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Cytoplasmic Control of Premature Activation of a Secreted Protease Zymogen: Deletion of Staphostatin B (SspC) in *Staphylococcus aureus* 8325-4 Yields a Profound Pleiotropic Phenotype

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The cytoplasmic protein SspC of *Staphylococcus aureus*, referred to as staphostatin B, is a very specific, tightly binding inhibitor of the secreted protease staphopain B (SspB). SspC is hypothesized to protect intracellular proteins against proteolytic damage by prematurely folded and activated staphopain B (M. Rzychon, A. Sabat, K. Kosowska, J. Potempa, and A. Dubin, Mol. Microbiol. 49:1051–1066, 2003). Here we provide evidence that elimination of intracellular staphopain B activity is indeed the function of SspC. An isogenic *sspC* mutant of *S. aureus* 8325-4 exhibits a wide range of striking pleiotropic alterations in phenotype, which distinguish it from the parent. These changes include a defect in growth, a less structured peptidoglycan layer within the cell envelope, severely decreased autolytic activity, resistance to lysis by *S. aureus* phages, extensively diminished sensitivity to lysis by lysostaphin, the ability to form a biofilm, and a total lack of extracellular proteins secreted into the growth media. The same phenotype was also engineered by introduction of *sspB* into an 8325-4 *sspBC* mutant. In contrast, *sspC* inactivation in the SH1000 strain did not yield any significant changes in the mutant phenotype, apparently due to strongly reduced expression of *sspB* in the sigma B-positive background. The exact pathway by which these diverse aberrations are exerted in 8325-4 is unknown, but it is apparent that a very small amount of staphopain B (less than 20 ng per 200 μg of cell proteins) is sufficient to bring about these widespread changes. It is proposed that the effects observed are modulated through the proteolytic degradation of several cytoplasmic proteins within cells lacking the inhibitor. Seemingly, some of these proteins may play a role in protein secretion; hence, their proteolytic inactivation by SspB has pleiotropic effects on the SspC-deficient mutant.

Staphylococcus aureus is a highly virulent and widely successful pathogen that is speculated to be the most common cause of human disease (39). Currently, *S. aureus* is the leading agent of nosocomial infections worldwide, causing a variety of ailments in a plethora of ecological niches within its host (19). These ailments range from minor complaints of superficial lesions to more serious systemic and life-threatening conditions, such as bacteremia. With the advent of antibiotic resistance and the emergence of clinical isolates resistant to last-resort glycopeptide antibiotics (41, 52), novel targets are crucial in the fight against a return to the preantibiotic era. The major focus in this area has been the characterization of extracellular virulence determinants produced by the organism, in the hope of determining possible targets for drug development.

The overall pathogenic diversity and success of *S. aureus* are largely due to the vast array of virulence determinants, which include hemolysins, toxins, adhesins, exoenzymes, and other extracellular proteins, such as staphylokinase and protein A (38, 39, 47). Moreover, in response to the changing host environment, *S. aureus* has the capacity to activate selected genes or groups of genes encoding virulence factors to enhance its chance of survival, dissemination, and proliferation (1, 47). This switching process is precisely controlled by global regulatory elements, which can broadly be divided into two major groups: two-component regulatory systems and the SarA protein family (10, 46, 47). Altogether, 16 two-component regulatory systems, including the widely studied *agr* (accessory gene regulator) locus, have been identified in *S. aureus*. The sensor proteins of these systems provide a means for environmental signaling, while the response regulators, in conjunction with other transcription factors (such as sigma B or any of the 12 members of the SarA protein family), function as effectors in overlapping, multifactorial feedback networks, responding to extracellular stimuli (10). Several of the loci affect the expression of proteases, and the strongest effect is exerted by the *agr* and *sarA* loci.

The *agr* locus strongly activates and SarA directly represses transcription of the four major extracellular proteases: aureolysin (Aur), a metalloprotease; staphopain A (ScpA) and staphopain B (SspB), two homologous cysteine proteases; and the V8 or SspA protease, a serine protease (9, 32, 70). It is believed that the temporal coordination of the expression of various groups of staphylococcal genes through the quorum-sensing system (*agr*), tuned by other regulatory loci, enables *S. aureus* to switch from the expression of adhesive molecules to the

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expression of more progressive virulence determinants, such as extracellular toxins and enzymes that can damage host tissues and the immune system (38, 39). Significantly, proteases have been shown to modulate bacterial surface adhesive molecules, changing the *S. aureus* phenotype from adhesive to invasive and possibly contributing to the dissemination of infection (33, 43, 44). In addition, these enzymes have multiple activities that may affect the host through inactivation of serpins, elastin degradation, prothrombin activation, and cleavage of immunoglobulins, fibronectin, fibrinogen, and high-molecular-weight kininogen (17, 42). Accordingly, it was shown that an *S. aureus* SspA protease-deficient mutant was severely attenuated in virulence in mouse abscess, bacteremia, and wound infection models (12). The reduced virulence of the *sspA* mutant is apparently due to the polar effect of the transposon insertion in *sspA* on the expression of *sspB*, which encodes a cysteine protease, located downstream in the same operon (55, 62). The inference that proteases secreted by *S. aureus* are crucial virulence factors was contradicted by a recent study which revealed no alteration in *S. aureus* virulence in a mouse model of septic arthritis when isogenic extracellular protease mutants were tested (6). However, it is typical of *S. aureus* that different sets of genes are important for showing a virulent phenotype in different models (12, 31), and thus the significance of staphopains for *S. aureus* pathogenicity is still an open question.

In addition to regulation at the transcriptional level, the proteolytic activity of *S. aureus* is also under posttranslational control, which occurs via an interdependent, hierarchical cascade of activation (14, 55, 62). The fidelity of this system of maturation (aureolysin → SspA → SspB) is further enhanced by the clustering of genes encoding two of the proteases in a single operon, *sspABC*. Apparently, however, this is not sufficient to control the activity of staphopain B, since the final gene in the operon (*sspC*) encodes a very specific, dedicated inhibitor of this enzyme, referred to as staphostatin B (58). A similar gene arrangement was also found in the case of the second cysteine protease operon (*scpAB*), in which the gene encoding staphopain A (*scpA*) is followed by *scpB*, which encodes a novel inhibitor homologous to staphostatin B (SspC) (15). Operon structures encoding a cysteine protease and its inhibitor are conserved in *Staphylococcus epidermidis* (16) and *Staphylococcus warneri* (69). Outside *Staphylococcus* spp., however, such a system is highly unusual and must be rare in the prokaryotic kingdom. Indeed, proteinaceous protease inhibitors have been described only in *Escherichia coli* (11), *Pseudomonas aeruginosa* (27), and *Streptomyces* species (65). Yet the regulation of proteolytic activity is not uncommon; several mechanisms are used to prevent premature activation, and the most common is the production of proenzymes. However, with the exception of *S. aureus*, an extracellular cascade of zymogen activation has been described only for *P. aeruginosa* (4, 35, 51).

In the case of *S. aureus* the physiological necessity of such elaborate systems, including a cytoplasmic inhibitor (SspC) to control the activity of an enzyme that is apparently secreted as a proteolytically inactive 40-kDa zymogen (proSspB) (21), is puzzling. It has been suggested that the specific, designated inhibitors of the staphopains are needed to protect the cytosol from the activity of prematurely activated staphopains (18, 58). In this study we generated an isogenic *sspC* mutant of *S. aureus* 8325-4 and demonstrated that in the absence of the inhibitory protein the growth and viability of the cells were impaired. In addition, major alterations were found in cellular physiology, including a decrease in autolytic activity, drastically reduced sensitivity to lysostaphin-mediated lysis, and elevated biofilm production. Furthermore, an apparent breakdown in protein secretion was noted, and no detectable extracellular proteins were found in culture supernatants. All these changes were most likely caused by proteolytic inactivation of a subset of cytoplasmic proteins by SspB in staphostatin-deficient cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *S. aureus* and *E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in Luria-Bertani medium (Fluka) at 37°C. *S. aureus* was grown in brain heart infusion (BHI) broth (Oxoid) (flask/medium volume ratio, 1:2.5) at 37°C (250 rpm) (8) unless otherwise indicated. When required, antibiotics were added at the following concentrations: 100 mg of ampicillin liter^{-1} and 12.5 mg of tetracycline liter^{-1} for *E. coli* and 5 mg of tetracycline liter^{-1}, 5 mg of erythromycin liter^{-1}, and 25 mg of lincomycin liter^{-1} for *S. aureus*.

