JOURNAL OF BACTERIOLOGY, Dec. 2006, p. 8079–8086 Vol. 188, No. 23
0021-9193/06/$08.00+0 doi:10.1128/JB.00858-06


# Microevolution of Cytochrome *bd* Oxidase in Staphylococci and Its Implication in Resistance to Respiratory Toxins Released by *Pseudomonas*†

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Received 15 June 2006/Accepted 24 June 2006

***Pseudomonas aeruginosa* and *Staphylococcus aureus* are opportunistic pathogens and frequently coinfect the lungs of cystic fibrosis patients. *P. aeruginosa* secretes an arsenal of small respiratory inhibitors, like pyocyanin, hydrogen cyanide, or quinoline N-oxides, that may act against the commensal flora as well as host cells. Here, we show that with respect to their susceptibility to these respiratory inhibitors, staphylococcal species can be divided into two groups: the sensitive group, comprised of pathogenic species such as *S. aureus* and *S. epidermidis*, and the resistant group, represented by nonpathogenic species such as *S. carnosus*, *S. pisciferm entans*, and *S. gallinarum*. The resistance in the latter group of species was due to *cydAB* genes that encode a pyocyanin- and cyanide-insensitive cytochrome *bd* quinol oxidase. By exchanging *cydB* in *S. aureus* with the *S. carnosus*-specific *cydB*, we could demonstrate that CydB determines resistance. The resistant or sensitive phenotype was based on structural alterations in CydB, which is part of CydAB, the cytochrome *bd* quinol oxidase. CydB represents a prime example of both microevolution and the asymmetric pattern of evolutionary change.**

A number of bacterial infections result from the colonization of more than one microorganism (12). *Pseudomonas aeruginosa* and *Staphylococcus aureus* are associated with diseases such as chronic lung infections in cystic fibrosis patients and peritoneal infections in patients undergoing dialysis (20). *P. aeruginosa* is an opportunistic pathogen that produces an arsenal of virulence factors that may act against the commensal flora as well as host cells. These virulence factors include a number of secreted and surface-associated molecules such as pilus adhesins (25), phospholipase (40), proteases (7, 36), ADP-ribosylating enzymes (37), and rhamnolipid biosurfactants (10, 29). Also among the secreted molecules are enzymes such as LasA, a staphylolytic endopeptidase (23), as well as small respiratory inhibitors like pyocyanin (19), hydrogen cyanide (5), and a mixture of quinoline N-oxides (28). Although it is thought that the antistaphylococcal activity in *Pseudomonas* supernatants is due mainly to LasA, our knowledge of the compounds and their antistaphylococcal activities is still fragmentary.

As most studies have focused primarily on monoculture infections, little is known about how and whether the *Pseudomonas* and staphylococcal strains interact with each other. Here, we show that *P. aeruginosa* exhibits an antagonistic relationship with *S. aureus* and other pathogenic staphylococci through its secreted respiratory inhibitors pyocyanin and cyanide. The nonpathogenic staphylococcal species resist these respiratory toxins released by *P. aeruginosa* due to *cydAB* genes that encode a pyocyanin- and cyanide-insensitive cytochrome *bd* quinol oxidase that oxidizes ubiquinol and reduces oxygen as part of the electron transport chain (6).

## MATERIALS AND METHODS

**Transposon mutagenesis and selection of mutants.** *Staphylococcus carnosus* TM300, harboring plasmid pTV1ts, was grown overnight at 30°C in basic medium (BM) containing 5 μg ml$^{-1}$ erythromycin and 10 μg ml$^{-1}$ chloramphenicol. The culture was subsequently diluted 1:1,000 in BM broth containing 2.5 μg ml$^{-1}$ erythromycin and was incubated twice at 40°C for 12 h. Appropriate dilutions of the bacterial suspension were spread onto BM agar containing 2.5 μg ml$^{-1}$ erythromycin and incubated at 37°C. Erythromycin-resistant and chloramphenicol-sensitive mutants were further screened on BM agar plates containing 30% (vol/vol) filter-sterilized culture supernatant of *P. aeruginosa* PAO1 and monitored for impaired growth. Tn*917*-specific primers were used to sequence genomic DNA upstream and downstream from the transposon insertion sites.

**Construction of the *S. aureus cydAB* deletion mutant.** Primers SAcydA up F (5′-TACATTGCTAGCAAATGAATCCATTCTTAGG-3′; introduced restriction site is underlined) and SAcyd up R (5′-TATCATAAGCTTCGCAGAATG ATTGTCCACC-3′) were used to amplify the upstream flanking region of *cydAB* from the chromosomal DNA of *S. aureus* SA113. The PCR product was cloned into the NheI/HindIII sites of pBT2, creating plasmid pBT2-F1. Primers SAcydB downF (5′-TACATTGGATCCTTGAGACGATACCCCAAC-3′) and SAcydB down R (5′-TATCATGAATTCCCAGTCATTATGAAGGTAAAC-3′) were used to PCR amplify the downstream flanking region of *cydB.* The PCR product was cloned into the BamHI/EcoRI sites of pBT2-F1, yielding pBT2-F1-F2. The erythromycin cassette (*ermB*) from pEC2 was ligated into the HindIII/BamHI sites of pBT2-F1-F2, generating pBT2-KO1. All recombinant plasmids were introduced into *Escherichia coli* DH5α. Plasmids were introduced into staphylococci via electroporation (2). Allelic replacement of wild-type *cydAB* genes by *ermB* was carried out as described previously by Brückner (4). The sequence of the altered genes of the resulting strain *S. aureus cydAB*::*ermB* was confirmed by PCR and DNA sequence analyses.

