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CtaA of *Staphylococcus aureus* Is Required for Starvation Survival, Recovery, and Cytochrome Biosynthesis

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A *Staphylococcus aureus* mutant (SPW3) apparently unable to survive long-term starvation was shown to have a transposon insertion within a gene homologous to *ctaA* of *Bacillus subtilis* which encodes a heme A synthase. Analysis of the cytochrome profiles of SPW3 revealed the absence of heme A-containing cytochromes compared to the parental 8325-4 strain. SPW3 demonstrated a 100-fold reduction in the ability to survive starvation induced by glucose limitation, under aerated conditions, compared to 8325-4. Analysis of starved cultures revealed that greater than 90% of the cells which demonstrated metabolism (as shown by rhodamine 123 accumulation) were unable to recover and form colonies on agar. Analysis of the lag phase and initial growth kinetics of those cells which could recover also showed a defect. This recovery defect could be partially alleviated by the inclusion of catalase in the recovery medium, indicating the probable involvement of oxidative stress. SPW3 also exhibited reduced colony size similar to that of a small-colony variant, increased resistance to aminoglycoside antibiotics, and reduced hemolysin and toxic shock syndrome toxin 1 production, but no alteration in the ability to form lesions in a subcutaneous mouse infection model.

To survive in the environment, bacteria must be able to adapt to continually changing conditions. One of the major fluctuations is in the availability of nutrients, which are often limiting for growth. Bacteria have evolved complex starvation survival responses enabling them to persist (16). Once conditions become permissive, the surviving bacteria rapidly resume growth in order to compete with other organisms for the available nutrients.

Staphylococcus aureus is a medically important human pathogen capable of causing a wide range of infections, some of which are life threatening (41). Recently we characterized the starvation survival and recovery responses of *S. aureus* (7, 43). A starvation survival state was shown to be induced by either glucose limitation or resuspension of cells in water, whereupon only 1 to 0.1% of the original population survived prolonged starvation (43). Analogous to the starvation survival responses characterized in other bacteria, the surviving cells become smaller and develop increased resistance to environmental stress, both of which are dependent on differential gene expression. Starved cells, however, respond rapidly to the addition of nutrients with an immediate increase in RNA synthesis, followed by protein synthesis, which leads to the reversal of many of the cellular changes that occurred during starvation (7). During this recovery period, proteins are expressed in a specific temporal order, and some appear only transiently.

Several transposon (Tn) insertion mutants defective in starvation survival have been isolated recently from *S. aureus* (42). By this approach, several loci involved in starvation survival, including genes involved in oxidative stress resistance, DNA repair mechanisms, and nutrient scavenging, were identified. In this report, we describe the characterization of a starvation survival mutant, SPW3, which is defective in heme A biosynthesis and determine its role in starvation survival, recovery, and other cellular processes.

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MATERIALS AND METHODS

Cloning and sequencing of *ctaA*. Plasmid pSPW3, which contained chromosomal DNA flanking the *lacZ* proximal region of the Tn insertion in *S. aureus* SPW3, was generated in a previous study (42). The insert, which contains 750 bp of chromosomal DNA, was excised, digoxigenin (DIG) labeled according to the protocol of Boehringer GmbH (Mannheim, Germany), and used to probe a λZAP Express library of a partial *Sau*3A digest (2 to 10 kb) of *S. aureus* 8325-4 genomic DNA (10). A clone containing a 3.5-kb genomic DNA fragment, spanning the pSPW3 insert, was identified, and the stable phagemid pSPW30 was excised from λZAP Express in *Escherichia coli* XLOLR (Stratagene). A primer-walking-based approach was used to sequence a 1,100-bp region.

Strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. *S. aureus* 8325-4 (23) and SPW3 (42) were grown in either a chemically defined media (CDM) containing 0.1% glucose (14, 43) or brain heart infusion (BHI) broth (Oxoid). Agar plates were prepared by the addition of 1% (wt/vol) agar to the above. Catalase plates were prepared by overlaying 4 ml of CDM agar (1% [wt/vol]) containing bovine catalase (5 mg ml^{-1}; Sigma) onto CDM agar. Sheep blood agar plates were made by the supplementation of blood agar base (Difco) with 5% (vol/vol) sheep blood (TCS Biologicals, Buckingham, United Kingdom). Starvation cultures were prepared by the inoculation of CDM to an optical density at 600 nm (OD_{600}) of 0.01, which was then incubated at 37°C with shaking (250 rpm). Stationary phase was reached after about 8 to 10 h. After 18 h of growth, cultures were either incubated statically or shaken further. Starved cells were recovered by the addition of 1/10 volume of glucose (1% [wt/vol]) and a mixture of 18 amino acids (29.4 mg ml^{-1}). This gives a final concentration of glucose and amino acids identical to that in the original CDM in which the cells were grown. Viable counts were determined by serial dilution of cultures with phosphate-buffered saline (PBS), plating on CDM agar, and incubated overnight (37°C). *E. coli* strains were grown in LB or on LB agar (1% [wt/vol]). Results of starvation experiments are representative of at least three independent experiments which showed no more than 10-fold variability between equivalent time points.