Construction of the *sspC* mutant strain. Primers OL101 and OL102 were used to PCR generate the *sspC* coding region along with approximately 1 kb of upstream and downstream flanking DNA. The 2.2-kb DNA fragment was digested with BamHI and SphI and cloned into pAZ106 (34) to generate pLES101 by using standard cloning techniques (59). A naturally occurring XbaI site approximately 50 bp 3′ of the *sspC* start codon was used as a target site for insertion of a tetracycline resistance cassette that was generated from pDG1515 (26) by using the OL105-OL106 primer pair. The XbaI-digested cassette was cloned into pLES101 to obtain pLES102. Electrocompetent *S. aureus* RN4220 was transformed by the method of Schenk and Ladagga (61). Integrants were confirmed by Southern blotting (LES42) and were used as donors for transduction with phage φ11. Transductants were selected on the basis of their resistance to tetracycline (indicating the presence of the cassette) and sensitivity to erythromycin (indicating loss of the plasmid and the associated functional copy of *sspC*) and were confirmed by Southern blot analysis in order to create strain LES43 (Δ*sspC*).

Construction of *sspB and sspC* complementation strains. The OL1136-OL1137 primer pair was used to generate a 191-bp fragment containing the natural *ssp* promoter located upstream of *sspA* (62). This fragment was digested with PstI/BamHI and ligated to pMK4 (64), creating pLES103. The OL1138-OL1139 and OL1134-OL1135 primer pairs were used to generate fragments containing the coding regions of *sspB* (1,261 bp) and *sspC* (501 bp), respectively. These fragments were digested with BamHI and EcoRI and ligated separately to pLES103 to create complementation constructs pLES104 (*sspB*) and pLES105 (*sspC*). These constructs were then transformed into RN4220 before φ11 phage transduction was used to transduce pLES104 into LES17 (Δ*sspBC*) to create LES46 (Δ*sspBC sspB*$^+$). As LES43 (Δ*sspC*) is phage resistant, an RN4220/pLES105 lysate was generated and used to transduce 8325-4 to create strain LES47, before it was used as the recipient in a transduction with a LES42 lysate. The strain was then resolved based on its resistance to tetracycline (*sspC* mutation) and chloramphenicol (pLES105) and its sensitivity to erythromycin, creating strain LES48 (Δ*sspC sspC*$^+$). All strains were confirmed by Southern blotting.

Analysis of cellular morphology by electron microscopy. The cellular morphology of strains was analyzed by using scanning electron microscope (SEM) and transmission electron microscope (TEM) techniques. Strains were grown under standard conditions until the stationary phase (approximately 15 h), and the cells were harvested by centrifugation. The pellets were washed three times with phosphate-buffered saline (PBS) and fixed in 2.5% (wt/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Cells were analyzed at the Center for Ultrastructural Research (University of Georgia, Athens) by using a Philips/FEI Technai 20 TEM or a LEO 982 field emission SEM.

Biofilm production assay. Strains were grown for 24 h in BHI media containing 0.25% (wt/vol) glucose in the wells of a 96-well plate at 37°C. The cells were washed twice with PBS, fixed with absolute ethanol, and stained with a 2% (wt/vol) crystal violet solution for 2 min (3). The stain was aspirated, and the wells were washed several times with PBS. One hundred microliters of absolute ethanol was added to each well and incubated for 10 min at room temperature; then 50 μl of the eluate was removed, and its absorbance at 570 nm (A_{570}) was determined by using a microplate reader (SpectraMax; Molecular Devices).

***S. aureus* culture fractionation.** The optical densities at 600 nm (OD_{600}) of *S. aureus* cultures were standardized, and the cells were separated from the culture

TABLE 1. Bacterial strains, plasmids, and primers

Strain, plasmid, or primer	Genotype or description	Reference or source
E. coli DH5α	ϕ80 Δ(*lacZ*)M15 Δ(*argF-lac*)*U169 endA1 recA1 hsdR17* (r_k^- m_k^+) *deoR thi-1 supE44 gyrA96 relA1*	59
S. aureus strains		
8325-4	Wild-type strain (NCTC 8325 cured of prophages)	Lab stock
RN4220	Restriction-deficient transformation recipient	Lab stock
SH1000	Functional *rsbU* derivative of 8325-4 *rsbU*$^+$	29
SH108	8325-4 *atl*::pAZ106 *atl*	22
LES17	8325-4 *sspB*::pAUL-A *sspBC*	62
LES22	8325-4 *sspA*::pAZ106 *sspABC*	62
LES42	RN4220 pAZ106::*sspC*::*tet*	This study
LES43	8325-4 *sspC*::*tet sspC*	This study
LES46	8325-4 *sspB*::pAUL-A (LES17)/pLES104 *sspBC sspB*$^+$	This study
LES47	8325-4/pLES105	This study
LES48	8325-4 *sspC*::*tet* (LES43)/pLES105 *sspC sspC*$^+$	This study
Plasmids		
pAZ106	Promoterless *lacZ erm* insertion vector	34
pMK4	Shuttle vector, Cm^r	64
pDG1515	Shuttle vector harboring tetracycline cassette	26
pLES101	pAZ106 containing a 2.2-kb OL101-OL102 *sspC* PCR fragment	This study
pLES102	pLES101 containing a tetracycline cassette within *sspC*	This study
pLES103	pMK4 containing the *ssp* promoter region	This study
pLES104	pLES103 containing the *sspB* coding region	This study
pLES105	pLES103 containing the *sspC* coding region	This study
Primers[a]		
OL101	ACTGGATCCCAAACTTCATCGCTAAAG	
OL102	AGCTAGGCATGCGGAACGCCGTCTTGTTGATGC	
OL105	ACTTCTAGACGGATTTTATGACCGATGATGAAG	
OL106	TGATCTAGATTAGAAATCCCTTTGAGAATG	
OL1134	ATGGGATCCGATTAAAGGCAGGTAAAACT	
OL1135	ATGGAATTCATAAGAATTTAAAAGGGC	
OL1136	ATGCTGCAGCCATTCGCTCTCAATTCC	
OL1137	ATGGGATCCCAAGTTAAATATAACACT	
OL1138	AGTGGATCCTCAGACAATCCAGATGCAGCT	
OL1139	AGTGAATTCCCTATCATTGAACCATACC	

[a] Restriction sites are underlined.

media by centrifugation (5,000 × *g*, 30 min). The supernatants were filtered through 0.22-μm-pore-size membrane filters, while the cell pellets were washed with PBS. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis, and covalent labeling of the active site cysteine residue of staphopain [by using a biotinylated derivative of the cysteine protease inhibitor L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino) butane (E-64), referred to as DCG-04 (25)], proteins in the filtered supernatants were concentrated 10-fold by trichloroacetic acid (TCA) precipitation or membrane ultrafiltration (10-kDa cutoff; VivaSpin Devices, Viva Science, Beverly, Mass.). Cell wall fractions were obtained by the method of Rzychon et al. (58). Whole-cell protein extracts were obtained by breaking cells in a French press, followed by centrifugation (10,000 × *g*, 10 min, 4°C) to remove unbroken cells and large debris.

SDS-PAGE, gelatin zymography, and Western blotting. Exoprotein sample preparation and analysis were performed by SDS–12% PAGE (60). Gelatin zymography was performed by the method of McAleese et al. (43), based on the original method of Heussen and Dowdle (28). Western immunoblotting was performed by the method of Towbin et al. (66). Briefly, proteins were blotted onto a polyvinylidene difluoride membrane (Bio-Rad) and were detected by using mouse antisera raised against Atl (1:1,000 dilution) or SspB (1:500 dilution). Horseradish peroxidase-conjugated goat anti-mouse secondary antibody (diluted 1:25,000) and chemiluminescent substrates (ECL plus; Amersham Biosciences, Little Chalfont, United Kingdom) were used for detection of proteins on the membrane. Mouse monoclonal antibodies specific for the SspB protein were developed at the University of Georgia Monoclonal Antibody Facility by using a recombinant protein.