**Construction of $cydAB_{Sa}$ and $cydAB_{Sc}$ expression plasmids.** Primers SAcydA F (5′-TACATTGGATCCAAAAGGTGATGTTTTTAAATG-3′) and SAcydB R (5′-TATCATCTGCAGTTATGATTTCTTTCCTTC-3′) were used to amplify the *cydAB* genes from *S. aureus* SA113 genomic DNA. The PCR product after digestion with BamHI at one end was ligated to BamHI/SmaI-digested pCX19, resulting in plasmid pCXcydAB$_{Sa}$. Expression of *S. aureus cydAB* (*cydAB$_{Sa}$*) genes was induced by the addition of 0.5% xylose to cultures at an optical density

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† Supplemental material for this article may be found at http://jb.asm.org/.

at 578 nm ($OD_{578}$) of 0.5. *S. carnosus* TM300 *cydAB* (*cydAB*$_{Sc}$) genes with their native promoter were amplified by PCR from genomic DNA using the primers SCcydA F (5′-TACATTAGTACTCTTTATTAAAAGTGA-3′) and SCcydB R (5′-TTATTGCTGCAGTTAATAATGACCTTCTTC-3′). The resulting PCR product was cloned into ScaI/PstI sites of pCX19, generating plasmid pCcydAB$_{Sc}$; the recombinant plasmid lacked the *xylR* regulation gene. This plasmid was used to complement *S. carnosus cydAB* mutants. Ligation mixtures were transferred into staphylococci by protoplast transformation (15).

**Exchanging *cydB* of *S. aureus* with *cydB* of *S. carnosus*.** The primer pair SaCydA promF (5′-ATTATAAGATCTCACAATTTCATAGCGC-3′) and SaCydABR (5′-TGAACCTGGTACCAACAATACCGTGCC-3′) was used to amplify *cydA* and the first part of the *cydB* gene (including the first 81 amino acids) of *S. aureus*. The PCR product obtained was restricted with BglII/KpnI and ligated with plasmid pRB473 precut with BamHI/KpnI. The plasmid obtained was designated pRBcydA. *S. carnosus cydB* was amplified from codon 81 using the primer pair ScCydBF (5′-GTATTACTGGTACCAGGGTCTATTGGATTG-3′) and SchiscydBR (5′-TACATTGAGCTCTTAATAATGACCTTCTTCAC-3′). The amplicon obtained was restricted with KpnI/SacI and ligated into pRBcydA precut with the same enzymes. The plasmid generated was named as pRBcydA$_{Sa}$B$_{Sc}$. Primers SaCydABR and ScCydBF were designed to anneal to the overlapping sequence of the *cydB* of both *S. aureus* and *S. carnosus*, and a KpnI site was generated by a single nucleotide exchange without altering the amino acid sequence.

**Measurement of oxygen uptake.** *S. aureus* clones were precultivated aerobically in the presence of xylose to induce the plasmid-encoded *cydAB* genes. Cells were pelleted from a culture grown in BM broth for 12 h, washed, and resuspended in 33 mM potassium phosphate buffer (pH 7.0) to a final volume of 1.5 ml ($OD_{578}$ of 50) at 25°C. The washed cell suspensions were analyzed for oxygen consumption using a Clark-type oxygen electrode. Respiration was initiated by the addition of 50 mM succinate as an electron donor to the cell suspension. After 30 to 40% of the oxygen was consumed, i.e., approximately 5 min after the addition of succinate, freshly prepared sodium cyanide solution (1.5 mM) was added.

**Purification and analysis of pyocyanin.** Pyocyanin was isolated as described previously (8). For isolation of pyocyanin, *P. aeruginosa* was cultivated in various media: tryptic soy (TS) broth, pyocyanogenic medium (succinate minimal medium with 0.1 mM potassium phosphate buffer), and apyocyanogenic medium (succinate minimal medium with 4 mM potassium phosphate buffer) (3). Purified pyocyanin was analyzed by reverse-phase high-performance liquid chromatography (HPLC) using a Hypersil ODS $C_{18}$ column (5 μm, Grom. 125 by 4.6 mm) with a solvent flow rate of 350 μl $min^{-1}$. The flow consisted of 2 min of 8% acetonitrile–25 mM ammonium acetate followed by a 25-min linear gradient to 80% acetonitrile–25 mM ammonium acetate. Pyocyanin was detected at 313 nm.

**Agar disk diffusion assay.** *P. aeruginosa* strains PAO1 and SH1 were incubated in TS agar medium at 37°C for 24 h. Cells were pelleted by centrifugation, and the culture supernatant was filtered through 0.22-μm-pore-size filters and stored at 4°C. Staphylococcal cells (100 μl of a culture grown overnight at 37°C in TS broth medium) were mixed with 4 ml of TS soft agar (TS broth plus 0.75% agar) and poured onto TS agar plates. Sterile filter disks spotted with 25 μl of *P. aeruginosa* culture supernatant were placed on top of the soft agar. Plates were incubated at 37°C and photographed after 12 h.

**CydAB partial purification.** Cell pellets obtained from a 1-liter culture were resuspended in 30 ml digestion buffer (30% raffinose, 25 mM Tris-HCl [pH 7.5], 145 mM NaCl) containing 200 μg/ml lysostaphin, 5 μg DNase, and 1 mM phenylmethylsulfonyl fluoride. The protoplasts obtained after incubation at 37°C were broken by two passages through a French pressure cell. All further steps were carried out at 4°C. Unbroken cells were removed by centrifugation at 11,200 × *g* for 10 min. Membranes were pelleted at 100,000 × *g* for 1 h, resuspended in 50 mM Tris-HCl (pH 7.5) containing 2% sodium cholate, and stirred for 1 h before centrifugation at 100,000 × *g* for 1 h. The pellet obtained was resuspended in 100 mM Tris-HCl (pH 7.5), and dodecyl maltoside was added to a final concentration of 1.5% (wt/vol). The suspension was stirred for 1 h before centrifugation at 45,000 × *g* for 30 min.

**Heme staining.** The partially purified CydAB proteins were subjected to native polyacrylamide gel electrophoresis without boiling and stained for heme-dependent peroxidase activity (31). Briefly, the gel was incubated in 10% trichloroacetic acid for 30 min at 25°C. The gel was rinsed with water before it was incubated in the dark for 1 to 2 h in the heme stain. This stain contained 3 parts of 6.3 mM 3,3′,5,5′-tetramethylbenzidine dissolved in methanol and 7 parts of 0.25 M sodium acetate, pH 5.0. The gel was developed for 10 to 20 min by adding 60 mM $H_2O_2$. Finally, the gel was placed into a stop solution containing 30% isopropyl alcohol and 0.25 M sodium acetate, pH 5.0. Lastly, the gel was photographed.