Construction of reporter fusion strains. The *ctaA*::Tn*917*-LTV1 locus was transduced from SPW3 into PC6911 (*agr*::*tet*) (3), PC1839 (*sar*::*km*) (4), and PC400 (*sigB*::*tet*) (5) by phage transduction using ϕ11 as the carrier (35), selecting for transductants on erythromycin (5 μg ml^{-1})-containing BHI agar; the resultant strains were named MC61, MC62, and MC63. The *tst*::*lux* reporter fusion was transduced from PC1072 into SPW3, to generate MC70. Transductants were then confirmed by selection on appropriate antibiotic-containing media.

Antibiotic MIC determination. Antibiotics were serially diluted in BHI broth and then seeded to an OD_{600} of 0.001. Cultures (200 μl) were grown in 96-well microtiter dishes at 37°C with shaking at 150 rpm.

Flow cytometry. Viable cell numbers were measured by flow cytometry after staining with rhodamine 123 (Rh123) as described by Watson et al. (43).

Cytochrome profile analysis. For spectrophotometric studies, strains were grown in CDM, and cells were harvested by centrifugation (5,000 rpm 10 min) and resuspended at a final OD_{600} of ≈100 in PBS. Electronic visible difference

TABLE 1. Bacterial strains used in this study

S. aureus strain	Genotype or relevant characteristics	Origin (reference)
RN4220	Restriction minus, modification plus	R. Novick (17)
8325-4	Wild-type strain cured of known prophages	R. Novick (23)
SPW3	*ctaA*::Tn*917*-LTV1 Ery^r	Laboratory stock (42)
PC6911	*agr*Δ::*tetM* Tc^r	Laboratory stock (3)
MC61	*ctaA*::Tn*917*-LTV1 *agr*Δ::*tetM* Tc^r Ery^r	This study
PC1839	*sarA*::*km* Km^r	Laboratory stock (4)
MC62	*ctaA*::Tn*917*-LTV1 *sarA*::*km* Km^r Ery^r	This study
PC400	*sigB*::*tet* Tc^r	Laboratory stock (5)
MC63	*ctaA*::Tn*917*-LTV1 *sigB*::*tet* Tc^r Ery^r	This study
PC1072	*tst*::*lux* Tc^r	Laboratory stock (3)
MC70	*ctaA*::Tn*917*-LTV1 *tst*::*lux* Tc^r Ery^r	This study

spectra were recorded in a custom-built Johnson Foundation SDB3 dual-wavelength scanning spectrophotometer in cuvettes of 1-cm path length as described by Williams and Poole (45). Oxidation was performed by shaking the sample in air, while reduction of the samples was performed by the addition of sodium dithionite. CO-liganded samples were prepared by bubbling CO gas through the sample for 1 min prior to analysis.

β-Galactosidase and luciferase assays. β-Galactosidase assays of cell lysates, using 4-methylumbelliferyl-β-D-galactoside as the substrate, were performed as previously described (3). Luciferase activity was determined by adding 20 μl of *n*-decyl aldehyde (1% [vol/vol] in ethanol) to 1 ml of bacterial culture with rapid mixing, and luminescence was measured on an Optocomp1 luminometer (Celsis) (13). Assays were performed as described by Chan and Foster (3).

Pathogenicity study. A single colony from an overnight sheep blood agar plate was inoculated into 100 ml of BHI broth in a 250-ml flask, which was then incubated at 37°C for 16 h. Cells were harvested by centrifugation (5,000 *g*, 10 min, room temperature) and washed twice in an equal volume of PBS. Cells were diluted to 5×10^8 CFU ml^{-1} with PBS, and 200 μl was injected subcutaneously into BALB mice. After 7 days, the mice were sacrificed and the lesion at the site of injection was harvested. The lesion was stored at −70°C until processed. The lesions were thawed, homogenized, resuspended in 5 ml of PBS, vortexed vigorously for 1 min, and stored on ice for 30 min. The homogenate was then serially diluted in PBS and plated onto BHI agar to determine the number of CFU per lesion. Results from six mice were recorded, and their significance determined by the Mann-Whitney test.

α-Hemolysin assays. α-Hemolysin levels in bacterial cultures were determined by the method of Rowe and Welch (29) as described by Chan and Foster (3).

Protein gel analysis. Samples were prepared, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (9). Samples for Western blot analysis were separated by SDS-PAGE on a 10% (wt/vol) polyacrylamide gel, then transferred to nitrocellulose, and treated with anti-Hla antibody at a 1:500 dilution as described by Burnette (2). Antigen-antibody complexes were detected by reaction with goat anti-rabbit immunoglobulin G (1:30,000) conjugated to alkaline phosphatase (Sigma).

Northern blot analysis. RNA was extracted from 1 ml of cells centrifuged (5,000 rpm for 5 min) and resuspended in PBS to an OD_{600} of 10, using a FastRNA BLUE kit (Bio 101) as described by the manufacturer. RNA was separated by electrophoresis through a 1% (wt/vol) agarose gel containing formaldehyde and blotted onto a nitrocellulose membrane as described by Sambrook et al. (30). Probes generated from internal PCR fragments of the *hla* and *agr* RNAIII genes were DIG labeled, hybridized, and detected by using a DIG DNA labeling and detection kit (Boehringer) as described by the manufacturer.

Nucleotide sequence accession number. The 1,100-bp region sequenced in this study has been assigned GenBank accession no. AF072726.