Autolysin extraction and zymography. Analysis of extracellular cell wall-associated murein hydrolases was carried out essentially as described by Qoronfleh and Wilkinson (54). Autolysin extracts prepared from 1 liter of exponential-phase cultures of *S. aureus* were concentrated 10-fold with a VivaSpin concentrator (Viva Science, Beverly, Mass.), and the amounts of total protein loaded were standardized by a bicinchoninic acid assay (Sigma). Autolysin zymography was performed as described previously (22).

Triton X-100-induced autolysis assay. Lysis induction assays were performed as described by Mani et al. (40). Overnight cultures of *S. aureus* were subcultured in fresh media and grown until the mid-log phase. Cells were pelleted and washed twice with ice-cold water, before they were resuspended to an OD_{600} of 2.0 in 10 ml of 0.05 M Tris-HCl (pH 7.6)–0.05% Triton X-100. The suspensions were incubated at 30°C with shaking (150 rpm), and the OD_{600} was measured every 30 min.

Peptidoglycan lysis kinetics assays. Cells were harvested from stationary-phase cultures, washed with PBS, and resuspended in 20 mM Tris-HCl (pH 8.0)–2 mM EDTA–1.25% Triton X-100 to obtain standardized OD_{600} values. Lysis was then performed in the presence of excess lysostaphin (50 μg/ml; Sigma), and OD_{600} values were determined at specific times by using a microplate reader (SpectraMax; Molecular Devices).

Protein extraction with LiCl. Cells harvested from the exponential growth phase were washed with 50 mM Tris-HCl (pH 7.5) and pretreated with 0.5 mM phenylmethylsulfonyl fluoride before incubation with 3 M LiCl for 1 h on ice (54). The supernatant was collected by centrifugation and concentrated by TCA precipitation.

Shedding of surface proteins with V8 protease. Exponential-phase cells were washed with PBS, resuspended in 50 mM Tris-HCl (pH 7.5)–20 mM $MgCl_2$–30% (wt/vol) sucrose, and treated with the V8 protease at 37°C for 2 h. The proteins in the supernatants collected were precipitated with TCA and resolved by SDS-PAGE.

Assays for adherence of bacterial cells. Assays for adherence of *S. aureus* to immobilized fibrinogen, fibronectin, and collagen (all obtained from Sigma) were performed as described by McAleese et al. (43).

Hemolysin assays. Blood agar plates (containing 10% [vol/vol] defibrinated rabbit or sheep blood) were used to detect hemolysin activity of single colonies. For quantitative hemolysin assays culture medium supernatants pretreated with

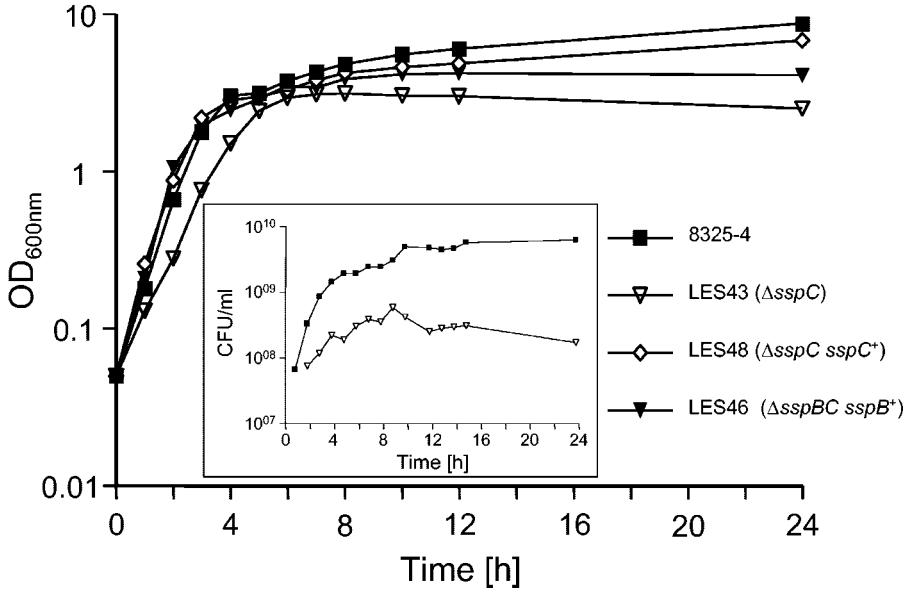

FIG. 1. Growth analysis of LES43 (Δ*sspC*) and its complemented derivatives. Strains were grown in BHI media at 37°C (250 rpm; volume/flask ratio, 1:2.5). The results are representative of at least three separate experiments.

0.025 mM phenylmethylsulfonyl fluoride and diluted in 145 mM NaCl–20 mM $CaCl_2$ were mixed with defibrinated blood (ratio, 1:40 [vol/vol]) and incubated for 15 min at 37°C. Hemolytic activity was measured at OD_{412} by using a microplate reader (SpectraMax; Molecular Devices).

Phage absorption assay and determination of MICs. Suspensions of exponential-phase cells of *S. aureus* 8325-4 or the *sspC* mutant were incubated with phage ϕ11 or ϕ85 at 30°C for 20 min. The bacterial cells were removed by centrifugation (5,000 × *g*, 10 min), and 100-μl portions of serial dilutions of the supernatant were mixed with 400 μl of the 8325-4 cells in the exponential phase of growth and 50 μl of 1 M $CaCl_2$. Following 10 min of incubation at room temperature, 100-μl samples were plated, and the number of plaques (number of PFU per milliliter) was determined after overnight incubation at 37°C. Phage stocks that were not incubated with bacteria were used as controls.

S. aureus (10^5 CFU/ml) was inoculated into Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) and dispensed (0.2 ml/well) into 96-well microtiter plates. MICs were determined in triplicate by serial twofold dilution of the antibiotics tested by following the recommendations of the National Committee for Clinical Laboratory Standards. The MIC was defined as the concentration of an antibiotic that completely inhibited cell growth during an 18-h incubation at 37°C. Growth was assayed with a microtiter plate reader by monitoring the optical density at 600 nm. The effects of the following antibiotics were tested: vancomycin, teicoplanin, penicillin, oxacillin, and ampicillin (Becton Dickinson, Mountain View, Calif.)

RESULTS

Insertional inactivation of *sspC* in *S. aureus* results in a defect in growth. Initial experiments to isolate the *sspC* mutant strain (LES43) revealed that overnight cultures had markedly reduced densities compared to parental strain 8325-4 cultures (Fig. 1). More detailed analysis demonstrated that LES43 (Δ*sspC*) grew very differently than 8325-4 and had a curious growth defect. Consistently, the mutant had a longer lag phase that was clearly seen when the growth was plotted on an arithmetic scale (data not shown). In addition, the *sspC* mutant had lower growth rates and yields during exponential growth, as reflected by statistically important ($P < 0.05$) differences in the exponential generation times (24.7 ± 12.6 and 33.3 ± 10.5 min for the mutant and the parent strain, respectively). Furthermore, the growth of the mutant appeared to stop in the postexponential phase (5 h), and this was followed by a period of stasis that lasted until approximately 8 h (Fig. 1). At this point the OD_{600} declined, and the culture density of the mutant was one-half the culture density of 8325-4 after 24 h.

These results were consistently observed, and while the trend remained the same, the severity of the defect was more pronounced in cultures grown in tryptic soy broth than in cultures grown in BHI medium and in cultures grown with increased aeration (a culture-to-flask volume ratio of 1:10 rather than 1:2.5) (data not shown). In order to confirm that the decline in the cellular density of LES43 (Δ*sspC*) was a result of cell lysis and death, a viability curve was produced for the mutant and its parent strain. A direct correlation between the decrease in OD_{600} and cellular viability was found, and the values for CFU per milliliter reflected the growth trends observed for 8325-4 and LES43 (Δ*sspC*) (Fig. 1, inset).