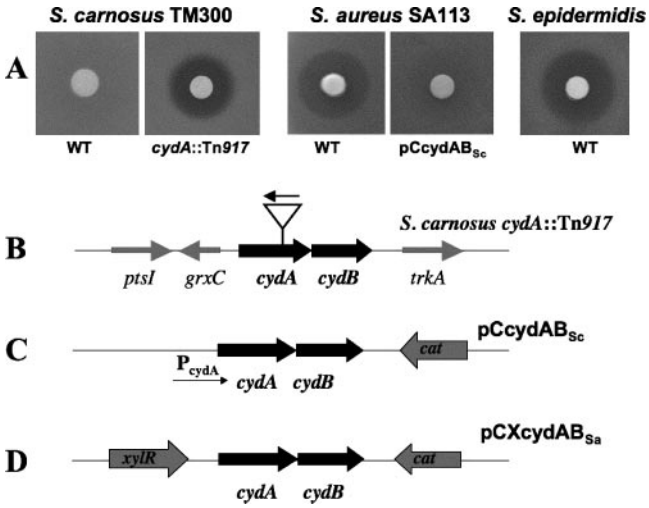


FIG. 1. (A) Agar disk diffusion assay with wild-type (WT) *S. carnosus* (17) and its *cydA*::Tn*917* mutant, wild-type *S. aureus* SA113, *S. aureus* harboring pCcydAB$_{Sc}$, and wild-type *S. epidermidis*. Filter disks were impregnated with 25 μl filter-sterilized culture supernatant of *P. aeruginosa*. (B) Gene organization of the *S. carnosus cydAB* region with the Tn*917* insertion site. (C) Relevant part of plasmid pCcydAB$_{Sc}$ encoding *cydAB*$_{Sc}$ under the control of its native promoter. (D) Relevant part of plasmid pCXcydAB$_{Sa}$ encoding *cydAB*$_{Sa}$ under the control of a xylose-inducible promoter.

**Phylogenetic analysis of CydA and CydB proteins.** CydA and CydB protein sequences of *S. aureus*, *S. carnosus*, *S. epidermidis*, *S. haemolyticus*, *S. pisciferm entans*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Chromobacterium violaceum*, *Escherichia coli*, *Listeria monocytogenes* F2365, *Mycobacterium tuberculosis*, and *Pseudomonas aeruginosa* were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The sequences were aligned using the program MUSCLE (13, 14). We calculated a distance-based tree with the neighbor-joining (NJ) method (33) using protdist and neighbor from the PHYLIP package (J. Felsenstein, University of Washington) and a character-based (maximum-likelihood [ML]) tree using IQPNNI (38). To estimate the significance of the NJ tree, a 1,000-fold bootstrap analysis was carried out using PHYLIP's seqboot. The tree was visualized with SplitsTree4 (21); the same program was used to calculate the majority-rule consensus of the 1,000 bootstrap trees.

For comparison, pairwise global sequence similarities of subsets were computed with the program stretcher of the EMBOSS program package (32).

## RESULTS

**Distribution of resistance/sensitive phenotype in various staphylococcal species.** While studying the antagonistic activity of *P. aeruginosa* against *S. aureus* and other staphylococcal species, we found that stationary-phase culture supernatants of *P. aeruginosa* PAO1 and another clinical isolate, SH1, inhibited the growth of all *S. aureus* and *S. epidermidis* strains tested, including their clinical isolates, as well as species representatives of *S. haemolyticus*, *S. saprophyticus*, *S. hyicus*, *S. muscae*, and *S. lugdunensis.* However, the growth of apathogenic staphylococcal species such as *S. carnosus*, *S. pisciferm entans*, *S. simulans*, *S. lentus*, and *S. gallinarum* was not inhibited. *S. carnosus* was resistant even when a 10-fold excess of *P. aeruginosa* culture supernatant was used. The resistance of *S. carnosus* and the susceptibility of *S. aureus* and *S. epidermidis* are illustrated by the agar disk diffusion assay (Fig. 1A).

**Role of *cydAB* in resistance to *P. aeruginosa* culture supernatants.** In order to identify the gene locus responsible for the resistance to the *P. aeruginosa* culture supernatant, *S. carnosus*

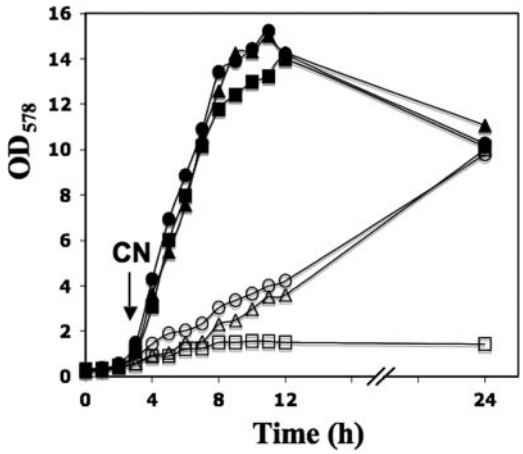


FIG. 2. Growth of *S. carnosus cydA*::Tn*917* is completely inhibited by 1.5 mM cyanide. ■, *S. carnosus cydA*::Tn*917*; ●, wild-type *S. carnosus*; ▲, *S. carnosus cydA*::Tn*917*(pCcydAB$_{Sc}$). Open symbols indicate growth in presence of 1.5 mM sodium cyanide. Closed symbols indicate growth in the absence of cyanide. The arrow indicates the time point of the addition of 1.5 mM sodium cyanide (CN) to the growing cultures.

was mutagenized with transposon Tn*917*. Around 1,500 mutants were screened on BM agar plates containing 30% (vol/vol) filter-sterilized culture supernatant of *P. aeruginosa* PAO1 and monitored for impaired growth. Six sensitive mutants were identified; an agar disk diffusion assay of one representative mutant (*cydA*::Tn*917*) is shown in Fig. 1A. The analysis of chromosomal sequences flanking the Tn*917* insertion site revealed that all the insertions were in the *cydAB* genes, which encode the two subunits of the cytochrome *bd*-type quinol oxidase. Three of the Tn*917* mutations were located within *cydA*, two were in the *cydA* promoter region, and one was within *cydB*. One mutant with the Tn*917* insertion in *cydA*, *cydA*::Tn*917* (Fig. 1B), was chosen for further studies. The mutant could be complemented with vector pCcydAB$_{Sc}$ (Fig. 1C), containing the entire *S. carnosus cydAB* operon (*cydAB*$_{Sc}$ genes) with its native promoter. These data indicate that in *S. carnosus*, CydAB$_{Sc}$ confers resistance to secreted toxins of *P. aeruginosa*, which produces various toxic compounds such as cyanide (5), pyocyanin (27), and quinolone signal molecules (11) as secondary metabolites.