RESULTS

SPW3 has a Tn insertion located in a homologue of *B. subtilis ctaA*. The Tn*917*-LTV1 insertion mutant SPW3 was isolated in a previous screen for starvation survival mutants of *S. aureus* (42). Chromosomal DNA flanking the Tn insertion was cloned and sequenced as described in Materials and Methods. Sequence analysis of this region (1.1 kb) with the Staden and Genetics Computer Group packages (SEQNET; Daresbury Laboratory, Warrington, United Kingdom) revealed a single open reading frame of 306 amino acids sharing 39.7 and 35.7% identity to the *Bacillus subtilis* and *Bacillus firmus* CtaA proteins encoding heme A synthases (21, 28) (Fig. 1). Like in *Bacillus subtilis*, the *S. aureus ctaA* gene appears to be monocistronic, since potential *rho*-independent transcription terminators were located 9 bp downstream of the putative translation stop codon and 526 bp upstream of the predicted start codon, with ΔG values of −36.4 and −3.6 kJ mol^{-1} respectively. Recently, Vasilieva et al. (38) isolated a *ctaA* mutant of *S. aureus* during a screen for mutants with altered β-lactam

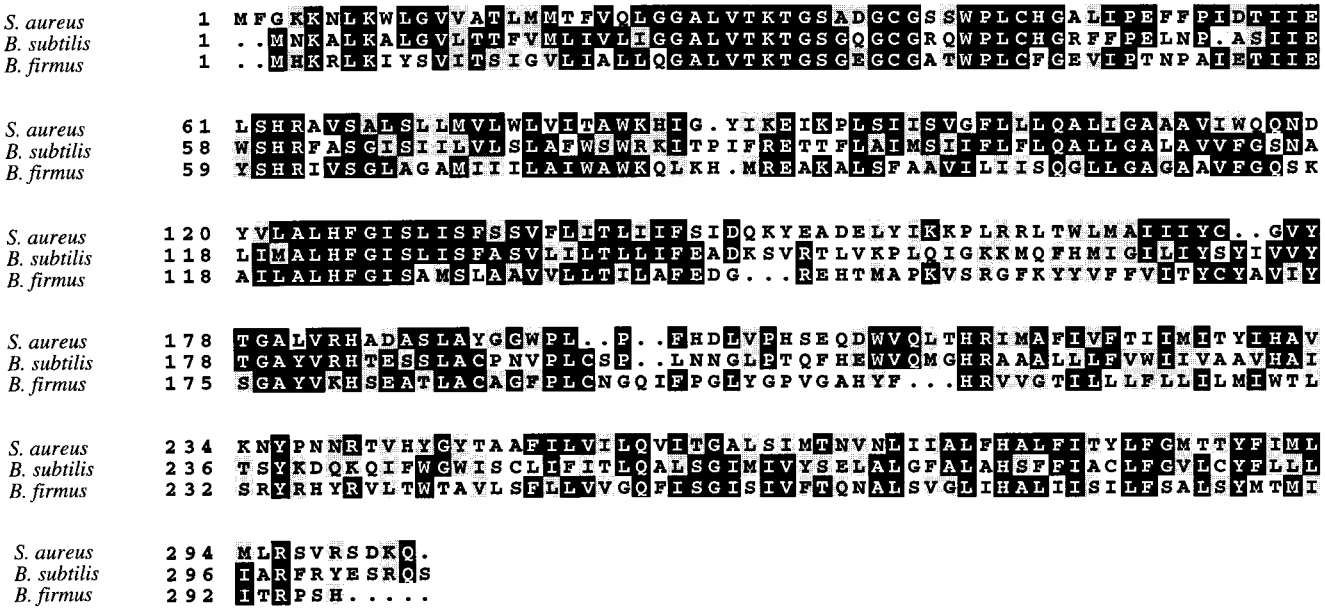

FIG. 1. Alignment of the *S. aureus* CtaA amino acid sequence with those of *B. subtilis* (22) and *B. firmus* (28). Numbers represent positions in the amino acid sequence. Identical residues are indicated by black boxes; conserved residues are shown in grey boxes.

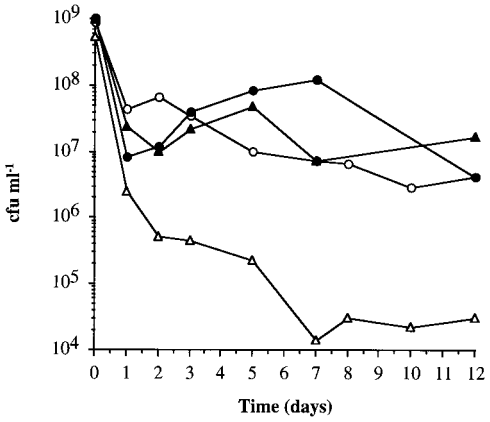

FIG. 2. Effects aeration on the starvation survival kinetics of *S. aureus* 8325-4 and SPW3. *S. aureus* 8325-4 (○, ●) and SPW3 (△, ▲) were grown in glucose-limiting CDM for 16 h at 37°C with shaking (250 rpm) and then incubated either statically (●, ▲) or with shaking (○, △). Viability was determined by plating on CDM agar. Results are representative of three independent experiments.

resistance in *S. aureus*. Their deduced partial (105 residues) amino acid sequence of CtaA is identical to ours except for their first five and last three amino acids.

