The *Staphylococcus aureus* *scdA* gene: a novel locus that affects cell division and morphogenesis

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A new *Staphylococcus aureus* gene termed *scdA* was found upstream of the autolysis regulatory genes, *lytS* and *lytR*, and was shown to potentially encode a hydrophilic 25 kDa protein. Analysis of *scdA* transcription revealed that it is transcribed as a monocistronic message and is *lytSR*-independent. A role in cell wall metabolism was indicated by examination of the *scdA* mutant *S. aureus* KB323, which had a grossly aberrant cellular morphology and formed large cell clusters when grown in liquid culture medium. Furthermore, KB323 exhibited a reduced rate of autolysis and had increased peptidoglycan cross-linking compared to the parental strain, NCTC 8325-4. These data suggest that *scdA* plays an important role in staphylococcal cell division.

Keywords: *Staphylococcus aureus*, *scdA*, peptidoglycan cross-linking, septum formation

INTRODUCTION

Recently, a new two-component regulatory system, comprising *LytS* and *LytR*, has been identified in *Staphylococcus aureus* and shown to be involved in the regulation of autolysis (Brunskill & Bayles, 1996a). This was first demonstrated by analysis of the *S. aureus* mutant strain KB300, which contained a disruption of the *lytS* gene (Brunskill & Bayles, 1996a). Autolysis assays revealed that KB300 lysed at a much faster rate than the parental strain, NCTC 8325-4 (Brunskill & Bayles, 1996a). These data suggested that *LytS* and *LytR* regulate the expression of genes encoding murein hydrolases or genes involved in murein hydrolase activity. This prediction was supported by a zymographic analysis which demonstrated that the *lytS* mutation caused a dramatic reduction in expression of cytoplasmic and extracellular murein hydrolases, with a corresponding increase in murein hydrolase activity in the cell wall fraction (Brunskill & Bayles, 1996a). It was hypothesized that the increased rate of autolysis in the

lytS mutant was a result of the overexpression of the cell wall-associated murein hydrolases.

The sequence analysis of the *lytSR* region revealed the presence of two additional open reading frames, designated *lrgA* and *lrgB*, that are located immediately downstream of and are oriented in the same direction as *lytS* and *lytR* (Brunskill & Bayles, 1996b). A Northern blot analysis of RNA isolated from NCTC 8325-4 and KB300 revealed that *lrgA* and *lrgB* are cotranscribed and that their transcription is dependent on *lytS* and *lytR* (Brunskill & Bayles, 1996b). Studies of *lrgA* and *lrgB* function suggested that these genes encode a holin-like protein and a gene involved in murein hydrolase activity, respectively (Brunskill & Bayles, 1996b). Upstream of *lytS* and *lytR* was another open reading frame of unknown function. To determine if this gene (designated *scdA*) was also involved in cell wall metabolism, the *scdA* open reading frame was insertionally inactivated and the resulting mutant strain was analysed. The results of this analysis, reported here, demonstrate that *scdA* affects peptidoglycan cross-linking and is required for normal cell growth and development.

METHODS

Strains, plasmids and growth conditions. The *S. aureus* strains and plasmids used in this study are listed in Table 1. The *Escherichia coli* strain used was DH5 α . L-broth and Trypticase-soy broth (TSB) were used for cultivation of *E. coli* and *S. aureus*, respectively. Ampicillin (50 μ g ml⁻¹) and ery-

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The GenBank accession number for the nucleotide sequence determined in this work is U57060.

Table 1. Strains and plasmids

Strain or plasmid	Description	Reference
<i>S. aureus</i>		
NCTC 8325-4	Wild-type strain cured of prophage	Novick (1990)
RN4220	NCTC 8325-4 r^-	Novick <i>et al.</i> (1990)
KB320	RN4220(pORF-2)	This study
KB321	NCTC 8325-4(pORF-2)	This study
KB323	<i>scdA</i> ::pORF-2 integrant	This study
KB300	<i>lytS</i> ::pKIN1 integrant	Brunskill & Bayles (1996a)
Plasmids		
pER924	Ap ^r Erm ^r ; shuttle/integration vector	Brunskill & Bayles (1996a)
pORF-2	pER924 containing a 211 bp internal <i>scdA</i> fragment	This study

thromycin (2 µg ml⁻¹) were used as indicated for selection in *E. coli* and *S. aureus*, respectively.

