Advantage of Upregulation of Succinate Dehydrogenase in *Staphylococcus aureus* Biofilms[▽]

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Previous studies have demonstrated that various tricarboxylic acid (TCA) cycle genes, particularly the succinate dehydrogenase genes (*sdhCAB*), are upregulated in *Staphylococcus aureus* biofilms. To better study the role of this enzyme complex, an *sdhCAB* deletion mutant (Δ*sdh*) was constructed. Compared to the wild type (wt) the mutant was impaired in planktonic growth under aerobic conditions, excreted acetic acid could not be reused and accumulated continuously, succinate was excreted and found in the culture supernatant, and metabolome analysis with cells grown in chemically defined medium revealed reduced uptake/metabolism of some amino acids from the growth medium. Moreover, the mutant was able to counteract the steadily decreasing extracellular pH by increased urease activity. The addition of fumarate to the growth medium restored the wt phenotype. The mutant showed a small-colony variant (SCV)-like phenotype, a slight increase in resistance to various aminoglycoside antibiotics, and decreased pigmentation. The decreased growth under aerobic conditions is due to the interruption of the TCA cycle (indicated by the accumulation of succinate and acetic acid) with the consequence that many fewer reduction equivalents (NADH and $FADH_2$) can fuel the respiratory chain. The results indicate that the TCA cycle is required for acetate and amino acid catabolism; its upregulation under biofilm conditions is advantageous under such nutrient- and oxygen-limited conditions.

Staphylococcus aureus is not only a major cause of acute nosocomial and community-acquired infections (1, 25, 43), it also causes chronic infections associated with medical devices due to its biofilm-forming ability (11, 14, 29). In persisting and recurrent *S. aureus* infections slow-growing subpopulations, called small-colony variants (SCVs), can be frequently isolated (19, 31, 47). SCVs are distinguished by reduced respiratory activity and toxin production, a higher tolerance to aminoglycoside antibiotics, and a longer intracellular persistence. These and very likely other properties are responsible for their frequent occurrence in latent and recurrent infections as well as in device-related infections (3, 36, 37, 50).

When grown in a biofilm population (14), staphylococci must adapt to rapid changes in the environment that require equally rapid changes in the demand for energy and biosynthetic intermediates necessary for bacterial growth and survival. Many environmental and nutritional signals alter the bacterial metabolic status and also regulate most virulence determinants in staphylococci (40). The tricarboxylic acid (TCA) cycle plays a central role in metabolism. Many nutrients, like sugars, amino acids, and fatty acids, can be metabolized into TCA intermediates and enter this cycle at several points; also, intermediates can be removed from the cycle for use in biosynthetic pathways. Additionally, the TCA cycle produces reducing equivalents that generate energy through the respiratory chain when a terminal electron acceptor is present. TCA cycle activity has been associated with different effects like survival, virulence, and the production of the main biofilm

slime substance, polysaccharide intercellular adhesin (PIA) (39, 51). Furthermore, different TCA cycle enzymes have been found to be upregulated in biofilm-grown cells compared to planktonically cultivated cells (33), but only the deletion of TCA cycle enzyme genes (*acn*, *citZ*, and *citC*) of the oxidative branch of the TCA cycle in *S. aureus* have been described in the literature (39; G. Somerville, personal communication).

Interruption of the respiratory chain leads to an electron transfer chain-deficient small-colony variant phenotype. Clinically occurring SCV strains require a specific substance, like hemin, menadione, and thymidine, to restore wild-type (wt) growth rates (26, 30, 31). Genetically stable mutants in the hemin biosynthetic gene, *hemB*, or menadione biosynthetic gene, *menD*, have been characterized as having lower capacities for oxidative phosphorylation. They revealed many phenotypic changes (e.g., decreased pigmentation) and unusual biochemical characteristics as consequences of altered metabolic and energy-dependent pathways (4, 5, 21, 38, 46, 48, 49).

In a previous study, it was found that some TCA cycle genes were upregulated in biofilms, particularly the succinate dehydrogenase genes (*sdhCAB*) but also the 2-oxoglutarate dehydrogenase (*odhA*) and the succinyl-coenzyme A (CoA) synthetase (*sucC*) genes (32, 33). The succinate dehydrogenase (EC 1.3.99.1) is part of the nonoxidative branch of the TCA cycle and is directly linked to the respiratory chain. This enzyme complex catalyzes the oxidation of succinate to fumarate, donating $FADH_2$ for oxidative phosphorylation. It consists of three subunits: membrane-bound cytochrome b_{558} (SdhC), a flavoprotein containing an FAD binding site (SdhA), and an iron-sulfur protein showing a binding region signature of the 4Fe-4S type (SdhB) (15, 16).

In this study we characterized an *sdh* deletion mutant of *S. aureus*. The results suggest that upregulation of Sdh and other

TCA cycle enzymes is advantageous under biofilm conditions, where nutrients and oxygen are limited. Increased TCA cycle activity provides optimal growth, excreted acetic and lactic acids as well as medium amino acids can be better consumed, oxygen consumption rate (respiration) with succinate is optimal, and a too-high acidification of the medium is circumvented by a better reconsumption of the fermentation products (increase of the urea cycle as a bypass is dispensable).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The following strains were used: *Escherichia coli* strain XL1-Blue (Stratagene), *Staphylococcus aureus* SA113, a derivative of NCTC8325 (18), HG001, also a derivative of NCTC8325 (RN1) but in which the *rsbU* mutation has been repaired (S. Herbert et al., submitted for publication), and *S. aureus* strain RN4220 (23). For DNA preparation and transformation, the strains were grown in basic medium (BM; 1% soy peptone A3 SC [Organotechnie], 0.5% yeast extract, 0.5% NaCl, 0.1% $K_2HPO_4 \cdot 3H_2O$, 0.1% glucose [pH 7.2 to 7.4]) or on BM plates containing 1.5% agar. Antibiotics were used, when appropriate, at the following concentrations: ampicillin at 100 μg/ml for *E. coli*, and erythromycin (2.5 μg/ml), spectinomycin (150 μg/ml), and chloramphenicol (10 μg/ml) for *S. aureus*.

Construction of the *S. aureus sdhCAB* deletion mutant in SA113 and construction of complementation plasmid pRBsdh. Primers sdhCAB_B_for (5′-TATTA GGTACCGGTTCAGACCATGAAGTAG-3′; the introduced restriction site is underlined) and sdhCAB_B_rev (5′-ATTATTCTAGACCCTTGATTATTCGA TGC-3′) were used to amplify the downstream flanking region (~1.1 kb) of *sdhCAB* from the chromosomal DNA of *S. aureus* SA113. The PCR product was ligated into the Acc65I/XbaI sites of pKO2 (a derivative of pBT2 [8], constructed by B. Krismer), creating plasmid pKO22. Primers sdhCAB_A_for (5′-TATAA TGAGCTCGAGTCCAGAACGATTAAAGC-3′) and sdhCAB_A_rev (5′-AA TAAAAGCTTCGCTTAACCCCCTAAAGTG-3′) were used to PCR amplify the upstream flanking region (~0.96 kb) of *sdhCAB*. The restricted PCR product and the spectinomycin cassette from pIC156 (HindIII/Acc65I) (42) were cloned into the SacI/Acc65I sites of pKO22, yielding pKO21spec2. All recombinant plasmids were introduced in *E. coli* XL1-Blue. Plasmids were introduced first into *S. aureus* RN4220 and then into *S. aureus* SA113 via electroporation (2). Allelic replacement of wild-type *sdhCAB* genes with *spc* was carried out as described previously by Brückner (8). The replacement of the genes in the correct manner to the resulting strain, *S. aureus sdhCAB::spc*(Δ*sdh*), was confirmed based on the antibiotic resistance phenotype, PCR, DNA sequence analyses, and enzymatic assays.