Complementational analysis studies. To assess whether the growth defect was functionally related to the absence of SspC, complementation studies were undertaken. *sspC* is the third of three genes in the polycistronic *ssp* operon (55), whose transcription is driven by a single promoter upstream of *sspA* (62). Therefore, in order to achieve complementation of the *sspC* mutation, it was necessary to fuse the *ssp* promoter to the *sspC* gene before it was introduced in *trans* into LES43 (Δ*sspC*), creating LES48 (Δ*sspC sspC*$^+$). Growth analysis of this strain revealed that complementation indeed restored the wild-type phenotype and that the growth closely mirrored that of 8325-4 (Fig. 1).

As SspC is hypothesized to act as a cytoplasmic inhibitor of SspB (58), we investigated whether the LES43 (Δ*sspC*) growth defect was a result of the loss of the capacity to inhibit SspB. An existing 8325-4 *sspBC* mutant (LES17) (62) was complemented with only *sspB*, in a manner similar to that described above for the *sspC* mutant strain. Studies with this strain, LES46 (Δ*sspBC sspB*$^+$) (Fig. 1), revealed that while its growth

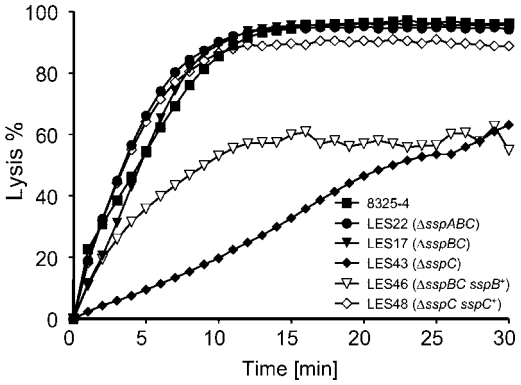

FIG. 2. Enzymatic reaction kinetics for lysostaphin lysis. Lysostaphin lysis reaction kinetics for a variety of strains were determined over a 30-min period by using an excess of enzyme. The efficiency of the lysis reaction is expressed as the percentage of cells lysed over time (the initial OD_{600} before addition of lysostaphin was defined as 100%).

was not impaired to the same degree as the growth of LES43 (Δ*sspC*), the growth was not like that of 8325-4. The initial growth rates and yields of this strain during exponential growth resembled those of 8325-4 (no statistical difference in the generation time was observed), yet when the strain entered the postexponential phase, the growth was retarded. A period of stasis between 5 and 10 h of growth was then observed, followed by a decrease in cellular density. The OD_{600} values for LES46 (Δ*sspBC sspB*$^+$) were found to be only one-half those of 8325-4 after 24 h. These changes in the growth pattern were apparently related to reconstitution of the *sspB* gene, since the 8325-4 *sspBC* mutant (LES17) had the same phenotype as the 8325-4 strain.

Loss of *sspC* in *S. aureus* 8325-4 results in profoundly reduced susceptibility of peptidoglycan to lysis by lysostaphin. During the early stages of identification of LES43 (Δ*sspC*), it was observed that the strain showed decreased susceptibility to enzymatic lysis by lysostaphin. Interestingly, the resistance was more apparent for cultures in the mid-exponential and stationary growth phases, and cells from earlier growth stages were susceptible to lysis. A detailed investigation of the lysis kinetics was undertaken by using this and a number of other strains to increase our understanding of this phenomenon (Fig. 2). It was determined that compared to the lysis rate of the wild-type strain in the late exponential or early stationary phase (50% lysis within 5 min after the start of incubation with excess of lysostaphin), the lysis rate of LES43 (Δ*sspC*) in the same phase of growth was consistently fivefold lower (50% lysis after 20 to 25 min). Furthermore, the initial reaction rate for 8325-4 was fourfold greater than that for LES43 (Δ*sspC*) during the first 15 min of lysis, and the 8325-4 reaction reached apparent completion within this time. Conversely, lysis of LES43 (Δ*sspC*) was slower and more gradual, and no reaction plateau was ever reached. None of the strains tested other than LES46 (Δ*sspBC sspB*$^+$), including LES17 (Δ*sspBC*) and LES22 (Δ*sspABC*), displayed similar characteristics. The lysis reaction of strain LES43 (Δ*sspC*) was very similar to that of LES46 (Δ*sspC*), indicating that expression of SspB within cells in the absence of SspC leads to this unusual phenotype. As a control, complemented strain LES48 (Δ*sspC sspC*$^+$) was also analyzed, and it displayed behavior identical to that of the wild type.

Phage-typing analysis of the *sspC* mutant strain. Despite numerous attempts, it proved to be impossible to lyse LES43 (Δ*sspC*) with either of the *S. aureus* transducing bacteriophages (ϕ11 or ϕ85). Phage typing was therefore performed (Department of Microbiology, Medical Academy, Gdansk, Poland) with the *sspC* mutant (LES43) along with parental strain 8325-4 (Table 2). Most strikingly, the *sspC* gene disruption rendered LES43 (Δ*sspC*) phage nontypeable, as demonstrated by resistance to lysis with all 19 phages tested, while the parental strain (8325-4) showed susceptibility to 9 phages. In contrast to the *sspC*$^+$ complemented mutant, the *sspC* mutant was unable to absorb phages (data not shown); thus, it was apparent that the resistance to lysis was due to changes in the cell envelope that prevented phages from binding to the LES43 (Δ*sspC*) cells.

Analysis of the cellular morphology of the *sspC* mutant (LES43). TEM and SEM analyses of stationary-phase cells of LES43 (Δ*sspC*) and 8325-4 were performed. TEM analysis (Fig. 3A and B) of more than 10 fields of both wild-type and mutant cells revealed that while the wild type had a tightly defined and smooth cell wall, the *sspC*-deficient LES43 (Δ*sspC*) mutant cell wall was highly diffuse and irregular. The *sspC* mutant (LES43) had an altered texture, with little or none of the obvious definition observed in 8325-4. Indeed, TEM analysis of LES43 (Δ*sspC*) revealed significant similarities to the cell wall structure of an *atl* (major autolysin) mutant of *S. aureus* (22). Further morphological differences between LES43 (Δ*sspC*) and the parental strain were revealed by SEM analysis (Fig. 3C and D). 8325-4 cells were found in characteristic grape-like clusters of rounded and smooth cells, yet LES43 (Δ*sspC*) cells appeared mottled and rough. Furthermore, a number of the cells appeared to have lysed, and the majority of the cells were coated with a great deal of extracellular debris or surface-associated material. Significantly, TEM and SEM analyses of the *sspC*$^+$ complemented strain (LES48) revealed that the cells were essentially indistinguishable from cells of the wild-type strain.

TABLE 2. Bacteriophage typing of *S. aureus* strains

Strain	Lytic phage[a]																		
	6	29	42E	47	52	52A	53	71	75	79	80	81	83A	84	88	89	94	95	96
8325-4	+		+	+			+		+				+	+	+	+			
SH1000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SH1000 Δ*sspC*	+	+	+	+	+	+			+	+		+	+	+	+	+	+	+	+

[a] +, lysis observed. Strain 8325-4 Δ*sspC* was not phage typeable.