DNA manipulations. DNA restriction digestions and ligations were performed as described by Sambrook *et al.* (1989), using enzymes obtained from Promega. The *scdA*-specific probe (nt 613–823) used in the Northern blot analysis (see below) was PCR-amplified from *S. aureus* genomic DNA using sequence-specific oligonucleotides and *Taq* polymerase (Promega). PCR products were gel-purified using β -agarase as described by the manufacturer (New England Biolabs) and radiolabelled with [γ -³²P]ATP (Sambrook *et al.*, 1989). Plasmid transformations of *E. coli* were carried out using the SEM method (Inoue *et al.*, 1990). *E. coli* plasmid DNA was isolated using the alkaline lysis method followed by CsCl/ethidium bromide density-gradient centrifugation (Sambrook *et al.*, 1989). *S. aureus* genomic DNA was isolated as described by Dyer & Iandolo (1983) and plasmid DNA transformations of *S. aureus* were carried out using a Bio-Rad Gene Pulser (Kraemer & Iandolo, 1990). Transduction in *S. aureus* strains was performed using ϕ 11 as described by Shafer & Iandolo (1979).

Campbell integration. The *scdA* gene disruption was created using a Campbell-like integration mechanism by cloning the 211 nt *scdA* PCR product (see above) into the shuttle vector pER924 (Brunskill & Bayles, 1996a). The resulting plasmid, pORF-2, was electroporated into *S. aureus* RN4220 at the permissive temperature (30 °C) to generate strain KB320. The plasmid was then transferred into NCTC 8325-4 by transduction using the bacteriophage ϕ 11, generating *S. aureus* strain KB321. An overnight culture of KB321, grown at the permissive temperature (30 °C), was streaked on Trypticase-soy agar containing erythromycin and incubated overnight at the nonpermissive temperature (43 °C). Since plasmid replication is inhibited at 43 °C, only those cells that had undergone a Campbell-like integration event into the *scdA* gene were selected. After overnight growth, single colonies were restreaked and grown again at 43 °C. Chromosomal DNA from single colonies was isolated and a Southern analysis was used to confirm that the clones contained a *scdA* gene disruption (data not shown). One such clone was identified and designated KB323.

DNA sequencing and computer analysis. The nucleotide sequence of *scdA* was determined using the dideoxy chain-termination method (Sanger *et al.*, 1977) and the Sequenase DNA sequencing kit (United States Biochemical). Sequence-

specific oligonucleotide primers were synthesized using an Expedite 8909 nucleotide synthesizer and were utilized to sequence both strands. A computer analysis of the sequences generated was conducted using the GCG Wisconsin Computer Package Unix Version 8.0 (Devereux *et al.*, 1984).

RNA analysis. *S. aureus* RNA (20 µg) was isolated from exponentially growing and stationary-phase cells as described by Hart *et al.* (1993) and was analysed by Northern blotting (Sambrook *et al.*, 1989) using a *scdA*-specific probe. A primer extension analysis of the *scdA* promoter region was conducted using the procedure described previously (Brunskill & Bayles, 1996a) and an oligonucleotide primer that was complementary to the 5' end of the *scdA* gene (nt 407–437).

Transmission electron microscopy. Mid-exponential-phase NCTC 8325-4 and KB323 cells were resuspended in 2.5% glutaraldehyde in Dulbecco's phosphate-buffered saline (PBS) overnight. The cells were collected and resuspended in five drops of PBS. The cells were then vortexed and incubated for 5 min at room temperature. Next, the cells were pelleted, resuspended in 1.5 ml PBS containing 1% OsO₄, and incubated for 30 min at room temperature. This was followed by centrifugation for 5 min at 13 500 g. The cell pellet was washed with 1.5 ml PBS, dehydrated in a series of ethanol washes and then fixed in propylene oxide/Araldite 502 (1:1, v/v) for 30 min, followed by a treatment with propylene oxide/Araldite 502 (1:3, v/v) for 60 min. The pellets were then embedded in polyethylene embedding capsules and spun in a clinical centrifuge on an embedding trench for 3 min. The pellets were incubated at 60 °C for 18 h and cut on a Sorvall MT2B ultramicrotome at a thickness of 60 nm. The thin sections were stained with 1% uranyl acetate/lead acetate and viewed with a Zeiss 10-CA transmission electron microscope.

Autolysis assays. Autolysis assays were performed as described by Mani *et al.* (1993). Cell samples (50 ml) were collected from exponentially growing TSB cultures (OD₅₈₀ 0.7) containing 1 M NaCl and the cells were pelleted by centrifugation. The cells were washed twice with 50 ml ice-cold water and resuspended in 50 ml 0.05 M Tris/HCl (pH 7.2) containing 0.05% Triton X-100 (Sigma). The cells were then incubated at 30 °C with shaking, and the OD₅₈₀ was measured at 30 min intervals.

Peptidoglycan analysis. *S. aureus* peptidoglycan was prepared, digested into muropeptides and analysed using reverse-phase HPLC as described by de Jonge *et al.* (1992).

Fig. 1. Northern blot analysis. Total cellular RNA from strains NCTC 8325-4 (lane 1) and KB300 (lane 2) was separated in a formaldehyde-agarose gel, transferred to nitrocellulose paper and hybridized with a *scdA*-specific probe. Transcript size was determined by comparison with RNA size markers.