The *S. aureus sdhCAB* genes (based on the sequence of *S. aureus* NCTC8325; gene ID SAOUHSC_01103-01105) with their putative native promoter were amplified from chromosomal DNA of *S. aureus* SA113 by PCR using the primers sdhCAB_C_for (5′-TTATAGAGCTCGAAGGAAGCTACACTTGAAG-3′) and sdhCAB_C_rev (5′-TTATTAAGCTTCCAAGTTATTGTGTATCTCCAT TC-3′). The resulting PCR product was cloned into SacI/HindIII sites of the medium-copy-number plasmid pRB473 (9), generating plasmid pRBsdh. The plasmid was introduced into *E. coli* XL1-Blue and transferred into *S. aureus* Δ*sdh* by electroporation to complement the mutant. The PCRs were carried out with High-Fidelity PCR enzyme mix (Fermentas); used primers were obtained from biomers.net. Computer sequence analyses were performed with MacDNASIS Pro (Hitachi Software Engineering).

Creation of an *sdhCAB* deletion mutant in HG001 by phage transduction. The *sdhCAB::spc* region of the SA113Δ*sdh* mutant was transduced into HG001 by phage transduction using ϕ11. The HG001 *sdhCAB::spc* mutant was verified by PCR and DNA sequencing. This mutant was also complemented with the plasmid pRBsdh.

Phage transduction. For generating phage ϕ11 lysates, the staphylococcal donor strains were resuspended in 500 μl phage buffer (1 mM $MgSO_4$, 4 mM $CaCl_2$, 0.59% NaCl, 0.1% gelatin, 50 mM Tris-HCl, pH 7.8), infected with ϕ11 (50 μl), and grown for 5 to 6 h in 4 ml soft tryptic soy agar (TSA) top agar (tryptic soy broth [TSB] and 0.5% agar) on $CaCl_2$-agar plates (TSB, 0.5 mM $CaCl_2$, 1.5% agar). Then, the soft TSA top agar was resuspended in 4 ml TSB, warmed for 10 min at 50°C, and sterilized by passage through 0.45-μm-pore-size filters after centrifugation (5,000 × *g*, 10 min, 4°C). For transduction, *S. aureus* cells from a fresh overnight plate were resuspended in 0.2 ml phage buffer, infected with ϕ11 lysates (100 μl), and incubated for 10 min at 37°C. Afterwards, 3.5 ml of phage top agar (3 g/liter Casamino Acids, 3 g/liter yeast extract, 5.9 g/liter NaCl, 7.5

g/liter agar) was added and poured on TSA-agar plates supplemented with appropriate antibiotic (2.5 μg/ml erythromycin, 150 μg/ml spectinomycin).

Enzymatic assays. TSB (Sigma) was inoculated from overnight cultures to an optical density at 578 nm (OD_{578}) of 0.07. Six-well cell culture plates (Greiner) were filled with bacterial suspension and incubated under static conditions at 37°C for 24 h. Then the cells were scraped off the plates, harvested by centrifugation, washed, resuspended in buffer (20 mM Tris-HCl, pH 8), and disrupted by shaking with glass beads in a tissue lyser (Qiagen). After a first centrifugation step (15 min, 2,000 × *g*, 4°C), the supernatants were ultracentrifuged at 30,000 rpm for 1 h at 4°C. The membrane fractions were washed and resuspended, and protein concentrations were determined by the method of Bradford (7). Different protein patterns in Coomassie brilliant blue-stained SDS-PAGE gels according to the method of Schägger and von Jagow (35) indicated successful separation of cytoplasmic and membrane fractions. To reaction tubes containing 100-μl aliquots of diluted membrane fractions, 300 μl of reaction mixture (each at 1 part: 0.2 M sucrose, 0.1 M Tris-HCl [pH 7.5], 10 mM sodium azide, 8 mM iodonitrotetrazolium chloride; 2 parts H_2O) was added. The reaction was started by addition of 0.5 M sodium succinate, pH 7.5 (100 μl) and incubated at 37°C, and when a change in color was visible 1.5 ml of ethanol was added and well mixed. The samples were incubated on ice for 10 min and centrifuged (10 min, 800 rpm), and the absorption at 458 nm was determined. Samples that either exchanged the enzyme fraction or the substrate against buffer served as controls. The specific activity was calculated, and the wild-type activity was set as 100%.

Growth in TSB and determination of extracellular metabolites. For growth analysis, TSB overnight cultures were diluted to an OD_{578} of 0.07 (~1:7 culture-to-flask volume ratio) and incubated at 37°C and 150 rpm; no antibiotics were added. CFU were determined by a modified drop plate method (17). For determination of metabolites the culture supernatant was heated to 80°C for 15 min and then stored at −20°C until further analysis. Glucose, acetate, D/L-lactate and succinate were determined enzymatically (R-Biopharm) in flat-bottom 96-well microtiter plates, and the A_{340} was determined in a microtiter plate reader (SpectraMax 340; Molecular Devices); the metabolite concentration was calculated from a standard curve.

Growth in CDM and determination of extracellular metabolites. Nuclear magnetic resonance (NMR) analysis of extracellular metabolites is more reliable in a chemically defined medium (CDM). The used CDM consisted of 10 mM Na_2HPO_4, 10 mM KH_2PO_4, 0.5 g/liter NH_4Cl, 0.5 g/liter NaCl, 0.2 g/liter $MgSO_4 \cdot 7H_2O$, 0.14 mM trisodium citrate dihydrate, and 7.47 mM glucose. This was supplemented with amino acids (L-alanine, L-arginine, L-aspartic acid, L-cysteine, L-glutamic acid, L-histidine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-valine; all at 1 mM), the vitamins cyanocobalamine (0.05 μg/ml), 4-aminobenzoic acid (0.04 μg/ml), biotin (0.01 μg/ml), 0.1 μg/ml nicotinic acid, 0.1 μg/ml Ca–D-pantothenic acid, 0.16 μg/ml pyridoxamine dihydrochloride, 0.1 μg/ml thiamine hyrdochloride, and 0.1 μg/ml riboflavine, and with 0.75 μM $FeCl_3$, 0.069 μM $ZnCl_2$, 0.099 μM $MnCl_2$, 0.006 μM boric acid, 0.35 μM $CoCl_2$, 0.002 μM $CuCl_2$, 0.24 μM $NiCl_2$, and 0.036 μM Na_2MoO_4.

For growth analysis CDM overnight cultures were diluted to an OD_{578} of 0.2 (~1:7 culture-to-flask volume ratio) and incubated at 37°C and 100 rpm; no antibiotics were added. After 4 h, 6 h, 9 h, and 24 h samples were taken and filter sterilized, the resulting supernatants were stored at −20°C. The metabolites were analyzed with ^{1}H-NMR as described elsewhere (24).