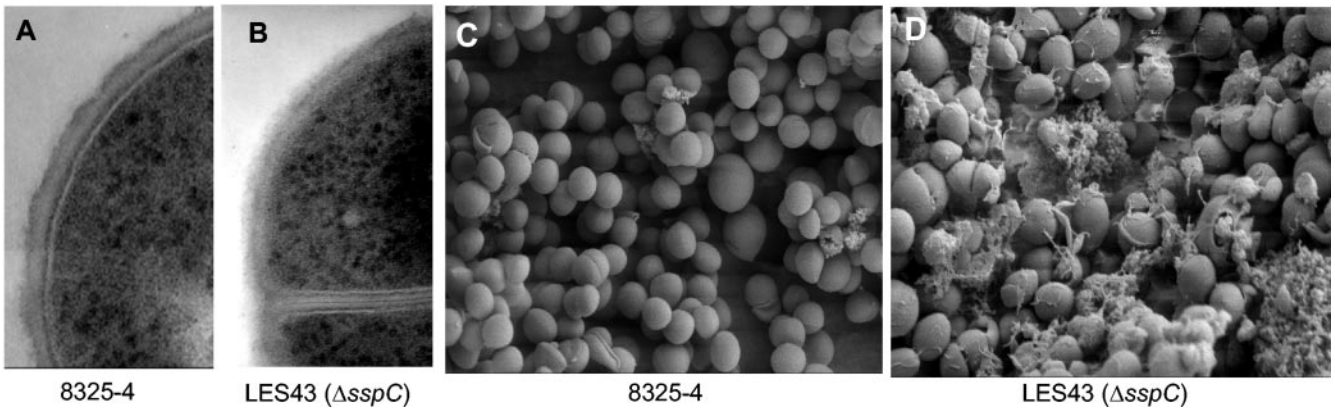

FIG. 3. Analysis of 8325-4 and LES43 (Δ*sspC*) by TEM (A and B) and SEM (C and D). Stationary-phase cultures (15 h) of 8325-4 (A and C) and LES43 (Δ*sspC*) (B and D) were harvested, washed with PBS, and resuspended in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Cells were analyzed with a Philips/FEI Technai 20 TEM (magnification, ×140,000) (A and B) or a LEO 982 field emission SEM (magnification, ×10,000) (C and D).

Insertional inactivation of *sspC* results in production of a biofilm by 8325-4. Based on increased aggregation and the altered cell surface of the mutant, assays were conducted to determine whether LES43 (Δ*sspC*) was capable of biofilm formation. 8325-4 is believed to be incapable of biofilm production; however, despite the lack of σ^B function in LES43 (Δ*sspC*), it was found that the mutant strain produced a biofilm. In contrast, a number of 8325-4 lineage strains also lacking *sspC*, including the LES22 (Δ*sspABC*) and LES17 (Δ*sspBC*) mutants, were incapable of biofilm formation. Complementation in strain LES48 (Δ*sspC sspC*$^+$) showed reversion to the 8325-4 phenotype, whereas LES46 (Δ*sspBC sspB*$^+$) displayed an *sspC* mutant-like phenotype (Fig. 4), indicating that the loss of SspC in the context of a functional *sspB* gene specifically resulted in biofilm formation.

Effect of *sspC* insertional inactivation on autolytic activity of *S. aureus*. As similar cellular morphologies were observed when we compared LES43 (Δ*sspC*) with an *atl* (major autolysin gene) mutant (22), autolysis assays in the presence of Triton X-100 were conducted (Fig. 5). *sspC* inactivation resulted in a significant decrease in autolysis in LES43 (Δ*sspC*) compared to that in 8325-4; a fivefold reduction in lysis was observed within a 1-h period. Indeed, the lysis of the *sspC* strain (LES43) closely followed that of the *atl* mutant (22). Furthermore, LES46 (Δ*sspBC sspB*$^+$) also exhibited decreased lysis, and there was a threefold reduction in the rate compared to the 8325-4 rate. Analysis of the *sspABC* (LES22) and *sspBC* (LES17) mutants and LES48 (Δ*sspC sspC*$^+$) revealed no difference in lysis from the parent strain.

Further analysis of peptidoglycan hydrolase activity was performed by using zymography with either *Micrococcus luteus* or *S. aureus* cells as substrates (Fig. 6A and B). Both the *sspABC* (LES22) and *sspBC* (LES17) mutants exhibited no apparent difference in autolysin activity compared to 8325-4. However, LES43 (Δ*sspC*) displayed a dramatic alteration in the activity profile. Discrete zones of activity at apparent molecular masses of 40, 35, 30, 22, and 20 kDa were replaced by a major 28-kDa band when *S. aureus* was used as a substrate (Fig. 6A). Furthermore, the 51-kDa activity against *M. luteus* was not observed with LES43 (Δ*sspC*) and was replaced by a band at

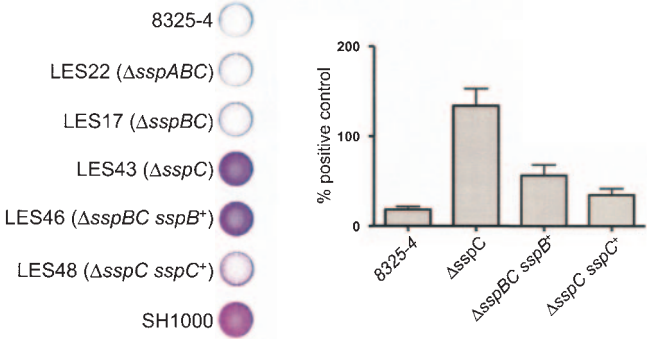

FIG. 4. Analysis of biofilm formation. Strains were analyzed for the ability to form a biofilm. Strain SH1000 was included as a positive control. Biofilm formation was quantified by using the intensity of staining with crystal violet and is expressed as a percentage of the positive control value.

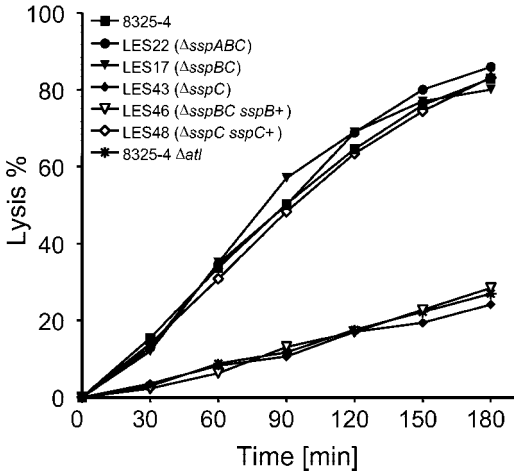

FIG. 5. Triton X-100-induced lysis assay. Triton X-100 (0.05%) was used to induce cellular lysis over a 180-min period. The efficiency of the lysis reaction is expressed as the percentage of cells lysed over time (the initial OD_{600} was defined as 100%).

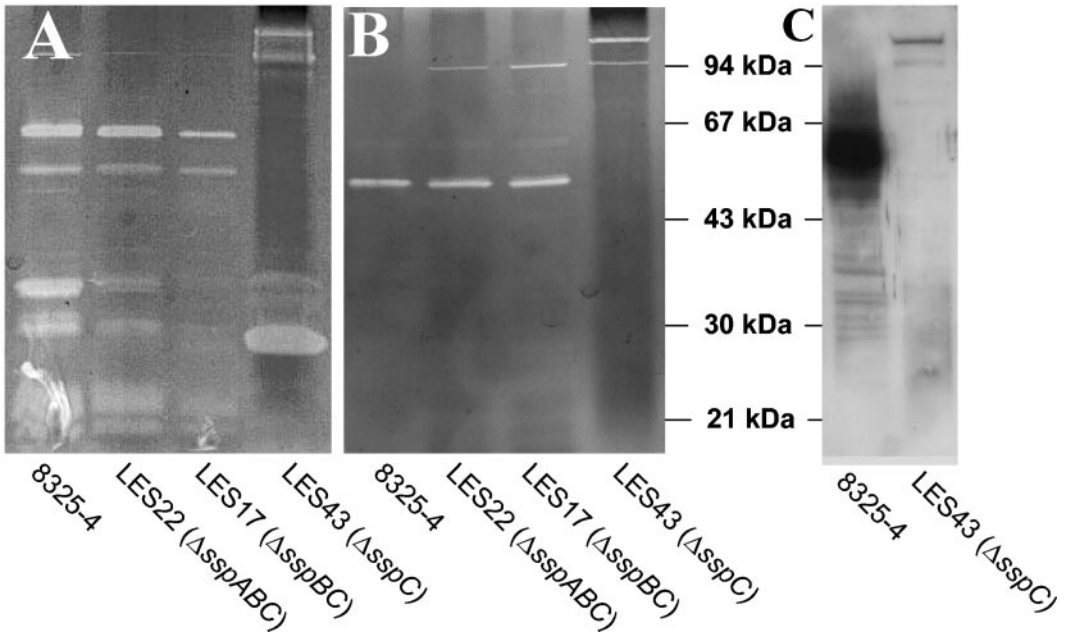

FIG. 6. Analysis of activity and processing of the major autolysin of *S. aureus*. (A and B) Autolysin activity zymography was performed by using *S. aureus* cell walls as a substrate for amidase activity (A) and *M. luteus* cell walls as a substrate for glucosamidase activity (B). (C) Western blot analysis with anti-Atl antibodies. Samples loaded on the gel were standardized to contain the same amount of protein from each strain.