SPW3 has altered growth characteristics. For both *B. subtilis* and *S. aureus*, mutations within *ctaA* have been reported to result in a small-colony morphology on solid media (21, 38). We clearly observed a small-colony morphology when *S. aureus* SPW3 was grown on either blood agar base (Difco) supplemented with sheep blood (5% [vol/vol]) or, after prolonged incubation, on CDM agar (37°C, 2 days), after which time the colonies were approximately 50% smaller than those of 8325-4 grown on the same plate (0.5 mm versus 1 mm in diameter). Although SPW3 formed smaller colonies on CDM agar, it grew at the same rate in liquid CDM as its parent, 8325-4 (data not shown). The *ctaA* mutation also led to a reduced ability to utilize carbon sources other than glucose, since SPW3 grew very poorly compared to 8325-4 on CDM with glucose replaced by 0.1% (wt/vol) lactose or succinate.

Alteration in antibiotic resistance. It was observed in *B. subtilis* that inactivation of *ctaA* resulted in increased resistance to aminoglycoside antibiotics (21). Upon comparing the MICs of a range of antibiotics for *S. aureus* 8325-4 and SPW3, we found that the resistance of SPW3 to aminoglycosides (gentamicin, streptomycin, and kanamycin) was two- to fourfold greater than that of 8325-4, while resistance to the cell wall biosynthesis inhibitor methicillin was unaltered (data not shown). The increased resistance properties were not due to the presence of Tn*917* per se.

***ctaA* is involved in starvation survival and recovery from starvation.** SPW3 was initially identified as a Tn insertion mutant which had a diminished ability to survive in response to carbon, amino acid, or phosphate starvation (42). Further characterization of the starvation survival response induced by carbon limitation revealed that the defect was more pronounced under highly aerated conditions. When SPW3 cultures were incubated statically during starvation, somewhat fewer cells survived ($T = 5$ days) compared to the parental strain 8325-4 (Fig. 2), but this difference was greater than 100-fold ($T = 5$ days) if the cultures were shaken (250 rpm) during starvation (Fig. 2).

The reduced ability of SPW3 cells to survive prolonged starvation was determined by the ability of starved cells to recover and form colonies on agar. It is therefore possible that the apparent reduction in the number of cells surviving starvation may be due not to cell death but to an inability of the cells to exit the starvation survival state and resume growth to form a colony. To distinguish between the two scenarios, a viability stain was used to estimate metabolically active cell numbers in parallel with agar plate viable counts, which enabled the presence of viable but nonrecoverable cells in the culture to be determined. The lipophilic dye Rh123, which is accumulated in cells in response to an active membrane potential, was used as an indicator of cell viability in association with flow cytometry (15, 43). For 8325-4 glucose-limited cultures, there was a good correlation between the number of viable cells detected by flow cytometry (2.8×10^6 fluorescent particles ml^{-1}) and plate counts (2.4×10^6 CFU ml^{-1}), but for SPW3 cultures there were >10-fold more cells demonstrating viability (Rh123 staining; 3.1×10^5 fluorescent particles ml^{-1}) than could recover to form colonies on agar (1.9×10^4 CFU ml^{-1}). It therefore appears that inactivation of *ctaA* results in approximately 100-fold reduction of cells surviving starvation under aerated conditions, and of those cells that do survive, more than 90% are unable to recover to form colonies on agar plates.

A defective recovery response of SPW3 was confirmed by the analysis of the recovery kinetics, as measured by increasing CFU per milliliter after the addition of nutrients to starved cells (Fig. 3). For 8325-4, an increase in cell numbers was observed after approximately 180 min; for SPW3, the lag period was extended by about 70 min, with cell numbers thereupon increasing more slowly than observed for 8325-4.

The recovery defect is due to increased oxidative stress. Several factors, such as temperature, reducing agents, media, oxygen tension, and inclusion of catalase in the recovery medium, have been identified as altering the recoverability of *E. coli* mutants defective in cytochrome biosynthesis (12). Varying these conditions had no effect on the number of starved SPW3 cells recoverable on agar plates (data not shown), except for the addition of catalase. When catalase was included in the recovery medium, the number of cells recovered was about twofold higher for SPW3 but unchanged for 8325-4 (data not shown). Therefore, the addition of catalase partially alleviated the recovery defect of the nonrecoverable SPW3 cells in a starved culture (as determined by Rh123 staining). Since catalase is involved in resistance to oxidative stress, this result suggests that the inactivation of *ctaA* leads to an increased oxidative stress on the cell during recovery.

SPW3 lacks cytochrome aa_3. In *B. subtilis*, *ctaA* is involved in the biosynthesis of heme A from heme O, which in turn is a

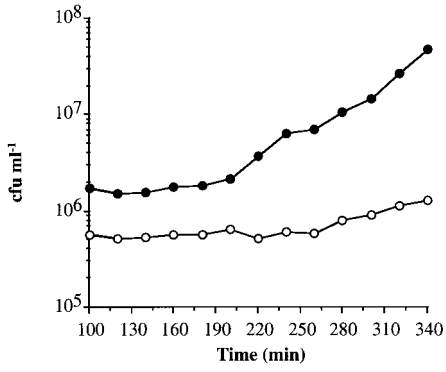

FIG. 3. Role of CtaA in recovery from starvation, determined from recovery kinetics of starved *S. aureus* 8325-4 (●) and SPW3 (○). Changes in CFU per milliliter were monitored after the addition of glucose and amino acids to glucose-starved cells (7 days, 37°C, shaking at 250 rpm).