**CydAB$_{Sc}$ is essential for growth of *S. carnosus* in the presence of cyanide.** Normally, the respiration of bacteria expressing cytochrome *bd* quinol oxidase is cyanide insensitive (1); therefore, we investigated whether the growth of *S. carnosus*, *S. carnosus cydA*::Tn*917*, and *S. carnosus cydA*::Tn*917*(pCcydAB$_{Sc}$) is affected by cyanide. In the presence of 1.5 mM sodium cyanide, *S. carnosus* and the complemented mutant, *S. carnosus cydA*::Tn*917*(pCcydAB$_{Sc}$), grew at a slow but constant rate. In contrast, the growth of the *S. carnosus cydA*::Tn*917* mutant was almost completely inhibited (Fig. 2). In the absence of cyanide, wild-type *S. carnosus* and the *cydA*::Tn*917* mutant grew equally well.

**CydAB$_{Sc}$ imparts resistance to *P. aeruginosa* culture supernatants in *S. aureus*.** When transformed with pCcydAB$_{Sc}$, *S. aureus* SA113 became almost as resistant to the *P. aeruginosa* culture supernatant as the *S. carnosus* wild type (Fig. 1A). Based on this result, we expected that *S. aureus* strains would lack the *cydAB* genes. Surprisingly, all *S. aureus* and *S. epidermidis* genomes available so far encode homologous *cydAB* genes at the same locus as that in *S. carnosus* (sequence similarities of the encoded proteins of 82% for CydA and 65% for CydB). In order to evaluate the role of *cydAB* genes in *S. aureus*, we replaced the genes with an erythromycin cassette (Fig. 3A) and also complemented this mutant with the recombinant plasmid pCXcydAB$_{Sa}$, which was constructed by cloning

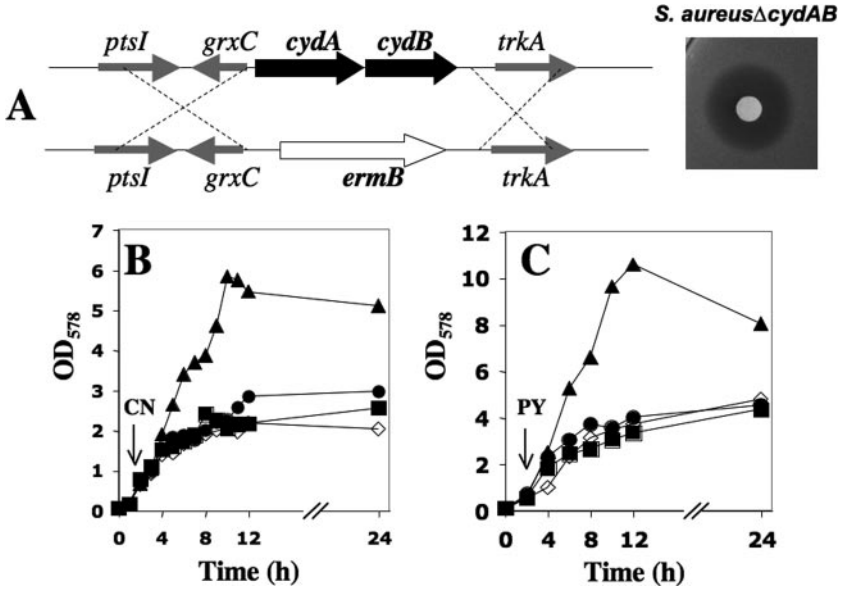


FIG. 3. Effect of cyanide and pyocyanin. (A) Deletion of *cydAB* in *S. aureus* and susceptibility of *S. aureus* Δ*cydAB* to the culture supernatant of *P. aeruginosa*. (B and C) Susceptibility of *S. aureus* clones to cyanide (B) and pyocyanin (C). ●, *S. aureus*; ■, *S. aureus* Δ*cydAB*; ◇, *S. aureus* Δ*cydAB*(pCXcydAB$_{Sa}$); ▲, *S. aureus*(pCcydAB$_{Sc}$). Cells were grown in BM broth to an OD$_{578}$ of approximately 0.5. Arrows indicate time points of the addition of 1.5 mM sodium cyanide (CN) and 10 μM pyocyanin (PY).

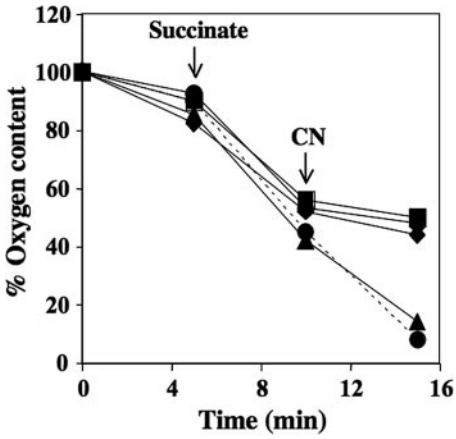


FIG. 4. Effect of cyanide on succinate-dependent $O_2$ consumption of *S. aureus* strains. The oxygen concentration was measured polarographically. Respiration was initiated with 50 mM succinate as a substrate. The dotted line represents oxygen consumption after the addition of succinate (control, without cyanide). Solid lines indicate the effect of sodium cyanide (CN) (1.5 mM) on respiration initiated by succinate. ●, *S. aureus*; ■, *S. aureus* Δ*cydAB*; ◆, *S. aureus* Δ*cydAB*(pCXcydAB$_{Sa}$); ▲, *S. aureus*(pCcydAB$_{Sc}$).

