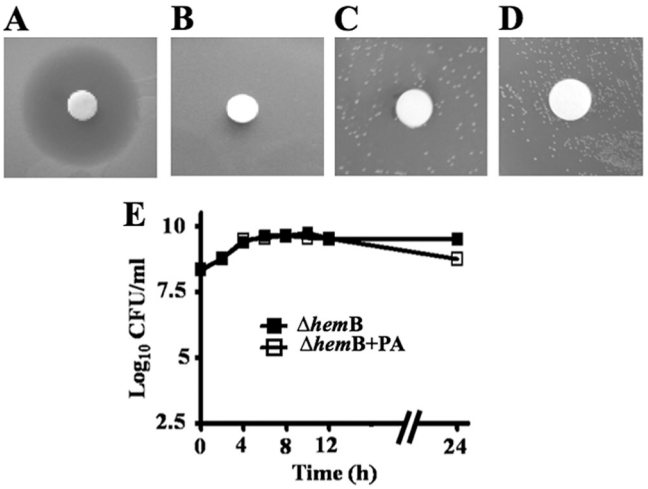

FIG. 3. Sensitivity of a defined electron transport-deficient SCV mutant, *S. aureus* Δ*hemB*. (A to D) Agar disc diffusion assay with filter discs containing 25 μl culture supernatant of *P. aeruginosa.* The agar contains embedded cells of wild-type *S. aureus* (A) or *S. aureus* Δ*hemB* (B). The effect of pyocyanin on *S. aureus* Δ*hemB* is shown, with discs containing buffer as a control (C) and purified pyocyanin (D). (E) Growth of *S. aureus* Δ*hemB* in monoculture (Δ*hemB*) and in coculture with *P. aeruginosa* (Δ*hemB*+PA).

placed on top of the soft agar containing *S. aureus* cells. *S. aureus* formed increasing numbers of smaller colonies with increasing pyocyanin concentrations. At the highest concentration (100 nM), most of the colonies appeared to be smaller and dispersed (Fig. 2E). At a concentration of more than 200 nM pyocyanin, a zone of inhibition surrounded by a zone of SCVs was observed (data not shown). The selection of electron transport-defective SCVs in the presence of pyocyanin allows *S. aureus* to coexist with *P. aeruginosa*.

Decreased pigment formation is one of the characteristics of SCV production of staphyloxanthin (3, 12) in *S. aureus* strains. Therefore, four different *S. aureus* strains (*S. aureus* Newman, *S. aureus* COL, *S. aureus* Mu50, and *S. aureus* USA300) were grown on tryptic soy agar (TSA) and TSA supplemented with a 25% (vol/vol) culture supernatant of *P. aeruginosa*. No staphyloxanthin production was observed in the TSA plates supplemented with the culture supernatant of *P. aeruginosa*; in addition, the colonies grew very slowly and had an SCV phenotype (data not shown).

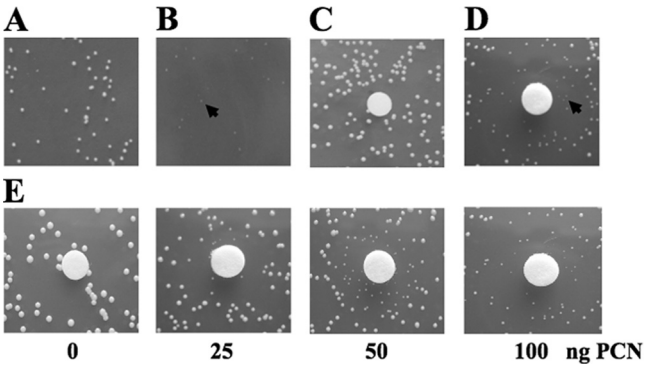

FIG. 2. Pyocyanin induces SCV selection in *S. aureus*. Arrows indicate SCVs. (A) *S. aureus* grown in monoculture exhibits a normal phenotype. (B) The *S. aureus* SCV phenotype is observed after 24 h of growth in coculture with *P. aeruginosa.* (C to E) Agar disc diffusion assay with *S. aureus.* Filter discs contained buffer as a control (C), a filter-sterilized culture supernatant of *P. aeruginosa* (D), and various concentrations of purified pyocyanin (PCN) (E).