Urease activity. Cells were grown in either TSB or CDM supplemented with 100 mM sodium fumarate as described above. After different times the cells were pelleted, washed, resuspended in 50 mM potassium phosphate buffer (pH 8), and disrupted by shaking with glass beads in a tissue lyser (Qiagen). After centrifugation (0.5 min, 13,500 rpm) the protein concentrations of the cell lysates were determined by the method of Bradford (7). Two different protein concentrations of each sample were used for urease activity determination. Cell lysate and buffer were mixed to a final volume of 800 μl in 1.5-ml reaction tubes. Incubations (30 min to 4 h) were started by the addition of 200 μl of 250 mM urea to the reaction tubes. The reaction was stopped by heat inactivation at 95°C for 1.5 min, and time zero samples (t_0) were treated the same way and used as background controls. Ammonium was determined by using Nessler reagent.

Construction of *hemB* and *menD* mutants in *S. aureus* SA113. The *hemB::ermB* mutation region in *S. aureus* 8325-4 (48) and the *menD::ermC* allele of *S. aureus* DB24 (4) were transduced with the help of phage ϕ11 into SA113. The successful integration of the erythromycin resistance cassettes in the two SA113 mutants was verified by PCR.

Colony size on Mueller-Hinton agar plates. Flasks filled with fresh Mueller-Hinton broth (MH; Applichem) were inoculated with overnight cultures to an OD_{578} of 0.07, grown to mid-exponential phase, and plated onto MH agar plates. Growth was assayed with and without supplementation of either glucose (5 mM),
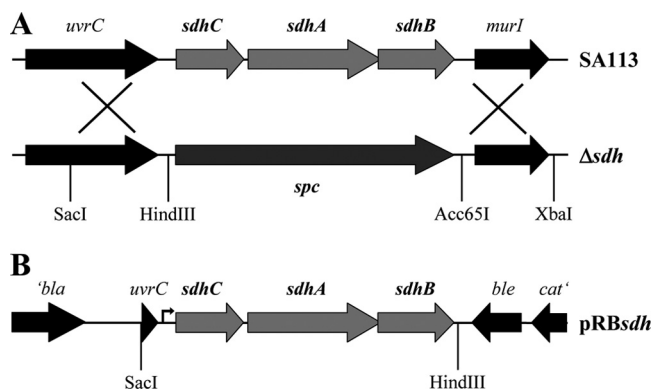
FIG. 1. Construction of the succinate dehydrogenase deletion mutant and complementation. (A) Location of the *sdh* operon in the chromosome of SA113 (upper panel) and the *sdhCAB* deletion mutant (Δ*sdh*; lower panel). (B) Gene arrangement in the complementation plasmid pRB*sdh*, including the putative native promoter region upstream (arrow). Plasmid-encoded genes: *bla*, beta-lactamase gene; *ble*, bleomycin resistance gene; *cat*, chloramphenicol resistance gene; *uvrC*, 3′ end of the *sdh* upstream gene.

sodium fumarate (5 mM), or sodium succinate (20 mM). The plates were incubated at 37°C and checked with respect to colony size and pigmentation.

Pigment extraction. *S. aureus* produces staphyloxanthin and diaponeurosporen as the main carotenoids (10, 28). Strains were cultivated for 24 h in TSB, and the pelleted cells were treated with ethanol (2.4 μl per mg [wet weight] of cells) and incubated for 20 min at 40°C in the dark. Cells were pelleted, and the majority of the pigments was in the ethanol phase, which was used to record an absorption spectrum (range, 350 to 550 nm). The absorption maxima of staphyloxanthin and intermediary products are between 450 and 470 nm, depending on the oxidation state of the carotenoids.

Determination of oxygen uptake. For determination of oxygen uptake, *S. aureus* clones were grown in TSB for 20 to 24 h. Cells were centrifuged, washed three times, and resuspended in 33 mM potassium phosphate buffer (pH 7.0) to an OD_{578} of 20. This suspension (1.8 ml) was analyzed for oxygen consumption using a Clark-type oxygen electrode. Respiration was initiated by the addition of either 100 mM succinate or 1 mM glucose as electron donor. The basal oxygen consumption rate (in the absence of electron donor) was subtracted. The oxygen consumption rate of the wt was set as 100%.

Susceptibility to antibiotics. Antibiotic susceptibility was determined in two ways: (i) agar diffusion assay and (ii) microdilution method. In the agar diffusion assay the following antibiotics (and concentrations) were tested: gentamicin, tobramycin, and kanamycin (10 μg, 5 μg, 2.5 μg, 1.25 μg, and 0.625 μg), chloramphenicol (10 μg), erythromycin (5 μg), fusidic acid (10 μg), mupirocin (5 μg), novobiocin (5 μg), ofloxacin (5 μg), oxacillin (1 μg), penicillin (1 μg), rifampin (5 μg), tetracyclin (5 μg), trimethoprim (2.5 μg), and the combination of trimethoprim (1.25 μg) and sulfamethoxazole (23.75 μg). For the aminoglycosides gentamicin, tobramycin, and kanamycin, the MICs were additionally determined by the microdilution method.

Statistical analyses. For determination of *P* values, paired Students *t* tests were performed.

RESULTS

Construction and characterization of the Δ*sdh* mutant. In *S. aureus* succinate dehydrogenase (Sdh) of the TCA cycle shows the typical subunit organization of SdhA, -B, and -C. As Sdh is involved in the electron transfer of the respiratory chain, it is postulated that the enzyme is membrane associated. The corresponding genes are organized in an operon that in *S. aureus* SA113 was completely deleted and replaced by a spectinomycin resistance cassette (Fig. 1A). The Δ*sdhCAB* mutant (designated herein as the Δ*sdh* mutant) showed no Sdh activity in either the cytoplasm or membrane fraction, indicating that no

other Sdh-like enzyme is present, while both SA113 and the complemented mutant showed activity in the membrane fraction (data not shown). For complementation, plasmid pRB*sdh* was constructed (Fig. 1B). However, the plasmid was not able to fully restore the Δ*sdh* mutant, although it encodes the structural genes *sdhCAB* together with a 408-bp upstream region with the putative native promoter region. To rule out a potential influence of second-site mutations in Δ*sdh*, the *sdhCAB*::*spc* allele was transduced into HG001 as an alternative strain; this strain has never undergone chemical mutagenesis and, in contrast to SA113, the global regulators Agr and RsbU (SigB) are functional. However, most of the experiments were carried out with SA113.

Growth and pH profile. Following growth in TSB medium under aerobic conditions (vigorous shaking) for over 48 h, one can see that SA113Δ*sdh* reached only half the OD values as SA113 (Fig. 2A). Also interesting was the course of the extracellular pH values. With SA113, the pH decreased steadily from 7.2 to 6.0 to reach a minimum at the end of the exponential growth phase, and then the pH values climbed to 8.4 in the late stationary growth phase. The pH profile of the mutant was significantly different. Like SA113, it decreased steadily from 7.2 to 6.0, but the pH remained at this level. After some delay, the complemented mutant reached the wt phenotype; however, the tendency can be clearly seen in later growth stages, where both pH and growth increased as in SA113 (Fig. 2A). A comparison of growth curves and pH courses revealed some differences in SA113 and HG001 (Fig. 2A and B). SA113 reached a higher OD value than HG001, while on the other hand HG001 grew faster (the generation time was only 46 ± 3 min, compared to 59 ± 7 min in SA113). The onset of alkalization of the culture supernatant was earlier in SA113 than in HG001. These differences very likely reflect the influence of the global regulators. Despite these general differences in parent strains, the corresponding Δ*sdh* mutants were impaired in growth and realkalization of the culture medium. In both Δ*sdh* mutants the complementing plasmid pRB*sdh* was unable to fully restore the wt phenotype, ruling out a side effect by point mutations in SA113Δ*sdh*.