the *cydAB*$_{Sa}$ genes in the xylose-inducible vector pCX19 (Fig. 1D). *S. aureus* was also transformed with pCcydAB$_{Sc}$. *S. aureus* Δ*cydAB*::*ermB* and *S. aureus* Δ*cydAB*::*ermB*(pCXcydAB$_{Sa}$) were sensitive to *P. aeruginosa* culture supernatant (Fig. 3A), cyanide (Fig. 3B), and pyocyanin (Fig. 3C), indicating that the *cydAB*$_{Sa}$ genes were unable to confer resistance. However, *S. aureus* cells expressing CydAB$_{Sc}$ became resistant to cyanide and pyocyanin (Fig. 3B and C) and to *P. aeruginosa* culture supernatant (Fig. 1A). Therefore, only the *S. carnosus cydAB* genes impart resistance. In order to determine whether insufficient expression of the *cydAB* genes in *S. aureus* is responsible for its susceptibility, the CydAB complex was partially purified from various *S. aureus* and *S. carnosus* clones and monitored for CydAB production by using zymograms stained for heme-dependent peroxidase activity. The *S. aureus* wild type produced low levels of CydAB but clearly increased amounts in the presence of pCXcydAB$_{Sc}$ or pCXcydAB$_{Sa}$ (see Fig. S1 in the supplemental material).

**Effect of cyanide on cellular respiration.** Cyanide caused an almost immediate cessation of oxygen consumption in *S. aureus cydAB*::*ermB* and *S. aureus cydAB*::*ermB*(pCXcydAB$_{Sa}$) but not in *S. aureus*(pCcydAB$_{Sc}$) (Fig. 4). As expected, the respiration of *S. carnosus* was insensitive to cyanide, whereas its *cydA*::Tn*917* mutant was sensitive (data not shown). These results showed that only the *S. carnosus* cytochrome *bd* quinol oxidase (CydAB$_{Sc}$) functions as a cyanide-resistant alternative terminal oxidase.

**Pyocyanin is an important antistaphylococcal compound in *P. aeruginosa* extracts.** The antibacterial mechanism of pyocyanin is less clear than that of cyanide. Pyocyanin is secreted into the medium by *P. aeruginosa* in copious amounts. Pyocyanin was isolated and purified to study its effect on the growth inhibition of *S. aureus*. The bluish-green pigment, pyocyanin, was produced in phosphate-poor medium (pyocyanogenic medium) and in TS broth but not in phosphate-rich (apyocyanogenic) medium. Purified pyocyanin was analyzed by reverse-phase HPLC (Fig. 5A), and its spectrum revealed the characteristic absorption maxima for pyocyanin (Fig. 5B). *S. aureus* growth was inhibited by purified pyocyanin and culture supernatants of *P. aeruginosa* cells grown in TS broth and pyocyanogenic medium but not by supernatants from apyocyanogenic medium (Fig. 5C). These results indicate that pyocyanin, which is active at concentrations that are almost 100-fold lower than those of cyanide, is an important antistaphylococcal compound produced in abundance by *P. aeruginosa*.

**Sequence alterations in CydB are responsible for the resistant or sensitive phenotype in pathogenic and nonpathogenic staphylococcal species.** Unlike CydA, we find no homologues

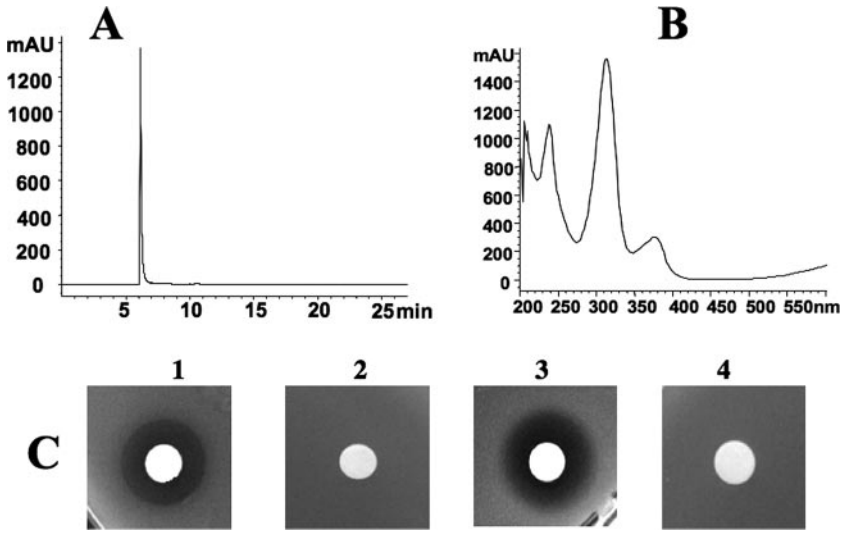


FIG. 5. (A) HPLC analysis of purified pyocyanin from pyocyanogenic medium. (B) UV spectra of purified pyocyanin obtained from pyocyanogenic medium. mAU, milli-absorption units. (C) Agar disk diffusion assay with *S. aureus*. Filter disks contain culture supernatant of *P. aeruginosa* grown in pyocyanogenic medium (plate 1), apyocyanogenic medium (plate 2), purified pyocyanin (plate 3), and chloroform extract from apyocyanogenic culture (plate 4). The retention time for pyocyanin was 6.2 min, and the typical absorption maxima were at 237, 313, and 376 nm.