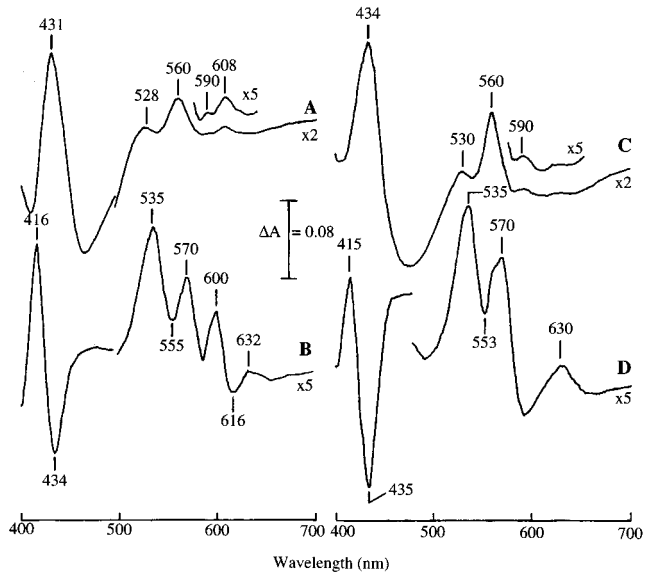

FIG. 4. Effect of *ctaA* on cytochrome profile. Electronic visible difference spectra of exponential-phase (OD_{600} = 0.6) whole cells of wild-type strain 8325-4 (spectra A and B) and *ctaA* mutant SPW3 (spectra C and D) are shown. Spectra A and C are reduced-minus-oxidized difference spectra; spectra B and D are CO-reduced-minus-reduced difference spectra. Distinctive features of the spectra are shown. The vertical bar represents ΔA = 0.08, except where individual scans show increased sensitivity (×2 or ×5). In spectra A, B, and D, the sensitivity changes at about 500 nm. The OD_{600}s of samples used for spectra A and B and for spectra C and D were 85.5 and 111.75, respectively.

functional component of the cytochrome *a*-type respiratory terminal oxidases (20–22, 33–35). To investigate if the *S. aureus* CtaA has a similar role in cytochrome biosynthesis, we compared the cytochrome complement of whole cells of SPW3 to that of 8325-4.

The reduced-minus-oxidized difference spectrum of wild-type strain 8325-4 showed bands in the g, b, and a regions attributable to cytochrome(s) *b*, with absorption maxima centered around 431, 528, and 560 nm, respectively (Fig. 4A). The a band at 608 nm is assigned to cytochrome aa_3, presumably a major oxidase in this bacterium. A small peak at 590 nm could not be assigned to a particular hemoprotein. The CO difference spectrum (CO-reduced minus reduced [Fig. 4B]) showed bands in the a region consistent with the presence of the CO-binding heme, i.e., a_3, of cytochrome aa_3. The peak at 600 nm is probably due to the CO adduct of this heme, and the trough at 616 nm is likely due to the loss of absorbance of the reduced form on binding CO; however, both bands are red-shifted (about 10 nm in the case of the peak) by comparison with the classical spectrum for cytochrome a_3 (44). The Soret region did not reveal clearly the anticipated bands (peak around 430 nm, trough near 445 nm) of cytochrome a_3 and its CO adduct; these may have been masked by the prominent peak at 416 nm and trough at 434 nm which can be attributed to cytochrome *o*. The undulations in the a and b regions of the CO difference spectrum between 500 and 600 nm are similar to those observed upon CO binding by *E. coli* cytochrome *o* as observed by photodissociation spectroscopy (26). The peaks at 535 and 570 nm are due to the CO adduct of heme O, and the shallow trough is due to the loss of absorbance of the reduced form. A small peak was seen at 632 nm (see below). In stationary-phase cultures (spectra not shown), wild-type cells contained significantly lower concentrations of cytochrome aa_3.

In marked contrast, spectra of the *ctaA* mutant SPW3 lacked all features that could be attributed to cytochrome aa_3. The reduced-minus-oxidized difference spectrum (Fig. 4C) lacked a clear 608-nm band, but the small 590-nm feature persisted. In the CO difference spectrum (Fig. 4D), the bands at 600 and 616 nm were also missing, and the remaining features between 400 and 600 nm can be attributed largely to cytochrome *o*. Mutant cells showed a prominent band at 630 nm in the CO difference spectrum (Fig. 4D) which was much more marked than in the wild-type strain (Fig. 4B). Its identity is unknown, and we are unaware of its observation before in this bacterium. In stationary-phase cultures (spectra not shown), mutant cells showed spectral features similar to those in exponentially growing cultures, but with minor variations (up to 3 nm) in the observed band positions.