Extracellular metabolites. To obtain more information regarding the metabolic state of the cells, we also determined the concentrations of extracellular metabolites during growth of SA113 and its isogenic Δ*sdh* mutant. In TSB, all strains consumed glucose within the first 5 h. Glucose consumption was correlated with an increase of acetate and lactate in both strains (Fig. 2C and D). The major difference was that in SA113 acetate is catabolized after prolonged growth, while the mutant was unable to utilize acetate that accumulated further to finally reach a concentration of 30 mM. The complemented mutant showed a similar acetate course as the wt. In contrast to SA113 and SA113Δ*sdh*(pRB*sdh*), SA113Δ*sdh* accumulated succinate in the culture supernatant, which was expected, as Sdh is missing in the mutant. In both SA113 and SA113Δ*sdh*, maximal lactate concentrations in the supernatants were reached after 4 h and declined gradually with time. When cultivated in TSB under aerobic conditions, the wt reached maximal lactic acid content after 4 h of incubation with 1.4 ± 0.3 mM D-lactic acid and 1.3 ± 0.5 mM L-lactic acid; obviously, lactate production is counteracted by lactate reoxidation. The SA113Δ*sdh* mutant reached a much higher lactic acid peak (2.9 ± 0.5 mM D-lactate and 5.0 ± 0.6 mM L-lactate). After 4 h
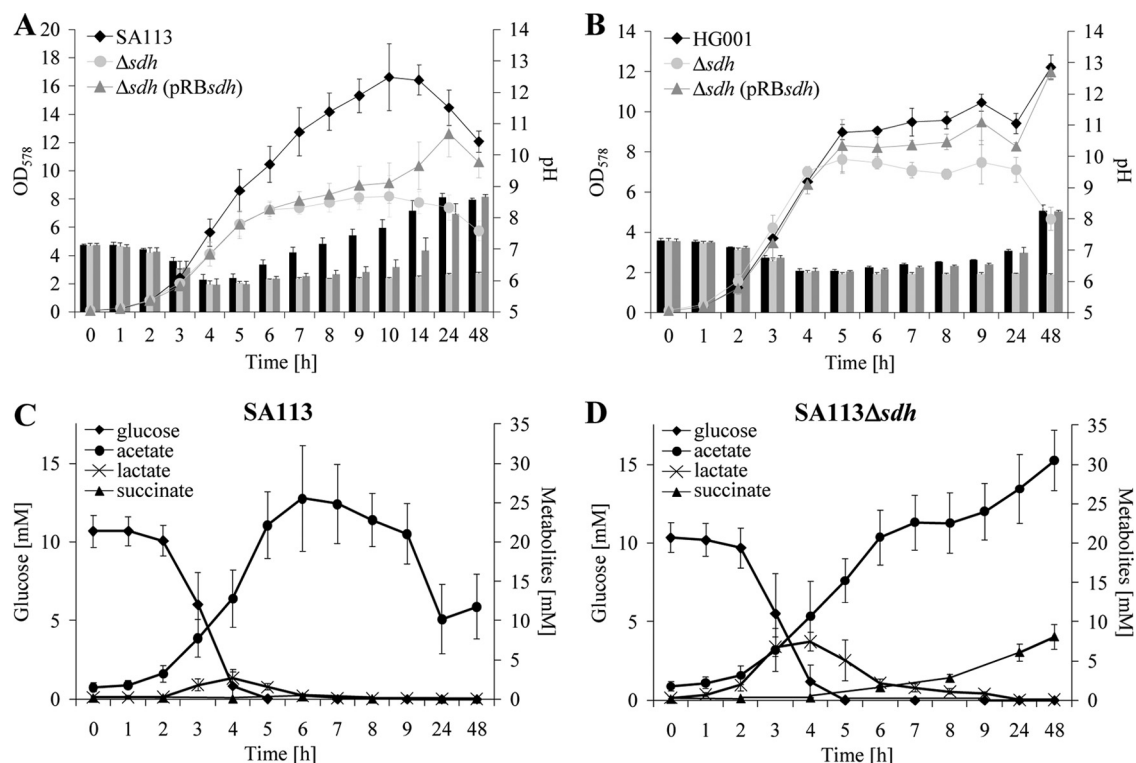
FIG. 2. Comparison of growth parameters and extracellular metabolites in *S. aureus* and the Δ*sdh* mutant. (A and B) Growth curves and pH profile (bars) of SA113 (A) and HG001 (B). (C and D) Metabolite concentrations in the culture supernatant of SA113 (C) and its Δ*sdh* mutant (D). Strains were aerobically cultivated in TSB at 37°C. Error bars indicate standard deviations from the means of three experiments.

the complemented mutant [SA113Δ*sdh*(pRB*sdh*)] was not yet able to reach the wt pattern; it still revealed a similar high lactic acid content as the mutant (data not shown).

By ^{1}H-NMR measurements of extracellular medium, the number of small-molecule metabolites could be extended. As such studies were not possible in detail with a complex medium like TSB, we used a CDM without interfering peptides or other macromolecules, as are included in TSB. Growth in this medium was poor for both SA113 and the mutant; a maximal OD_{578} of only 2.2 could be reached. In contrast to TSB, all strains grown in CDM maintained low extracellular pH values after the exponential growth phase (data not shown). The extracellular concentrations after 24 h of some selected metabolites are shown in Table 1. After 9 h of cultivation under

TABLE 1. Accumulated metabolites in culture supernatant of CDM after 24 h

Metabolite	Concn (mM) in culture supernatant[a]		
	SA113	Δ*sdh*	Δ*sdh* (pRB*sdh*)
Acetate	10.7 ± 0.7	9.4 ± 0.23	10.7 ± 0.6
Succinate	0.0 ± 0.0**	1.2 ± 0.2**	0.2 ± 0.2
Lactate	0.4 ± 0.1	0.6 ± 0.1	0.4 ± 0.1
Acetoine	1.8 ± 0.1	2.1 ± 0.2	2.0 ± 0.1

[a] Metabolite concentrations were determined in the culture supernatant of cultures grown aerobically in CDM for 24 h. Values are the averages of three independent experiments ± standard deviations. **, $P < 0.05$ for the Δ*sdh* mutant versus SA113.

aerobic growth, glucose (8 mM) was entirely consumed, while citrate, the second carbon source in the chemically defined medium, was hardly degraded after 24 h (data not shown). In CDM there was no marked difference in acetate and lactate accumulation, which is different from the results obtained in TSB (Table 1); even SA113 was unable to reutilize acetate. Only the accumulation of succinate in the culture supernatant of the mutant was comparable with the results in TSB. Other typical fermentation products, such as acetoine, 2,3-butanediol, ethanol, and formate, were not altered in SA113Δ*sdh*. The highest accumulated metabolite beside acetate was acetoine, with a concentration of circa 2 mM; there was no significant difference between SA113 and its mutant. In all strains traces of 2-oxoglutarate, fumarate, and ornithine as well as some amino acid degradation products (isovaleric acid, 2-methyl propionic acid, and 2-methyl butyric acid) were detected in similar amounts in the culture supernatant.