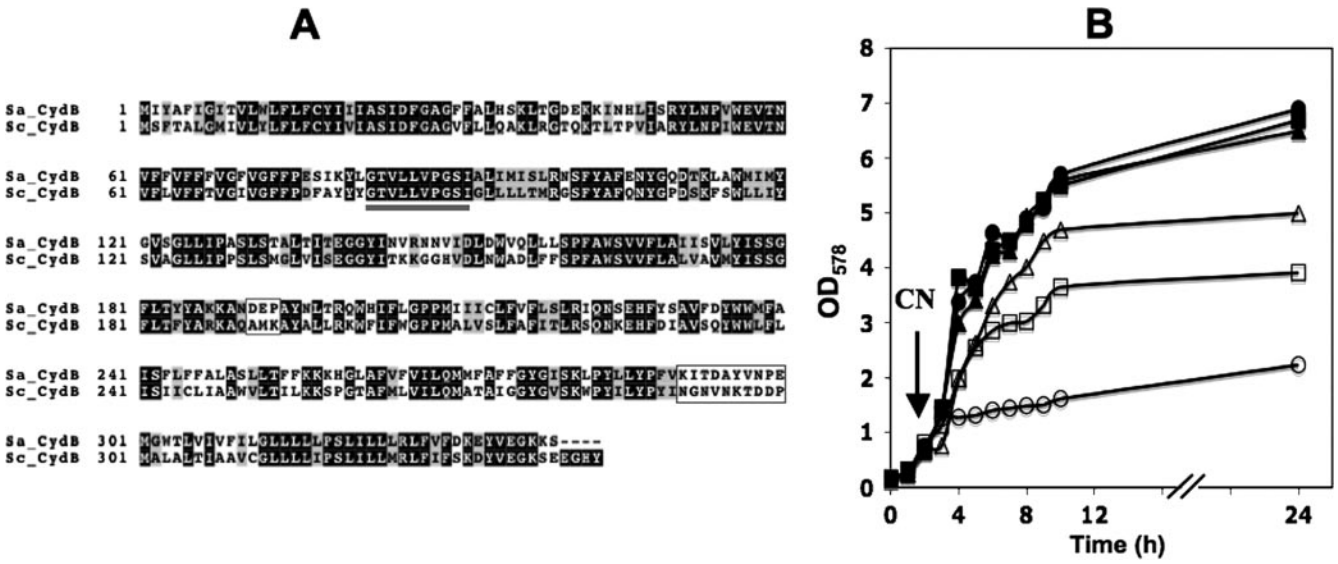


FIG. 6. (A) Multiple sequence alignment of the protein sequences of subunit CydB using ClustalW. Sequences shown are $CydB_{Sc}$ (Sc_CydB) and $CydB_{Sa}$ (Sa_CydB). Identical amino acid residues are shaded in black, whereas similarly charged amino acids are shaded gray. Amino acids that differ between the two strains are boxed. The part where CydB of *S. aureus* is exchanged with that of *S. carnosus* is underlined. (B) Growth of *S. aureus* and its transformants in the presence of 1.5 mM cyanide. ■, *S. aureus*(pRBcyd$A_{Sa}B_{Sc}$); ●, wild-type *S. aureus*; ▲, *S. aureus*(pCcydA$B_{Sc}$). Open symbols indicate growth in the presence of cyanide. Closed symbols indicate growth in the absence of cyanide. The arrow indicates the time point of the addition of 1.5 mM sodium cyanide to the growing cultures.

of *Staphylococcus*-specific CydB in *E. coli* or *B. subtilis*. Even within the various staphylococcal species, the primary sequence reveals marked differences at various sites in the CydB sequence (Fig. 6A). The substitution of the C-terminal portion of CydB of *S. aureus* (from amino acid position 82 to the C terminus) (Fig. 6A, underlined sequence) with the corresponding CydB sequence of *S. carnosus* conferred enhanced cyanide (1.5 mM) resistance to *S. aureus*(pRBcyd$A_{Sa}B_{Sc}$) (Fig. 6B). In the absence of cyanide, the *S. aureus* parent strain and its transformants grew equally well. This result indicates that the slight differences in sequence and structure of the CydB subunit are responsible for the resistant or sensitive phenotype of the various species mentioned.

**Phylogenetic analysis of CydA and CydB proteins: CydB represents a prime example of microevolution.** CydA is more conserved in staphylococci than CydB. Amino acid sequence identity within the pathogenic and apathogenic species lies in the range of 79 to 96%; the values between the two groups were around 70%. This is in line with the few changes observed in the amino acid sequence alignment (see Fig. S2 in the supplemental material). CydB also appears to be conserved within the pathogenic and apathogenic species. However, the amino acid sequence identity between the two groups is only between 56 and 59%, which represents rather high sequence variation, implying that CydB suffered a higher evolutionary rate than CydA. This is surprising, as *cydAB* are organized in the same operon, are cotranscribed (start and stop codons are overlapping), and are under the same evolutionary pressure. Indeed, it has been found that the subunits within the cytochrome *bd* complex vary considerably among different species and that subunit II (CydB equivalent) evolved 1.2 times faster on most of the branches of its phylogeny than subunit I genes (18). The phylogenetic trees of CydA and CydB protein sequences obtained with the NJ method and ML method had the same topologies (ML tree not shown). The mean mutual global sequence similarity of CydA and CydB proteins of the selected nonstaphylococci is very close (54% and 53%, respectively), whereas the homologous proteins from staphylococci resemble each other by 88% versus 81% (similarity), respectively, indicating that staphylococcal CydA proteins are more similar to each other than are CydB proteins of the same genus (Fig. 7). Whereas the subtrees of nonstaphylococcal CydA and CydB were almost superimposable, staphylococcal CydB sequences appear to have more divergence from each other than do staphylococcal CydA sequences. *S. carnosus* and *S. piscifermentans* CydB are more distant from the other CydBs of the same genus than they are in the CydA subtree. The mean similarity between CydA of nonstaphylococci and CydA of staphylococci is 46%, whereas the CydB of nonstaphylococcal species and that of staphylococcal species have a mean similarity of only 36%. This again is easily observable on the phylogenetic tree (Fig. 7). CydB represents a prime example of microevolution (evolution on a small scale at the species level). It also represents an interesting example of an asymmetric pattern of evolutionary change.