Regulation of *ctaA* expression. The Tn insertion inactivating *ctaA* in SPW3 also generated a *lacZ* fusion with the promoter of this gene. Expression of *ctaA* could therefore be monitored throughout growth by measuring the level of β-galactosidase within the cell, using the fluorogenic substrate 4-methylumbelliferyl-β-D-galactoside. The expression of *ctaA*::*lacZ* during growth was very low, with maximal levels of expression occurring during the transition from the exponential to the stationary phase of growth (42). To investigate the role of several well-characterized *S. aureus* regulators of gene expression on the expression of *ctaA*, the Tn*917*-LTV1-generated *ctaA*::*lacZ* was introduced into a set of strains bearing a defined mutation, *agr*Δ (PC6911) or *sarA* (PC1839), which are regulators of virulence determinant expression, and the alternative sigma factor gene *sigB* (PC400), generating strains MC61, MC62, and MC63, respectively. No alteration in the pattern of *ctaA*::*lacZ* expression was observed in any of the backgrounds (data not shown).

Role of *ctaA* in toxin production and pathogenicity. Small-colony variants (SCVs) of *S. aureus* show decreased synthesis and secretion of the α-hemolysin toxin compared to the wild-type strain from which they were derived (40). To determine if the *ctaA* mutation altered the production of α-hemolysin, culture supernatants were assayed for α-hemolysin activity by a rabbit blood cell lysis assay (Fig. 5). For 8325-4, α-hemolysin is synthesized preferentially during the post-exponential phase of growth (25), but interestingly the appearance of α-hemolysin activity in SPW3 was delayed by 2 h, with maximum levels of activity only 50% of that observed for 8325-4. α-Hemolysin activity can also be detected as a zone of clearing when *S. aureus* is cultured on agar plates containing rabbit blood. About

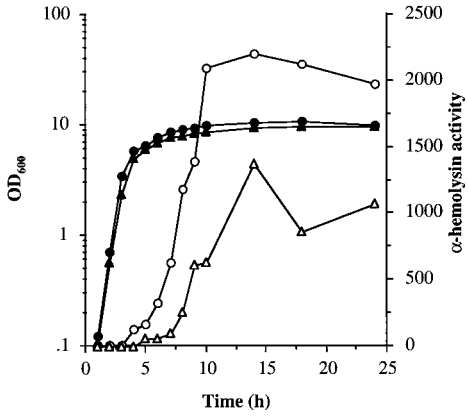

FIG. 5. Role of CtaA in α-hemolysin production. α-Hemolysin activities in culture supernatants of *S. aureus* 8325-4 (○, ●) and SPW3 (△, ▲) were determined. Cells were grown in BHI broth, and growth was monitored by OD_{600} (●, ▲). ○, △, specific α-hemolysin activity measured by rabbit blood lysis.

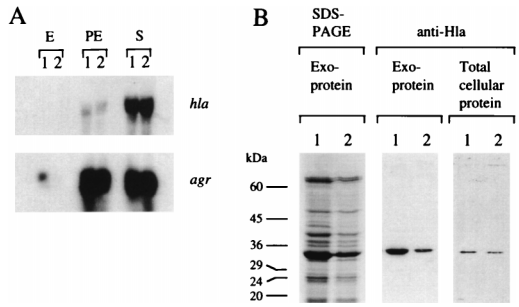

FIG. 6. α-Hemolysin regulation by CtaA. *hla* transcription and Hla protein levels in *S. aureus* 8325-4 and SPW3 are shown. (A) Northern blot analysis of total RNA from 8325-4 (lane 1) and SPW3 (lane 2). RNA samples of exponential-phase cells (T = 2 h [E]), post-exponential-phase cells (T = 5 h [PE]), and stationary-phase cells (T = 8 h [S]) were probed with an internal fragment from either *hla* or *agr* as described in Materials and Methods. (B) Exoproteins, and total cellular proteins of stationary-phase cultures (equivalent of 0.05 OD_{600} units of original culture), of 8325-4 (lane 1) and SPW3 (lane 2) were separated by SDS-PAGE (10% [wt/vol] gel) and then analyzed by Coomassie blue staining or Western blotting with antisera raised against Hla as the probe.

a 10-fold reduction in the zone of clearing surrounding colonies of SPW3 compared to 8325-4 was observed after overnight incubation in aerobic conditions, but no difference was observed when cells were grown in either microaerobic or anaerobic conditions.

To determine if the decreased levels of α-hemolysin activity were due to either reduced *hla* transcription, translation, or posttranslational modification of the protein, Northern and Western blot analyses were performed. Northern blot analysis of total cellular RNA during exponential (T = 3 h), post-exponential (T = 5 h), and stationary (T = 10 h) phases of growth in BHI broth showed that *hla* transcripts could be detected during post-exponential and stationary phases but not during the exponential phase of growth (Fig. 6A), as previously observed (39). The major positive regulator of *hla* transcription, RNAIII (25), showed a similar pattern of expression (Fig. 6A). There was no apparent difference in the level of transcription of either *hla* or RNAIII between SPW3 and its parent, 8325-4. Western blot analysis of exoproteins and total cellular proteins in stationary phase (T = 10 h) of 8325-4 and SPW3 was performed with antisera raised against Hla as the probe. Approximately twofold less Hla was present in the SPW3 exoprotein sample than in the 8325-4 sample (Fig. 6B). This reduction in Hla in the supernatant was not due to defective secretion, since no accumulation of Hla was observed in the total cellular proteins extract of SPW3 (Fig. 6B). Interestingly, comparison of the total exoprotein profiles of 8325-4 and SPW3 revealed a reduction in the amounts of all proteins secreted into the culture supernatant by SPW3.