The CDM contained 15 amino acids, each at 1 mM. Cysteine, phenylalanine, and tryptophan concentrations stayed more or less constant in the culture supernatants, indicating that they were not metabolized. Histidine, lysine, valine, leucine, isoleucine, proline, alanine, arginine, aspartate, glutamate, serine, and threonine concentrations were decreased in the culture supernatants after 24 h. The SA113Δ*sdh* mutant showed a decreased consumption of some amino acids, particularly of proline (Table 2).

Stationary-phase survival assay. Inactivation of the TCA cycle enzyme aconitase resulted in enhanced stationary-phase survival (39). To address the question of whether this also

TABLE 2. Remaining amino acids in culture supernatant of CDM after 24 h

Amino acid	Concn (%)[a] in culture supernatant		
	SA113	Δsdh	Δsdh (pRBsdh)
Alanine	22.0 ± 4.7**	47.4 ± 2.0**	33.8 ± 4.0
Glutamate	19.1 ± 6.0**	43.8 ± 3.3**	30.0 ± 5.3
Histidine	76.5 ± 0.4*	90.7 ± 7.1*	83.9 ± 6.5
Isoleucine	30.4 ± 2.7*	48.0 ± 5.4*	40.5 ± 0.7
Proline	29.0 ± 14.6**	91.4 ± 13.9**	52.9 ± 12.9

[a] Amino acid concentrations in the culture supernatant of cultures grown aerobically in CDM for 24 h. Of the 15 amino acids contained in CDM, the differentially consumed amino acids are listed. An initial amino acid concentration of 1 mM was set as 100%. Values are the averages of three independent experiments ± standard deviations. *, $P < 0.1$; **, $P < 0.05$ (Δsdh mutant versus SA113).

occurs in the Δsdh mutant we performed a long-term survival assay. SA113, its Δsdh mutant, and the complemented mutant were cultivated in TSB medium aerobically for 7 days, and the CFU were determined daily. In the course of this study, SA113Δsdh survived better than wt or the complemented mutant (Fig. 3). A similar effect was also seen with HG001 and its mutant (data not shown).

Urease activity. As mentioned above, in SA113Δsdh the decrease in medium pH corresponded with glucose consumption. While in the wt the pH increased with time, it stayed constant in the mutant, at approximately 6.2, after depletion of glucose (Fig. 2A, C, and D). This was astonishing, as in the mutant acetic acid constantly increased with time. During exponential growth, lactic acid was excreted and reused, very likely to form acetic acid via oxidation to pyruvate. In the course of this process, the medium pH should become more alkaline as the comparatively strong lactic acid (pK_a 3.9) is replaced by the weaker acetic acid (pK_a 4.8). Furthermore, the glucose (10 mM) in the medium allows theoretically only the formation of 20 mM acetate. As we found, however, of the 30 mM acetate produced, part of the acetate must be derived from pathways such as amino acid or fatty acid catabolism. The ammonia concentration in TSB culture supernatants of SA113 and its Δsdh mutant were increased only after 48 h (wt, 1.24 ± 0.29 mM, versus Δsdh, 2.99 ± 0.66 mM per OD_{578} unit of 1.0). In the complemented Δsdh mutant the ammonia concentration was between that of the wt and the mutant (1.85 mM ± 0.32 mM per OD_{578} unit of 1.0). One explanation for the continuous low pH levels in SA113Δsdh could be the upregulation of a pathway influencing medium pH. One promising candidate is urease, which produces ammonia and CO_2 from urea as a substrate. Indeed, we found that in cell lysates of the mutant the urease activity was 2- to 5-fold increased compared to the wt. In the presence of fumarate, this effect was largely relieved. The enhanced urease activity in the mutant was more pronounced in TSB than in CDM (Table 3). The complemented mutant showed a wt phenotype when grown in CDM, but not in TSB.

We assume that in Δsdh the urea cycle (all genes annotated in *S. aureus* NCTC8325) is more active. This makes sense, as the urea cycle produces fumarate and urea, thus closing the metabolic gap in the TCA cycle, and delivers at the same time the substrate for urease.

In the medium supplemented with fumarate (100 mM), urease activity was decreased in the Δsdh mutant (Table 3). Furthermore, the addition of fumarate led to markedly better growth of SA113Δsdh in TSB and to an increase in external pH values comparable to the wt without fumarate (data not shown). Taken together, we showed that the interruption of the TCA cycle in the Δsdh mutant led to an enhanced stationary-phase survival, an impaired catabolism of acetate and succinate as well as some amino acids, and a temporary shift to lactate fermentation. Moreover, the mutant revealed an increased urease activity, which could be indicative for an increased urea cycle.

SA113Δsdh has an SCV-like phenotype. Due to the linkage of Sdh to the respiratory chain, we asked if the Δsdh mutant shows a SCV phenotype. As controls, we constructed *hemB* and *menD* insertion mutants in SA113 to generate stable SCVs. In these mutants the biosynthesis of hemin and menadione, respectively, is interrupted, which causes an inactivation of respiration. The two mutants showed the typical pinpoint colonies on agar plates, with colonies of the *hemB* mutant slightly smaller than colonies of the *menD* mutant. Both mutants also showed impaired growth in liquid medium under aerobic conditions. Supplementation of the culture medium for the *hemB* mutant with 1 μg/ml hemin restored wt growth, as previously shown (48).

The SA113Δsdh mutant also displayed markedly smaller colonies than the wt on Mueller-Hinton agar plates with no glucose. While wt colonies showed yellowish pigmentation, the Δsdh mutant colonies appeared white. However, the small colonies and the decreased pigmentation of SA113Δsdh were restored to the wt phenotype by the addition of either glucose or fumarate to the MH medium. The supplementation of succinate had no influence, confirming the finding that the Δsdh mutant is unable to use succinate (Fig. 4). The colonies of the complemented mutant did not quite reach wild-type size (data not shown).
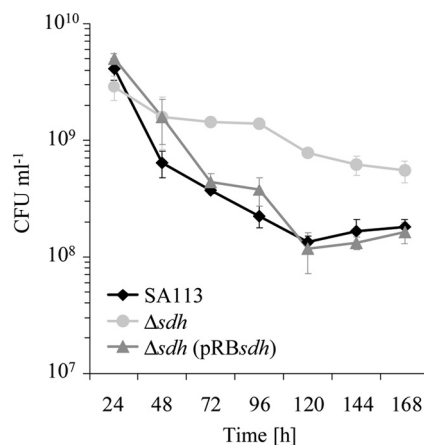

FIG. 3. Long-term survival assay results. Strains were aerobically cultivated in TSB at 37°C for 7 days. Cultural aliquots were removed daily, spotted on TSB agar plates, and incubated at 37°C for 24 h. The values (CFU per milliliter) were determined by the drop plate method (17) in six replicates. Error bars indicate standard deviations from the means of five experiments.