## DISCUSSION

In this study, we found that *Pseudomonas aeruginosa* exhibits an antagonistic relationship with *Staphylococcus* via its secreted respiratory inhibitors. The presence of a resistant or sensitive CydAB, the cytochrome *bd* oxidase, governs the impact of the respiratory inhibitors on the respiration and thereby the viability of staphylococci. Our results indicated that *S. aureus* produces low levels of CydAB, but even when the $cydAB_{Sa}$ genes were overexpressed, the corresponding clones

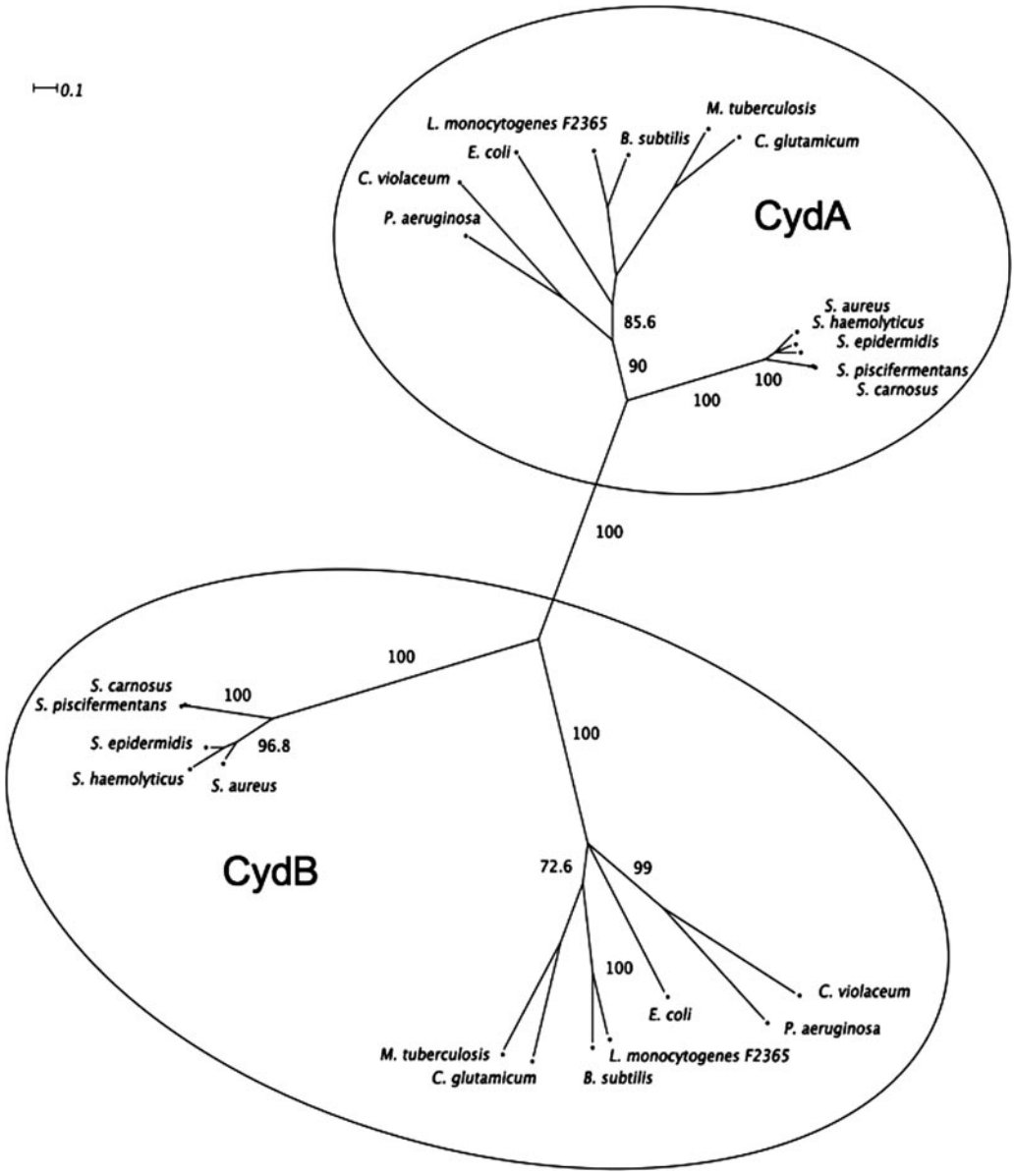


FIG. 7. Phylogenetic tree of CydA and CydB proteins of five staphylococcal species and seven other species (for details, see Materials and Methods). Numbers at the edges denote bootstrap support values. (All bootstrap values are >67.8 and at most >90). The evolutionary distance is proportional to the edge length. The tree reveals that CydB sequences of staphylococci are more distantly related to CydB sequences of nonstaphylococci than is the case in the subset of CydA sequences. Furthermore, staphylococcal CydB sequences are mutually more distant than are staphylococcal CydA sequences.

did not become pyocyanin or cyanide resistant. Therefore, even though the *S. aureus* and *S. carnosus* CydAB proteins have similar structural patterns, only the latter confers resistance. Both *S. carnosus* and *S. aureus* CydA proteins have characteristic amino acids found in *Escherichia coli* and *Bacillus subtilis* CydA proteins, which also encode two subunits of cytochrome *bd*-type quinol oxidase (6, 41). In *E. coli*, CydA, His-19, His-186, and Met-393 provide three of the four axial ligands to the iron of the three hemes in the cytochrome *bd* complex (35). These amino acid residues are conserved in *S. carnosus* and *S. aureus* CydA proteins (His-18, His-183, and Met-393). The 11-amino-acid stretch in *E. coli* and *B. subtilis* CydA proteins, thought to constitute part of the ubiquinol binding site (22), is also present in *S. carnosus* and *S. aureus* CydA (residues 252 to 262). The CydA heme binding and Q-loop motifs that are conserved in *B. subtilis* and *E. coli*, and also in *Corynebacterium glutamicum* (26) and *P. aeruginosa* (9), are also found in *S. carnosus* and *S. aureus* CydA (see Fig. S2 in the supplemental material). CydA is highly conserved in all the staphylococcal species and also in *Escherichia coli* and *Bacillus subtilis*. The *S. aureus* CydA is not only highly conserved, it is also functional together with the *S. carnosus*-specific CydB subunit.