Transcription of the toxic shock syndrome toxin 1 gene (*tst*) was also monitored during growth, via a *tst*::*lux* reporter fusion, in both SPW3 (MC70) and 8325-4 (PC1072) backgrounds. Expression of *tst*::*lux* in PC1072 occurs preferentially during post-exponential-phase growth (3) (Fig. 7); in the SPW3 background (MC70), however, *tst*::*lux* expression was delayed by about 1 h and reached only 20% of the levels observed for PC1072 after 13 h of growth. This profile was very similar to the accumulation of Hla activity observed in the SPW3 supernatant (Fig. 5).

In a mouse lesion model of infection (5), no significant difference was observed in the number of *S. aureus* CFU recovered from lesions or in the sizes of lesions formed by 8325-4 and SPW3 (data not shown). This finding suggests that *ctaA* does not have a significant role in the ability of *S. aureus* to cause infection in this model, even though the level of α-toxin synthesized is reduced.

DISCUSSION

We have characterized an *S. aureus* mutant, SPW3, in which a Tn insertion inactivated a gene, giving rise to pleiotropic effects on cell physiology including alterations in the ability to survive and recover from starvation. The Tn insertion was localized to a gene homologous to *B. subtilis ctaA*, which is involved in heme A biosynthesis required for cytochrome aa_3 and caa_3 synthesis (20–22, 33–35). Recent studies of *B. subtilis* have indicated that *ctaA* encodes a heme O monooxygenase which converts heme O to heme A (35). Analysis of SPW3 cytochromes confirmed that *ctaA* in *S. aureus* is involved in cytochrome biosynthesis since absorption profiles demonstrated the loss of bands with properties similar but not identical to those of heme A-containing cytochrome terminal oxidases. We predict that these bands correspond to cytochrome aa_3 and that the phenotypic characteristics of SPW3 are attributable to its inability to synthesize the *a*-type cytochrome terminal oxidases.

S. aureus has previously been shown by electronic visible absorbance spectroscopy (reduced-minus-oxidized spectra) to possess an *a*-type oxidase (1, 8, 37). Such a spectrum in Fig. 4A demonstrates this; in addition, we have used CO difference spectra (not used previously) to confirm the presence of such an oxidase and the absence of cytochrome *bd*. Spectra in Fig. 4C and D present unequivocal evidence for diminution of cytochrome aa_3 in mutant SPW3 to below spectroscopically detectable levels. These spectra do not provide clear evidence for the nature of the oxidase in this strain. However, in the absence of cytochromes aa_3 and *bd* (and the unclear role, if any, for the species identified by the 630-nm band [see below]), it is likely that the respiratory chain of this mutant is terminated by cytochrome *o*. The presence of this oxidase in *S. aureus* has been proposed earlier (37), and the spectral features in Fig. 4D are very similar to those (peaks at 416, 535 and 570 nm; trough at 432) identified by Smith in *Staphylococcus albus* (see reference 6 and references therein).

The CO difference spectra reveal in the wild-type strain an unidentified absorption maximum at 630 nm which is considerably more prominent in the mutant. Its position in the red region of the spectrum suggests carbon monoxycytochrome *d*

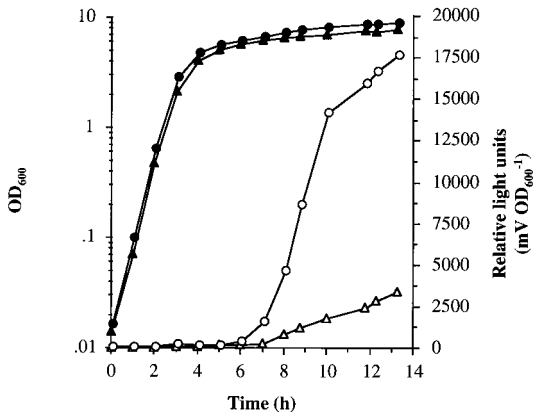

FIG. 7. Role of CtaA in *tst* expression. The expression of *tst*::*lux* in *S. aureus* 8325-4 (PC1072; ○, ●) and SPW3 (MC70; △, ▲) was determined. Cells were grown in BHI broth, and growth was monitored by OD_{600} (●, ▲). *tst*::*lux* expression was measured as described in Materials and Methods (○, △).

but, classically, such adducts absorb at about 636 to about 642 nm (45). If this species is a CO-ligated form, the unligated form should appear in the corresponding reduced-minus-oxidized spectra; however, no candidate signals were observed in Fig. 4C. Two explanations can be offered. First, the band may arise from an unusual cytochrome *d* whose turnover at room temperature is too rapid for the oxidized form to be stabilized; a similar phenomenon has been described for cytochrome *bd* in *Azotobacter vinelandii* (46). Alternatively, the 630-nm species may be the CO adduct of the unidentified hemoprotein with a maximum at 590 nm when reduced (Fig. 4A and C). An unidentified feature at 628 nm was also noted in CO difference spectra of *Helicobacter pylori* (18), but in that case the 590-nm band of a putative unligated reduced form was not described.