TABLE 3. Specific urease activity in cell lysates

| Medium | Culture time (h) | Urease sp act (mU/mg)[a] | | | | | |
| | | Without fumarate | | | With 100 mM fumarate | | |
		SA113	Δsdh	Fold difference, Δsdh vs SA113	SA113	Δsdh	Fold difference, Δsdh vs SA113
TSB	3	1.8 ± 0.8	3.7 ± 1.9	2.2 ± 0.8	1.6 ± 0.7	2.9 ± 1.8	1.8 ± 0.4
	5	2.4 ± 0.9	8.7 ± 1.6	4.0 ± 1.0	1.9 ± 0.4	5.5 ± 2.1	2.8 ± 0.6
	7	13.6 ± 7.3	50.4 ± 11.6	4.3 ± 1.7	9.9 ± 1.4	19.0 ± 6.8	1.9 ± 0.4
	9	14.5 ± 7.9	69.2 ± 16.1	5.3 ± 1.4*	7.6 ± 1.3	20.5 ± 3.3	2.7 ± 0.1*
	24	16.7 ± 8.8	59.6 ± 2.5	4.2 ± 1.7*	7.4 ± 1.6	15.3 ± 2.2	2.1 ± 0.2*
CDM	7	5.4 ± 1.2	7.7 ± 1.5	1.5 ± 0.6	2.5 ± 0.9	3.5 ± 0.4	1.5 ± 0.4
	9	6.2 ± 1.6	9.1 ± 0.7	1.5 ± 0.3	2.9 ± 1.0	3.7 ± 0.9	1.3 ± 0.1
	24	3.3 ± 0.6	8.6 ± 0.7	2.7 ± 0.3**	6.8 ± 1.9	4.9 ± 0.8	0.7 ± 0.1**

[a] Specific urease activities (with 1 U defined as 1 μmol NH_4^+ min^{-1} mg of protein^{-1}) were determined in cell lysates from cultures grown in TSB and CDM either with or without addition of 100 mM fumarate. Values are the averages of three independent experiments ± standard deviations. *, $P < 0.1$; **, $P < 0.05$ (urease activity ratio of cells cultivated in medium without fumarate versus activity ratio of cells cultivated in medium with 100 mM fumarate added).

Decreased pigmentation in the Δsdh mutants of SA113 and HG001. The Δsdh mutants of both strains produced much less carotenoids than the corresponding wt, as shown in the absorption spectra of crude ethanol extracts. Due to the *rsbU* defect in SA113, pigmentation in this strain was generally weaker than in HG001 (6). In both complemented mutants the wt carotenoid content was fully reached (Fig. 5).

Impairment of succinate-dependent oxygen consumption rate in SA113Δsdh. We also compared the oxygen consumption rate of resting cell suspensions with a Clark-type oxygen electrode with either glucose or succinate as electron donor. With succinate, the oxygen consumption rate was strong and rapid in SA113, while in the mutant it was less than 10% (Fig. 6A). With glucose the oxygen consumption rate was even slightly higher with the Δsdh mutant, indicating that the general electron transfer was not affected in SA113Δsdh (Fig. 6B).

Increased aminoglycoside antibiotic resistance. The SA113Δsdh mutant was slightly (two to four times) more resistant to various aminoglycoside antibiotics, such as gentamicin, tobramycin, or kanamycin, than the wt. This is far below the high resistance that was observed with a *hemB* or *menD* mutants, for which the MIC values were increased 32- to 64-fold (Table 4). In summary, the deletion of *sdh* genes in *S. aureus* leads to some properties similar to electron-deficient SCVs, even though the effects are not so pronounced as in classic SCVs.
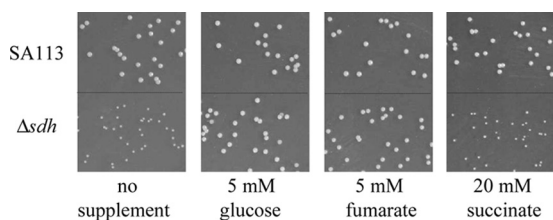
DISCUSSION

In a staphylococcal biofilm, TCA cycle enzymes (32) and corresponding genes (33) are upregulated compared to levels under planktonic growth, which suggests that this pathway is important in biofilm formation. Among the genes which were upregulated 3- to 4-fold in biofilms were the three subunit genes of the succinate dehydrogenase complex (*sdhCAB*). Sdh is the only enzyme of the TCA cycle that can directly feed electrons to the respiratory chain. It was therefore of general interest what impact this enzyme of the nonoxidative part of the TCA cycle has on the physiology in staphylococci. This could best be seen when an *sdhCAB* deletion mutant of *S. aureus* was compared with the wt. The most eye-catching differences of the *sdhCAB* mutant under aerobic growth conditions in TSB were decreased growth, inability to reuse exported acetate, leading to its continuous accumulation, and accumulation of succinate. There was also transiently a higher amount of lactate exported than from the wt, but as with the

FIG. 4. Size of colonies on Mueller-Hinton agar plates. The SA113Δsdh mutant displayed a SCV phenotype. Supplementation of the agar with 5 mM glucose or 5 mM sodium fumarate, but not with 20 mM sodium succinate, restored wild-type colony size.
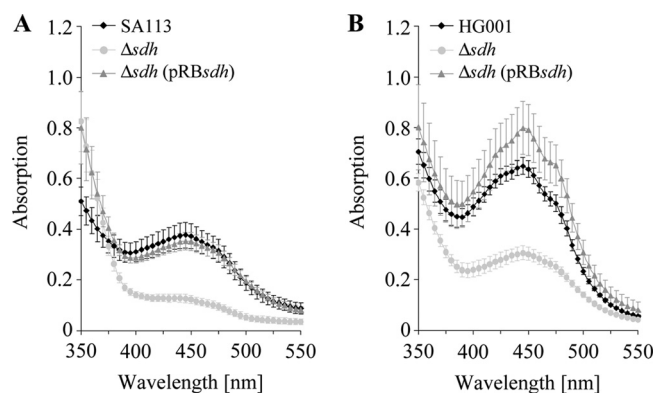

FIG. 5. Absorption spectrum of extracted pigments. SA113 (A) and HG001 (B) along with corresponding Δsdh mutants were aerobically cultivated for 24 h in TSB. Absorption spectra were determined in ethanol extracts (2.4 μl ethanol per mg [wet weight] of cells). Error bars indicate standard deviations from the means of four experiments.
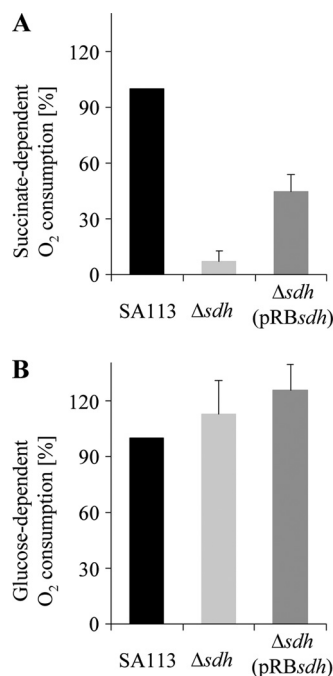
A

B

FIG. 6. Polarographic measurement of the oxygen consumption rate, determined with a Clark-type oxygen electrode. SA113 and isogenic Δ*sdh* mutants were grown aerobically for 20 to 24 h. Respiration of washed cell suspensions was initiated either with 100 mM succinate (A) or 1 mM glucose (B) as a substrate. The oxygen consumption rate of SA113 was set as 100%. Error bars indicate standard deviations from the means of three experiments.

wt, lactate could be reused and disappeared with time from the culture supernatant.