Our results indicate that subunit B governs cyanide and

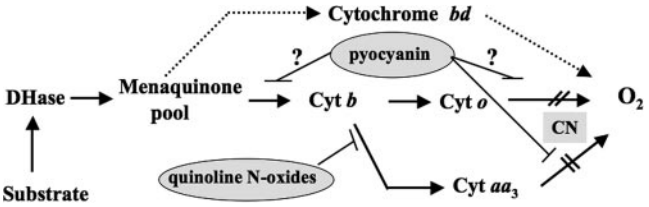


FIG. 8. Proposed pathway of electron transport in staphylococci. The meager information available on the respiratory components of staphylococci (16) suggests a branched respiratory system consisting of two alternative and menaquinol-dependent terminal oxidases, a cytochrome *bo* and a cytochrome $aa_3$ oxidase. NADH-dehydrogenase (DHase) delivers electrons to the menaquinone pool. From there, the electrons are transferred either to cytochrome *b* (Cyt b) or to the alternative electron acceptor, cytochrome *bd* quinol oxidase. Cytochrome *b* transfers the electrons to one of two terminal oxidases, cytochrome *o* or cytochrome $aa_3$. These terminal oxidases transfer the electrons to oxygen and are cyanide sensitive. Quinoline N-oxides interfere with electron transfer by inhibiting the oxidation of cytochrome $b_1$ and the reduction of cytochrome $a_2$. Pyocyanin also interferes with electron transfer, but it is not known whether it inhibits the electron transfer from the menaquinone pool to cytochrome *b* or from the two terminal oxidases to oxygen or whether it is involved in both steps. We showed that the bypass pathway through cytochrome *bd* quinoline oxidase is resistant to pyocyanin, cyanide, and also quinoline oxides in apathogenic staphylococci. Due to the low molecular weight, hydrophobic alkyl-hydroxyquinoline N-oxides (28) are also called the *Pseudomonas* quinolone signal molecules. These quinoline N-oxides are known to inhibit the growth of *S. aureus* and other gram-positive organisms. They inhibit the oxidation of cytochrome *b* and the reduction of cytochrome *c* in general. In *S. aureus*, they inhibit the oxidation of cytochrome $b_1$ and the reduction of cytochrome $a_2$ (24). The scheme is necessarily incomplete, since not all of the components of the respiratory chain of *S. aureus* and other staphylococci have been identified. CN, sodium cyanide.

pyocyanin resistance. Therefore, we speculate that there was a higher selective pressure on CydB than on CydA, leading to asymmetric evolution. The driving force very likely was the selection of *cydB* mutants that resist the respiratory toxic compounds produced by *Pseudomonas*. We assume that the resistant nonpathogenic staphylococcal species share their natural habitat with *Pseudomonas* species and have therefore evolved a resistant phenotype. Little is known with respect to the habitat of nonpathogenic staphylococcal species. Many of them can be isolated from food or are even used in food fermentation, e.g., *S. carnosus*, *S. xylosus*, *S. equorum*, *S. arlettae*, *S. condimenti*, and *S. piscifermentans.* For a long time, *S. carnosus*, *S. xylosus*, and *S. equorum* have been used as starter cultures for the production of raw fermented sausages and hams (34), and the cheese industry employs *S. succinus* subsp. *casei*, *S. equorum*, and *S. xylosus* as starter cultures in red smear cheese production. We assume that these staphylococcal species live in an environment that is also occupied by *Pseudomonas* and that they have learned to resist cyanide and pyocyanin to be able to coexist with *Pseudomonas*.

The mechanism of resistance of cytochrome *bd* oxidase to cyanide can be explained by bypassing the electrons to the cyanide-insensitive oxidase (Fig. 8). However, the resistance mechanism for pyocyanin is unclear. It has been described that in vitro pyocyanin oxidizes $NADH+H^+$, forming $O_2^-$ and $H_2O_2$ as by-products, and it was concluded that the production of these reactive oxygen species is responsible for the antibiotic activity of pyocyanin (19). However, it is unlikely that pyocyanin-resistant cytochrome *bd* quinol oxidase protects $NADH-H^+$ from oxidation by pyocyanin. We assume that in vivo pyocyanin interferes with components of the respiratory chain. Since cytochrome *bd* quinol oxidases oxidize dihydroubiquinol or dihydromenaquinol while reducing oxygen to water, we postulate that pyocyanin very likely becomes reduced to a toxic radical form. This reduction occurs somewhere between the electron flow from the menaquinol pool to cytochrome *b* and/or from the terminal oxidases (cytochrome $aa_3$ or cytochrome *o*) to oxygen, as indicated in Fig. 8.

It is intriguing that *S. aureus* and other pathogenic staphylococcal species have not evolved a cyanide- and pyocyanin-resistant respiration. Our hypothesis is that in their evolution, they have been so much adapted to their hosts (animals and humans) that they either have not gained or have lost the ability to resist the respiratory toxins released by *Pseudomonas* species. Probably, there was no real selective pressure, as pathogenic *Staphylococcus* and *Pseudomonas* species do not interact frequently during infection. For example, in the course of lung infection in cystic fibrosis patients, *S. aureus* infection frequently comes first and is later followed by *P. aeruginosa* infection. But even when they infect the same organ, they must not necessarily reside at the same site. The persistence of *S. aureus* in cystic fibrosis has previously been associated with the isolation of a subpopulation of *S. aureus* with small-colony variant (SCV) phenotypes (30, 39). Our preliminary studies reveal that *S. aureus* SCVs are indeed resistant towards pyocyanin and cyanide. A detailed study involving *S. aureus* SCVs and their susceptibility is in progress.

The apathogenic species representatives have evolved a higher tolerance, suggesting that they share their biotope more frequently with *Pseudomonas* species than with pathogenic staphylococci. It should be noted that *Pseudomonas* species that live in soil, such as *P. fluorescence* and *P. aureofaciens*, also produce these phenazine compounds. We don't know whether pathogenic staphylococcal species have developed backwards from an originally cyanide-resistant cytochrome *bd*-type quinol oxidase to a sensitive one or whether the originally sensitive *bd*-type quinol oxidases in nonpathogenic staphylococci have evolved to resistant ones. We favor the latter evolutionary pathway, as there is selective pressure as a driving force.

## ACKNOWLEDGMENTS

We thank Leo Eberl (Institute of Plant Biology, University of Zürich), Larry Gallagher (Genome Sciences, University of Washington), and Jeffery A. Hobden (Immunology and Microbiology, Wayne State University) for providing us with *P. aeruginosa* strains. We thank Rüdiger Hampp (Botanical Institute, University of Tübingen) for support in oxygen consumption assays.

This work was supported by the DFG, Graduate College, Infection Biology, GKI 685, and the BMBF PathoGenoMik (031U213B).

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