The persistence of *S. aureus* in CF has previously been associated with the isolation of a subpopulation of *S. aureus* with SCV phenotypes (11, 13). Therefore, a defined electron transport-deficient SCV mutant, *S. aureus* Δ*hemB*, which is auxotrophic for hemin (18), was tested for its sensitivity toward the culture supernatant of *P. aeruginosa* and purified pyocyanin. *S. aureus* Δ*hemB* was insensitive to both the culture supernatant and purified pyocyanin (Fig. 3A to D). The viability of *S. aureus* Δ*hemB* when grown in coculture with *P. aeruginosa* was found to be unaffected until 12 h; there was only a slight decrease of CFU after 24 h (Fig. 3E).

In patients with CF, *S. aureus* is often the initial pathogen colonizing the lungs (10, 16). Mainly, *S. aureus* infects the lungs of children up to 10 years; however, *P. aeruginosa* is the predominant pathogen in adolescents and adults (1, 8). It has been shown that *S. aureus* persists in the airways of patients with CF over extended periods by transforming into SCVs. *P. aeruginosa* produces an arsenal of virulence factors, which include respiratory inhibitors like pyocyanin, hydrogen cyanide, and alkyl-hydroxyquinoline *N*-oxides (HQNO). Pyocyanin is a blue redox-active secondary metabolite that is readily recovered in large quantities in sputum from patients with CF who are infected by *P. aeruginosa*. It is shown here that the culture supernatant of *P. aeruginosa* and purified pyocyanin can select for an electron transport-deficient SCV phenotype in *S. aureus* and that the defined electron transport-deficient SCV mutant *S. aureus* Δ*hemB* is resistant to both. HQNO have also been shown to target the electron transport chain (7) and have recently been shown to induce SCV selection in *S. aureus* (6). Our results have shown that the secreted respiratory inhibitors

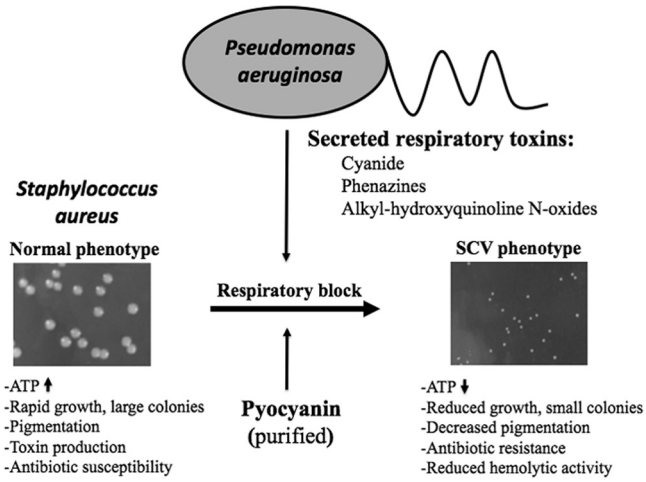

FIG. 4. Illustration of *P. aeruginosa*-induced *S. aureus* SCV selection. (Left) Colony size of *S. aureus* under normal conditions. (Right) In the presence of respiratory toxins, like HQNO or the *Pseudomonas* quinolone signal, pyocyanin or cyanide produced by *P. aeruginosa* leads to selection of the electron transport-deficient SCV phenotype in *S. aureus*.

of *P. aeruginosa* suppress the aerobic metabolism and growth of *S. aureus*. However, *S. aureus* as a versatile pathogen can evade this suppression with the help of selection of a respiration-defective subpopulation that is characterized by the SCV phenotype. The blockage of the electron transport pathway drives *S. aureus* to fermentative growth, which is accompanied by a decreased ATP yield and slower growth that finally results in the formation of smaller colonies (Fig. 4). SCVs are known to persist better than their normal counterparts within host cells, are resistant to aminoglycoside antibiotics, and are difficult to detect. This study explains why SCV phenotypes of *S. aureus* are frequently isolated from patients with CF.

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