It is well known that in staphylococci under aerobic conditions glucose is oxidized in two steps. In the first step glucose is only partially oxidized to acetate, which accumulates in the supernatant; in the second step, when glucose is almost completely consumed, the excreted acetate disappears from the medium and is further oxidized by the TCA cycle (13, 39, 41, 44). As shown in Fig. 2C the consumption of 12.6 mM glucose was correlated with an accumulation of 25 mM acetate in the supernatant, indicating that almost all glucose is converted to acetate. The TCA cycle comes into play after glucose consumption. The decreased growth of the Δ*sdh* mutant under aerobic conditions is due to the interruption of the TCA cycle (indicated by the further accumulation of acetate and succinate) with the consequence that much less reduction equivalents (NADH and FADH$_2$) can fuel the respiratory chain. The

glyoxylate pathway as a short-circuit of the TCA cycle also produces succinate, but there are no genomic or physiological hints for this pathway in *S. aureus* (51). As compensation for the limited reducing equivalents in the mutant, lactic acid production increased transiently, with a maximum after 4 h growth, when D- and L-lactic acids amounted to 3 mM and 5 mM, respectively (Fig. 2D). However, the excreted lactic acid can be reused by the mutant, and the question arises as to how this is done. It has been shown that L-lactate oxidation occurred via two terminal menaquinol oxidases, the ba_3 type, which is sensitive to low KCN, and the *bo* type, which is insensitive to cyanide, and that ATP synthesis with L-lactate was coupled only to the *bo*-type oxidase (45). Whether L- and D-lactic acids are actually directly oxidized by the *bo*-type oxidase or via lactate dehydrogenase (LDH) oxidation to yield NADH is unknown. However, in both cases the respiratory chain is fueled with reducing equivalents and is accompanied by pyruvate production. What is the fate of pyruvate? In the wt, pyruvate can be fed into the TCA cycle via the pyruvate dehydrogenase (PDH) complex. In the mutant, the TCA cycle is a dead end, and part of the pyruvate is metabolized and the larger portion is converted to acetic acid, which increased steadily in the culture supernatant (Fig. 2D). Pyruvate oxidation occurs probably via the phosphate acetyltransferase (Pta) and acetate kinase (AckA) pathways, generating reducing potential and ATP. Another candidate for such a pyruvate oxidase is CidC, which catalyzes the oxidative decarboxylation of pyruvate, yielding acetate and CO$_2$ (27). In a nonfunctional TCA cycle *S. aureus* is unable to further metabolize or to oxidize acetic acid, and it has been mentioned that accumulation of acetic acid contributes to cell death and lysis in stationary-phase cultures (27). We found, however, in the long-term stationary-phase survival assay that the viable counts of the Δ*sdh* mutant were increased compared to wt. A similar observation was also made with an aconitase mutant (39). Although acetic acid continuously accumulated in the Δ*sdh* mutant during growth, the medium pH stayed constant around 6.2. We think that the steady pH value was achieved by upregulation of urease, which counteracts further acidification via excretion of ammonia. Very likely not only urease but also probably the entire urea cycle is upregulated, although we have not yet examined this (Fig. 7). This is another example of how efficiently *S. aureus* can adapt survival strategies in cases of emergency.

In CDM, growth was severely affected, with a maximal OD of only 2.2. This was expected, as glucose was present at a lower concentration (7.5 mM versus 12.6 mM) to enhance amino acid consumption. In CDM, the growth and accumula-

TABLE 4. MICs of different aminoglycoside antibiotics

Antibiotic	MIC (μg/ml) for strain						
	SA113	Δ*sdh* (*sdhCAB::spc*)[a]	Δ*sdh* (pRB*sdh*)[a]	Δ*lip* (*lip::spc*)[a]	Δ*fdh* (*fdh::spc*)[a]	*hemB*	*menD*
Gentamicin	1	2–4	2–4	1	1	32	32
Tobramycin	0.5–1	2	2	0.5–1	0.5–1	32	32
Kanamycin	2–4	8	8	2–4	2–4	64–128	64–128

[a] These strains contain the same spectinomycin resistance cassette on the chromosome. The deletion mutants for the lipase gene (SA113Δ*lip*) and formate dehydrogenase gene (SA113Δ*fdh*) were constructed by G. Thumm and M. Leibig in our laboratory.
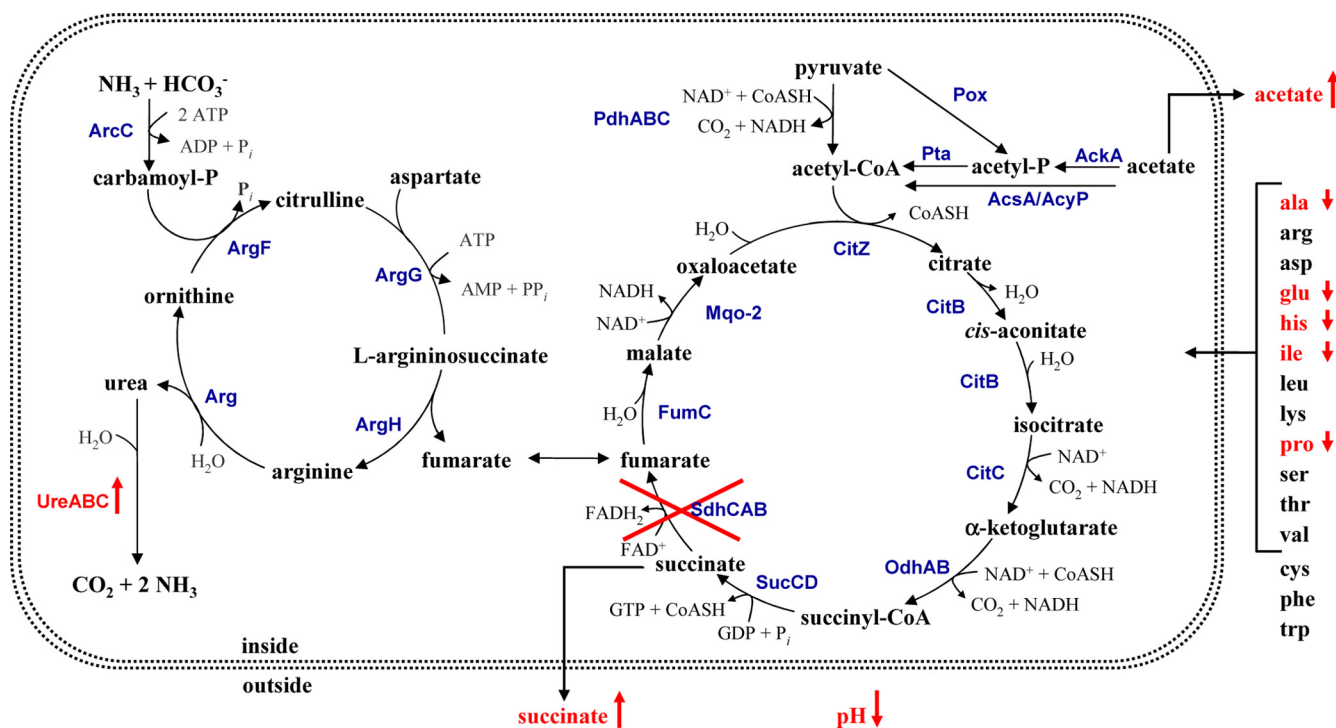
FIG. 7. Schematic representation of observed metabolic differences in the Δ*sdh* mutant. In the Δ*sdh* mutant urease activity was increased, succinate and acetate were excreted in higher amounts, the pH of the culture supernatant stayed low, and consumption of the amino acids alanine (ala), glutamate (glu), histidine (his), isoleucine (ile), and proline (pro) was affected. The corresponding genes of the indicated enzymatic reactions are annotated in *S. aureus* NCTC8325: ArcC, carbamate kinase (SAOUHSC_01129 and SAOUHSC_02965); ArgF, ornithine carbamoyltransferase (SAOUHSC_01128); ArgG, argininosuccinate synthase (SAOUHSC_00899); ArgH, argininiosuccinate lyase (SAOUHSC_00898); Arg, arginase (SAOUHSC_02409); UreA, urease subunit gamma (SAOUHSC_02558); UreB, urease subunit beta (SAOUHSC_02559); UreC, urease subunit alpha (SAOUHSC_02561); PdhA, pyruvate dehydrogenase complex E1 component, alpha-subunit (SAOUHSC_01040); PdhB, pyruvate dehydrogenase complex E1 component, beta-subunit (SAOUHSC_01041); PdhC, pyruvate dehydrogenase component E2 (SAOUHSC_01042); CitZ, citrate synthase (SAOUHSC_01802); CitB, aconitate hydratase (SAOUHSC_01347); CitC, isocitrate dehyrogenase (SAOUHSC_01801); OdhA/ Kgd, 2-oxoglutarate dehydrogenase E1 component/alpha-ketoglutarate decarboxylase (SAOUHSC_01418); OdhB, 2-oxoglutarate dehydrogenase E2 component/dihydrolipoamide acetyltransferase (SAOUHSC_01416); SucC, succinyl-CoA synthetase subunit beta (SAOUHSC_01216); SucD, succinyl-CoA synthetase subunit alpha (SAOUHSC_01218); SdhC, succinate dehydrogenase cytochrome b_{558} subunit (SAOUHSC_01103); SdhA, succinate dehydrogenase flavoprotein subunit (SAOUHSC_01104); SdhB, succinate dehydrogenase iron-sulfur subunit (SAOUHSC_01105); FumC, fumarate hydratase class II (SAOUHSC_01983); Mqo-2, malate:quinone oxidoreductase (SAOUHSC_02647 and SAOUHSC_02927); Pox, pyruvate oxidase (SAOUHSC_02849); AckA, acetate kinase (SAOUHSC_01820); AcyP, acylphosphatase (SAOUHSC_01406); Pta, phosphate acetyltransferase (SAOUHSC_00574); AcsA, acetyl-CoA synthetase (SAOUHSC_01846).