approximately 140 kDa (Fig. 6B). Significantly, both 51- and 62-kDa activities of *S. aureus* were also replaced by a higher-molecular-mass activity (Fig. 6A). Since the major autolysin is produced as a 140-kDa protein, which is proteolytically cleaved into 62-kDa (amidase) and 51-kDa (glucosamidase) activities, the zymography data suggest that inactivation of *sspC* hinders pro-Atl processing. Indeed, Western blot analysis confirmed that LES43 (Δ*sspC*) possessed only the unprocessed pro-Atl protein (Fig. 6C). Interestingly, cleavage of the Atl zymogen into the 62- and 51-kDa activities, as well other lower-molecular-mass activities, was not affected by the *sspABC* (LES22) and *sspBC* (LES17) mutations, eliminating the possibility that SspA or SspB functions as a pro-Atl processing enzyme.

Sensitivity to antibiotics. Since the lack of phage binding, altered autolytic activity, and increased resistance to lysis by lysostaphin may be indicative of some alteration of the peptidoglycan structure, we compared the susceptibilities of LES43 (Δ*sspC*) and the parent strain to a panel of antibiotics that affect cell wall synthesis, including vancomycin, teicoplanin, penicillin, oxacillin, and ampicillin. In this assay no difference was observed between the strains investigated (data not shown).

Disruption of *sspC* causes a total loss of secreted extracellular proteins but only a partial loss of peptidoglycan-associated proteins in *S. aureus* 8325-4. Further analysis was conducted to determine the effects of *sspC* insertional inactivation on the exoprotein profile of LES43 (Δ*sspC*). Interestingly, after up to 12 h of growth not a single protein could be found in the culture medium (as determined by SDS-PAGE). This is in stark contrast to the parental strain and the *sspBC* (LES17) mutant, which produced an array of extracellular proteins (Fig. 7A). Furthermore, compared to other strains, the *sspB*$^+$ complemented *sspBC* mutant (LES46) showed a highly aberrant profile of extracellular proteins. Moreover, an exoproteolytic activity analysis performed by using gelatin zymography revealed no trace of active protease in LES43 (Δ*sspC*) culture media and only a faint band of activity for LES46 (Δ*sspBC sspB*$^+$) (Fig. 7B). In order to confirm that the lack of extracellular proteolytic activity was not a result of cessation of transcription from the protease-encoding loci *aur*, *ssp*, and *scp* (62), reverse transcription-PCR was performed, which confirmed expression from these three loci (data not shown). In accordance with the lack of protease secretion, the LES43 (Δ*sspC*) culture medium was also devoid of hemolysin activity. Significantly, in *trans* restoration of *sspC* in LES48 (Δ*sspC sspC*$^+$) fully restored secretion of extracellular proteins (Fig. 7A), including proteolytic activity (Fig. 7B), and reverted the hemolytic phenotype to that of the wild type (Fig. 7C and D).

The presence of autolysins in LES43 (Δ*sspC*) indicates that a subset of secreted proteins, specifically cell wall-associated proteins, is still exported outside the cells in the *sspC* mutant. To verify this hypothesis, noncovalently associated proteins were extracted from the surface of *S. aureus* by LiCl treatment (Fig. 8A), whereas proteins bound covalently to peptidoglycan were obtained by V8 protease (SspA) treatment (Fig. 8B). Identical biomasses (wet masses of cultures) of 8325-4 and LES43 (Δ*sspC*) yielded similar amounts of solubilized proteinaceous matter (2.5 to 3.0 mg per g [wet weight]) when the preparations were subjected to extraction with LiCl. However, the SDS-PAGE profiles revealed that LES43 (Δ*sspC*) contained a high-molecular-mass major protein that was absent in 8325-4 and only a few proteins with electrophoretic mobilities equivalent to those of the parent proteins (Fig. 8A). On the other hand, preincubation of an *S. aureus* cell suspension with SspA resulted in release of an 80-kDa protein from the surface of LES43 (Δ*sspC*) but not from 8325-3 cells subjected to the

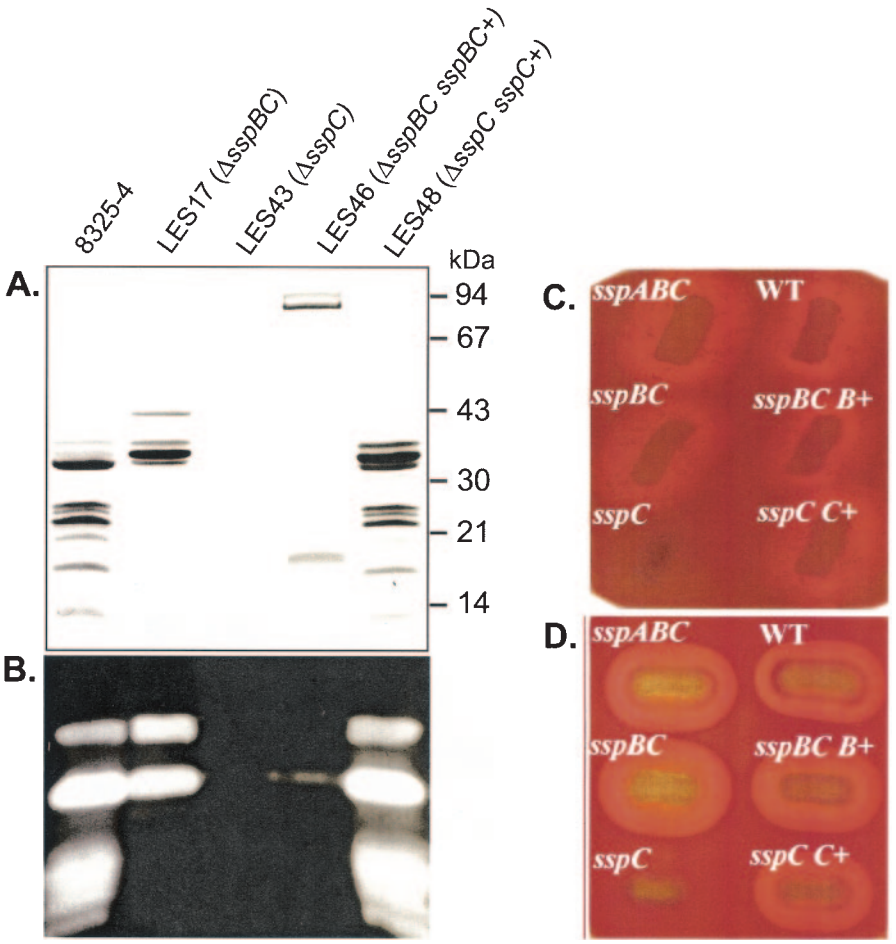

FIG. 7. Comparison of secreted proteins and enzyme activity profiles of *S. aureus* strains. (A and B) Extracellular protein fractions were obtained from the concentrated supernatants of stationary-phase cultures and were resolved by SDS-PAGE (A) or assayed for proteolytic activity by gelatin zymography (B). (C and D) Hemolytic activity was evaluated by growing *S. aureus* strains on rabbit (C) and sheep (D) blood agar for detection of α- and β-hemolysin activities, respectively. WT, wild type.

same treatment (Fig. 8B). The SspA shed polypeptide may have represented peptidoglycan-attached staphylococcal adhesins belonging to the MSCRAMM family of surface proteins. This hypothesis was corroborated by the fact that the *sspC* mutant (LES43) was able to bind to immobilized fibrinogen and fibronectin, although the efficiency was only 20 to 30% of the efficiency of 8325-4 (data not shown). Again, these data correspond well with the temporal difference in production of soluble extracellular and peptidoglycan-associated proteins. Apparently, before activation of transcription from the *ssp* operon in mid-exponential growth MSCRAMM secretion occurs normally; however, it is arrested in later growth phases, which seemingly accounts for the decreased level of adhesive molecules on the mutant cells.