The growth characteristics of SPW3 were broadly similar to that of 8325-4, except that growth was poor on medium in which glucose is not the main energy source. *B. subtilis ctaA* mutants have similar phenotypes since they are unable to utilize nonfermentable carbon sources (21). The reduced ability to utilize nonfermentable substrates is thought to be attributable to the inability of the respiratory chain to reduce NADH, NADPH, and FADH generated during their respiration, since this appears to be dependent on cytochrome caa_3 in the respiratory chain (21). An accumulation of reducing equivalents required for oxidative reactions during the tricarboxylic acid cycle would uncouple electron transport from oxidative phosphorylation.

Changes in the respiratory chain could also account for the starvation survival and recovery defects of SPW3. The starvation survival response of *S. aureus* is triggered by glucose limitation. Therefore, at the onset of starvation the growth rate is reduced and respiration becomes dependent on energy sources other than glucose. It is under these conditions that respiration appears to be dependent on the *a*-type cytochromes. This theory is supported by our observation that transcription of *ctaA* increases upon glucose limitation. Interestingly, the starvation survival phenotype was partially suppressed when starved cells were incubated statically, which suggests that molecular oxygen also plays a role in the toxicity of the *ctaA* mutation. *B. subtilis ctaA* mutants also show a starvation defect since mutants are unable to sporulate upon exhaustion of glucose (21), but the mechanism giving rise to the sporulation defect remains unclear since post-exponential-phase regulation of *ctaA* is independent of sporulation specific factors (22).

The insertional inactivation of *ctaA* in *S. aureus* SPW3 resulted in a pronounced defect during recovery from starvation. A large proportion of starved cells which had characteristics indicating viability, as evidenced by active metabolism, were unable to recover and resume growth. For these experiments, recovery was initiated by the addition of glucose to starved cells. Under these conditions, the *ctaA* cells may well experience a sudden burst of metabolic activity resulting in the generation of excess reducing equivalents, leading to the uncoupling of electron transport (21). The uncoupling of electron transport from substrate-level phosphorylation could then lead to increased oxidative stress, killing the recovering cell. This theory is supported by the observation that catalase, part of the cell's protective mechanism against oxygen free radicals, was able to partially alleviate the recovery defect.

Interestingly, a mutant of *E. coli* (*surB1*) which is also defective in resuming growth after starvation has been isolated (31). The recovery defect was shown to be due to inactivation of *cydC*, which encodes a putative heme transporter required for the biosynthesis of functional cytochrome *d* (11, 12, 32). Like the recovery defect that we have observed in *S. aureus* SPW3, the *cydC* growth defects could be suppressed by the inclusion of catalase in the recovery media, although unlike the case for SPW3, other reducing agents also increased the recovery efficiency (12). These results strengthen the role of specific cytochromes in the development and maintenance of the starvation survival response and their requirement for successful exit from this state by preventing the formation of toxic oxidizing products.

The phenotype of SPW3 is similar to that observed for SCVs of *S. aureus* which have been associated with persistent and relapsing infections (27). As their name suggests, SCVs show reduced colony size when grown aerobically on agar plates and, like SPW3, demonstrate increased resistance to aminoglycoside antibiotics and reduced levels of α-hemolysin production. SCVs have been shown to have defective electron transport due to a block in either hemin or menadonine synthesis leading to a loss of respiratory chain components (19). Recently a mutation in *hemB*, a biosynthetic gene involved in hemin biosynthesis, was characterized and was found to give rise to an SCV phenotype (40). The *hemB* mutant was shown to have an increased ability to persist within cultured endothelial cells which was attributed to a decrease in α-hemolysin production both at mRNA and protein levels.

Like *hemB*, SPW3 demonstrates decreased synthesis of α-hemolysin. Interestingly, while the amount of Hla protein and hemolysin activity in culture supernatants was approximately twofold lower in SPW3 than in the parental strain 8325-4, the level of *hla* and RNAIII (a positive regulator of *hla* expression) transcripts appeared unaltered, suggesting that the defect in Hla synthesis occurred at the level of translation. Analysis of *tst* transcription via a *tst*::*lux* reporter fusion, which is regulated similarly to *hla* (3), revealed that *tst* transcription was delayed and reduced in *ctaA* mutant compared to a wild-type background. This discrepancy between the level of *tst* transcription and *hla* transcription may be due to the higher sensitivity of the *lux* reporter fusion compared to Northern blot analysis or, alternatively, due to reduced translation of the *tst*::*lux* reporter fusion transcripts reflecting a general reduction in translational proficiency of SPW3. Therefore, the reduced level of Hla in culture supernatants is possibly due to reduced transcription and translation. Since the amounts of all exoproteins in the culture supernatants of SPW3 are lower than in those of 8325-4, the defect may not be specific to only toxin production. Exoproteins are preferentially synthesized in the post-exponential phase, when growth appears to be dependent on the heme A-dependent cytochromes; therefore, the reduced rate of protein synthesis in SPW3 probably reflects the general decreased energetic status of the cell. The increased resistance of the *ctaA* mutant to aminoglycosides may be due to the fact that they are accumulated within cells by active transport, and disruption of the respiratory chain results in a decreased membrane potential and hence reduces the active uptake of the antibiotic (36).

From this study, it can be seen that inactivation of *ctaA* has diverse physiological effects on the cell, which are attributable to the inability to synthesize heme A-containing cytochromes. This results in many of the characteristics of SCVs. The possible role of *ctaA* in chronic infection and SCV formation requires further examination.

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