tion of fermentation products (e.g., acetic acid, lactic acid, and acetoin) were very similar between the wt and mutant strains (Table 1). Surprisingly, in both strains acetic acid increased continuously throughout growth over 24 h, indicating that in CDM the wt was unable to reuse acetic acid. To drive the TCA cycle, anaplerotic reactions are needed to offset the extraction of intermediates for anabolic pathways. If there is a limited supply of C_4 or C_5 compounds, the TCA cycle shuts down, which could be an explanation for the inability to reuse acetate. Consistent with our observations in TSB, succinate accumulated only in the mutant. Another difference in the mutant was the retarded amino acid consumption of alanine, glutamate, histidine, isoleucine, and proline (Table 2).

In Δ*sdh*, the respiratory chain is still intact. NADH formed during glycolysis could fuel respiration. Even lactic acid, which is typically transiently excreted during aerobic growth in staphylococci, can directly feed the *bo*-type oxidase as mentioned above. Even glutamate oxidation via the *bo*-type oxidase and subsequent ATP synthesis have been described (45). There are

alternative pathways and substrates by which the respiratory chain can be fueled even in the absence of a functional TCA cycle. Nevertheless, the loss of Sdh is sufficient to cause an SCV-like phenotype. We name it SCV-like because the effect is not that pronounced as that described for the *hemB* (48) or *menD* (20) mutants, in which the respiratory chain is completely disrupted. These mutants have a typical anaerobic phenotype even under aerobic conditions, consistent with high accumulation of lactic acid and low excretion of acetic acid (21). Typically, the SCV phenotype displays an increased tolerance for aminoglycoside antibiotics, which require a certain membrane potential (Δψ) for uptake (5, 31, 48). The Δ*sdh* mutant shows only a 2- to 4-fold-higher resistance to aminoglycoside antibiotics, but this is much lower than the 32- to 64-fold increase in resistance observed for *hemB* and *menD* mutants. This indicates that the membrane potential of Δ*sdh* cannot be that low. Another characteristic feature of SCVs is decreased pigmentation (48). Indeed, the absorption spectra of wt (SA113 and HG001) and corresponding Δ*sdh* mutants showed

that carotenoid content in the mutants was significantly decreased; in the complemented mutants the wt level was restored.

The first clean TCA cycle deletion mutant was constructed in the aconitase gene (*acnA::ermB*) of *S. aureus* by Somerville and colleagues (39). This mutant shows some similarities but also differences compared to Δ*sdh*. The Δ*sdh* mutant appears more severely affected in growth; however, the catabolism of acetic acid was similarly affected in both mutants. Interestingly, the *acnA* mutant excreted no citrate, but the intracellular concentration was higher relative to the wt (G. Somerville, personal communication). In *Staphlococcus epidermidis*, *acnA* mutant citrate was also increased intracellularly (34).

The complementation of the *sdh* deletion mutant by pRB*sdh* was delayed. Such problems are often observed with complementing membrane proteins like Sdh with a medium-copy-number plasmid. Additionally, the Sdh complex consists of three subunits, which have to be organized in the right stoichiometry, which also might complicate complementation. In order to rule out that the phenotype of SA113Δ*sdh* is not influenced by some point mutations in this strain, the Δ*sdh* mutant was transduced into HG001. The resulting HG001Δ*sdh* showed similar properties as SA113Δ*sdh*: decreased growth, no alkalization of culture supernatant, decreased pigmentation, and enhanced long-term survival.

Why are various TCA cycle enzymes, particularly Sdh, upregulated in biofilms? In a biofilm nutrients are limited, oxygen is depleted, and *ica* genes are consequently upregulated under anoxic conditions (12, 33). Cells in a biofilm represent with respect to physiological activity a very heterogenous population due to the various gradients of oxygen, nutrients, and pH. This spectrum of different conditions affords a permanent adaptation of cells to better survive in such a complex environment. The upregulation of the TCA has the advantages (i) to make better use of traces of oxygen, (ii) to allow better metabolism of excreted fermentation products like acetic or lactic acid, and (iii) to permit better catabolism of amino acids. Staphylococcal infections where oxygen is limited are not that seldom, for example, in abscess formation or in the sputum of cystic fibrosis patients, where oxygen depletion is accelerated by the phagocyte NADPH oxidase (22).

ACKNOWLEDGMENTS

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