The lack of secreted proteins, including extracellular proteases and hemolysins, in the culture medium of LES43 (Δ*sspC*) implies that these proteins may accumulate in the cytoplasm or at the cell membrane-wall interface. Thus, we attempted to demonstrate the presence of protease and hemolytic activities in crude cell extracts or the fractionated cell envelope or cytoplasmic fractions of LES43 (Δ*sspC*). Remarkably, despite the very high sensitivity of the hemolysin assay, no

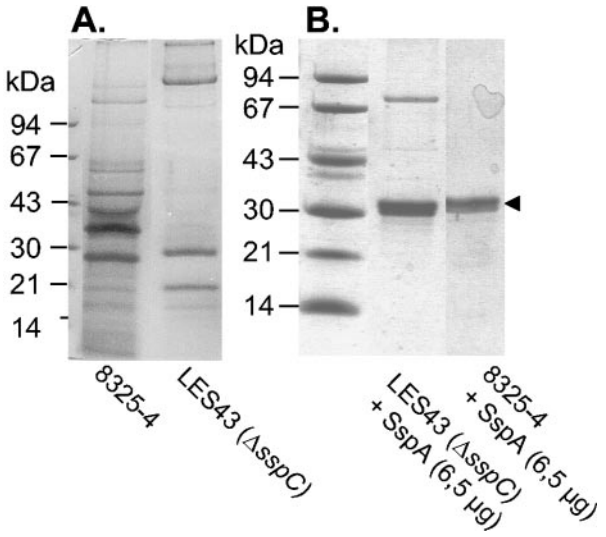

FIG. 8. Analysis of surface-associated proteins. Noncovalent cell envelope-associated proteins were extracted by LiCl treatment (A), while proteins covalently bound to the peptidoglycan were released by limited proteolysis with SspA (V8 protease) (B). The arrowhead indicates the position of the V8 protease used to shed surface proteins.

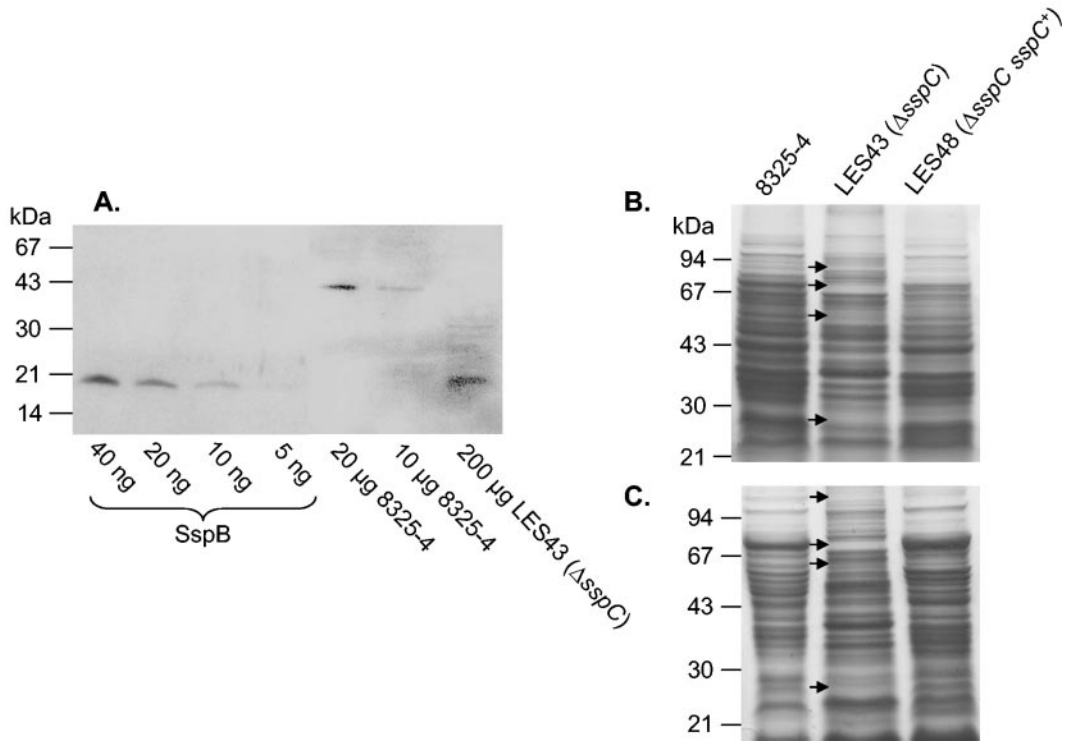

FIG. 9. Western blot analysis of *S. aureus* cell extracts for the presence of staphopain B (A) and SDS-PAGE profiling of intracellular protein during late exponential growth (4 h) (B) and early stationary growth (8 h) (C). Washed bacterial cells were suspended in PBS and disrupted with a French press. Debris was removed by centrifugation, and the proteins in the supernatant were analyzed by SDS-PAGE. The arrowheads in panels B and C indicate missing protein bands in LES43 (Δ*sspC*) compared to 8325-4. To determine a detection limit for staphopain B by using Western blotting (A), serial dilutions of purified protease were loaded and analyzed in parallel.

hemolytic activity was detected in the mutant-derived fractions. Also, it was not possible to detect any proteolytic activity in LES43 (Δ*sspC*) extracts, apparently due to the limited sensitivity of zymography and DCG-04 labeling. Nevertheless, Western blot analysis revealed an immunoreactive band at a molecular mass similar to that of mature SspB (20 kDa) in the cell extract of LES43 (Δ*sspC*). In stark contrast, SspB in the form of the unprocessed 40-kDa zymogen was detected in the 8325-4 cell extract (Fig. 9A). Taking into account that the detection limit of Western blot analysis was estimated to be 5 ng (Fig. 9A), we calculated that the amount of intracellular SspB in LES43 (Δ*sspC*) was exceedingly low (~20 ng per 200 μg of cell proteins). Even so, this amount is apparently sufficient to degrade several proteins inside cells lacking SspC. SDS-PAGE analysis of LES43 (Δ*sspC*) cell extract protein profiles revealed that one major protein and a few minor proteins were missing when this strain was compared to the parental strain and the complemented *sspC* mutant (LES48) (Fig. 9B and C). These data suggest that uncontrolled proteolysis within cells lacking *sspC* leads to the apparently pleiotropic change in the phenotype of LES43 (Δ*sspC*).

Insertional inactivation of the *sspC* gene in a *sigB*-positive background. Because expression of the *sspABC* operon is negatively regulated by the alternative sigma factor (σ^B), we transduced the *sspC* mutation into strains SH1000 and Newman. Surprisingly, insertional inactivation of *sspC* in these backgrounds had no effect on the mutant's growth or susceptibility to lysostaphin (data not shown). Some minor phenotypic differences between the SH1000 *sspC* mutant and the parent strain in susceptibility to lysis by specific phages were observed. The SH1000 parental strain was lysed by all of the phages tested, yet the SH1000 *sspC* mutant displayed susceptibility to 16 of the 19 phages used (Table 2). This indicates that despite the fact that SH1000 lacks any of the other phenotypic characteristics of the 8325-4 *sspC* mutant (LES43), inactivation of *sspC* in SH1000 still has some effect.

DISCUSSION

SspC was recently described as a very specific, tightly binding inhibitor of staphopain B (SspB) (20). Since SspB is a secreted protein and SspC is an intracellular protein, it was hypothesized that SspC functions as a cytoplasmic inhibitor that is required to protect cytosolic proteins from degradation by prematurely folded or activated SspB (58). Here we characterized an *sspC* mutant of *S. aureus* and obtained compelling experimental evidence that SspC does indeed function as a cytoplasmic inhibitor of the SspB protease, at least in the SigB-deficient-like background of the 8325-4 strain.

Insertional inactivation of *sspC* resulted in a growth defect in the 8325-4 background, which was defined by significantly shorter generation times during exponential growth, followed by an arrest in growth during the postexponential phase and a late-stationary-phase decline in cellular density and viability. After an approximately 5-h period of stasis, the cells began to lose viability and underwent apparent lysis. Although tran-

scriptional analysis of the *ssp* operon revealed that maximal expression from this locus occurs approximately 5 h into growth (62), corresponding to the time at which LES43 (Δ*sspC*) stops growing, SspC secretion is observed at the very beginning of exponential growth (data not shown). Therefore, it seems certain that the detrimental impact on growth and other changes in the phenotype of the 8325-4 *sspC* mutant (see below) are a direct result of uncontrolled activity of SspB. This postulate was corroborated by complementational analysis of LES46 (Δ*sspBC sspB*$^+$), an *sspBC* double mutant complemented with only *sspB*, and LES48 (Δ*sspC sspC*$^+$), an *sspC* mutant complemented with *sspC*. The *sspBC* mutant strain (LES17) displayed none of the phenotypic characteristics of LES43 (Δ*sspC*) until *sspB* was introduced under the control of its innate promoter (LES46), while complementation of LES43 (Δ*sspC*) with *sspC* resulted in reversion to the phenotype of 8325-4 in LES48 (Δ*sspC sspC*$^+$).

Furthermore, for the defect in growth, LES43 (Δ*sspC*) also displayed markedly altered cell wall-related properties, as demonstrated by profound differences in the rates of autolysis and by both resistance to lysis with innate staphylococcal phages and extensively diminished susceptibility to lysis by the specific lytic enzyme lysostaphin. All these changes become apparent before the culture enters the mid-exponential phase of growth. In *S. aureus* resistance to lysostaphin occurs as a result of a modification in the pentaglycine cross-linking of the cell wall; a decrease in the glycine content and an increase in the serine content are observed, as is the case with lysostaphin-resistant *Staphylococcus* spp. (56, 63). Alternatively, disappearance of a receptor for lysostaphin on the cell wall may lead to considerably decreased sensitivity to lysis (45). The second option is a more plausible explanation for the changes in lysostaphin sensitivity due to the *sspC* mutation and correlates with the loss of phage receptors, the altered patterns of cell surface-associated proteins in LES43 (Δ*sspC*), and the unchanged susceptibility to antibiotics that affect cell wall synthesis.

Autolysis was also found to be severely affected by the *sspC* insertional inactivation, and LES43 (Δ*sspC*) was much less susceptible to autolysis than the parent strain 8325-4. This correlates well with a profoundly changed profile of cell wall-associated autolysin activities. Alterations to pro-Atl processing are observed, resulting in aberrantly processed forms and accumulation of the 140-kDa zymogen. Also, the amount of processed Atl associated with the LES43 (Δ*sspC*) cell envelope was massively reduced compared to the amount in the wild-type strain. Analysis of transcription from the *atl* locus revealed that although there is a basal constitutive level of expression, there is an increase as the cells enter the exponential phase (22, 48). Thus, the loss of Atl function could be explained by modulation of *atl* transcription as the mutant cells stop growing, by a protein secretion defect (see below), or by hindered Atl folding in the presence of altered peptidoglycan and proteolytic degradation at the cell membrane-wall interfaces. Although it is unclear which mechanism is responsible for the decrease in Atl levels, the decreased activity of this autolysin explains the significant resistance of LES43 (Δ*sspC*) to autolysis.

The most fascinating phenotypic idiosyncrasy of LES43 (Δ*sspC*) is the apparent lack of detectable extracellular proteins in culture supernatants. *S. aureus* secretes a plethora of extracellular virulence determinants during the postexponential and stationary phases of growth (70). Among these proteins are numerous proteases, hemolysins, and toxins, many of which are regulated in a temporal manner by *agr* (2, 30, 53). The lack of such proteins in LES43 (Δ*sspC*) is unusual, indicating that there is a general breakdown in protein secretion in this mutant. Such an observation is in conflict with the presence of peptidoglycan-associated proteins, since both sets of proteins use the Sec translocation system for passage through the cell membrane (68). This contradiction can be explained in three mutually complementing ways. First, the cell wall proteins are predominantly secreted during early stages of *S. aureus* growth, before expression from the *sspABC* operon is induced. In this scenario intracellularly active SspB may damage an essential component of the Sec system and/or cytosolic factors involved in targeting proteins to the cell secretory machinery. Second, a recent study by Rosch and Caparon (57) with the gram-positive pathogen *Streptococcus pyogenes* revealed that the bulk of extracellular protein secretion in this organism does not occur indiscriminately throughout the cell wall but occurs at specific microdomains adapted to contain Sec translocons. A similar system was described in *Bacillus subtilis* (7) and probably functions in other gram-positive organisms. Such a system in *S. aureus* could represent a target for the uncontrolled SspB activity of the *sspC* mutant. Third, folding and/or maturation of polypeptide chains newly translocated across the cytoplasmic membrane into the interface with the cell wall peptidoglycan is hindered in the mutant, and misfolded proteins are degraded by quality control proteases (e.g., HtrA) located in this compartment (49, 68). It is also plausible that all three pathways contribute to the absence of protein secretion in the *sspC* insertional mutant.

The attachment of Δ*sspC* cells to solid surfaces and their subsequent aggregation into clusters also seem to be enhanced. Although biofilm production in *S. aureus* 8325-4 has previously been demonstrated (5, 13, 37, 67), the extent of this phenomenon is striking in the SspC-null strain. Biofilm formation in this strain could be a pleiotropic effect resulting from an increase in cellular clumping due to alterations in the outer structures or a lack of functional autolysins, hemolysins, or proteases.

Interestingly, LES44 (SH1000 Δ*sspC*) exhibited none of the phenotypic alterations of LES43 (8325-4 Δ*sspC*). SH1000 is identical to 8325-4 apart from the restoration of an 11-bp deletion in *rsbU* (29), which is required for full activity of the alternative sigma factor, σ^B (23, 36, 50). The lack of a LES44 growth defect is most likely explained by the observation that SH1000 expresses very low levels of extracellular proteases, including those of the *ssp* operon (29, 62). Therefore, although LES43 (8325-4 Δ*sspC*) and LES44 (SH1000 Δ*sspC*) were derived from the same lineage, it can be assumed that the nearly total lack of *ssp* synthesis in LES44 protects it from the damaging SspB-mediated phenotype of LES43. Moreover, in the context of the fact that SH1000 is a direct derivative of 8325-4 (29), it is interesting that the restoration of σ^B function reestablished susceptibility to lysis by phages in this strain (Table 2).

In summary, the range of phenotypic events brought into play by a mutation in the cytoplasmic inhibitor of staphopain B (SspB) is extensive. The exact pathway by which the changes

take place requires further study; however, it seems that all the changes described here are due to the deregulation of SspB control. Apparently, a minute amount of SspB can escape into the cytoplasm from its pathway for secretion to the extracellular environment. This tiny amount of active protease is evidently enough to modulate the activity of key intracellular proteins, including possibly those involved in biofilm formation and autolysin processing and, more generally, those required for efficient extracellular protein secretion. Identifying these targets is a matter of ongoing research in our laboratories. Here we only intend to stress as-yet-unknown functions of SspB and put forward a question. Why does *S. aureus* synthesize an enzyme which can potentially produce such widespread changes in the cell? In this context it is worth reiterating that it is no coincidence that SspB is tightly regulated both at the transcriptional level and at the posttranslational level and that additional control is guaranteed through the unique coexpression of a protease and an inhibitor from the same locus. Significantly, all these regulation mechanisms have evolved for an enzyme which is not even an essential housekeeping protein and has unproven importance as a virulence factor in mouse models of staphylococcal infections (6, 55, 62). Nevertheless, SspB is conserved in all of the *S. aureus* clinical isolates investigated to date (24). Taking all these facts into account, it seems evident that there must be selective pressure to maintain such a potentially harmful protein in vivo. For this reason we cannot resist speculating that SspB is an important factor for the *S. aureus* commensal-pathogen dichotomy with the human host, and as such the enzyme itself and its endogenous inhibitor could be attractive targets for the development of anti-staphylococcal therapies.

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