Fig. 2. Transcription start site of *scdA*. Total cellular RNA (25 µg) was isolated (Hart et al., 1993) from exponentially growing (lane 1) and stationary-phase (lane 2) cells of *S. aureus* NCTC 8325-4 and subjected to primer extension analysis (Brunskill & Bayles, 1996a). The sizes of the extended products were determined by comparison with a DNA sequencing ladder of the *scdA* promoter region that was generated using the same primer.

RESULTS AND DISCUSSION

Sequence analysis of the *scdA* gene

Analysis of the DNA sequence upstream of the *S. aureus* autolysis regulatory genes, *lytS* and *lytR* (Brunskill & Bayles, 1996a), revealed the presence of a previously uncharacterized open reading frame which we termed *scdA*. The *scdA* coding sequence is preceded by a consensus ribosome-binding site and potentially encodes a hydrophilic protein (ScdA) containing 224 amino acids and having a deduced molecular mass of 25 369 Da. Immediately downstream from *scdA* is a large 12 bp inverted repeat sequence that is a potential rho-independent terminator. Upstream of *scdA* is another open reading frame that is also followed by a potential rho-independent terminator. The ScdA protein shares

Fig. 3. Autolysis assay of *S. aureus* NCTC 8325-4 (□) and KB300 (○). The rate of autolysis was measured as the decline in OD₅₈₀ versus time. The data presented illustrate one of three independent experiments which exhibited the same difference between the autolysis rates of the two strains.

the greatest amino acid sequence similarity (26.9% and 27.2% identical residues, respectively) with the hypothetical *E. coli* and *Haemophilus influenzae* YtfE proteins (accession nos P39313 and P45312, respectively), whose functions have not been determined. The deduced amino acid sequence of the upstream open reading frame did not match any sequences in the GenBank database.

The transcription of *scdA*

To determine if *scdA* transcription is affected by *lytS* and *lytR*, a Northern blot analysis of RNA isolated from *S. aureus* NCTC 8325-4 and its *lytS* mutant derivative, KB300, was performed using a *scdA*-specific probe. This analysis revealed the presence of an approximately 0.7 kb *scdA*-specific transcript that was present in both NCTC 8325-4 and KB300 (Fig. 1, lanes 1 and 2, respectively), indicating that *scdA* transcription is independent of *lytS* and *lytR*. Furthermore, a primer extension analysis localized the *scdA* transcription start site to a guanine residue 16 nucleotides 5' relative to the putative *scdA* translation start codon (Fig. 2). Upstream of the transcription start site is a putative σ^A promoter region (TTCATT-N₁₈-TATACA), which matches 7 out of 12 bases of the prototypical σ^A promoter of *Bacillus subtilis* (Moran, 1993). These data, along with the location of the potential rho-independent terminator sequence, are consistent with the size of the *scdA* transcript and suggest that *scdA* is transcribed as a monocistronic message. This analysis also revealed that a much stronger signal was produced using RNA from stationary-phase cells (Fig. 2, lane 2) compared to mid-exponential-phase cells (Fig. 2, lane 1). As equal amounts of RNA were used in the primer extension experiments, these data indicate that significantly more *scdA* message is present in stationary-phase cells than in mid-exponential-phase cells and that this gene is subject to temporal regulation.

Fig. 4. Transmission electron micrographs of cells of *S. aureus* NCTC 8325-4 (a) and KB323 (b-d). Bar, 0.2 μ m (magnification is the same in each panel).

Analysis of a *scdA* mutation

To investigate the function of *scdA*, an internal fragment from this gene was used to construct an insertional *scdA* disruption in NCTC 8325-4, creating strain KB323 (see Methods). One striking feature of KB323 was that it formed large aggregated clumps of cells when grown in liquid culture medium, a feature that is characteristic of *S. aureus* mutants deficient in murein hydrolase production. For example, Oshida *et al.* (1995) have recently characterized the *atl* gene, which encodes a bifunctional, extracellular protein that is proteolytically processed to generate an endo- β -N-acetylglucosaminidase and an N-

acetylmuramyl-L-alanine amidase. A mutation within *atl* results in cells that form large clusters (Oshida *et al.*, 1995; Oshida & Tomasz, 1992) and exhibit a reduced rate of autolysis (Foster, 1992). It was hypothesized that these two murein hydrolases are required for the separation of cells by cleaving the peptidoglycan connecting the two adjoining daughter cells (Sugai *et al.*, 1989, 1995). An autolysis assay of KB323 demonstrated that this strain also undergoes autolysis at a lower rate than the parental strain (Fig. 3), indicating that *scdA* could affect murein hydrolase activity or that the mutation has a polar effect on the downstream autolysis regulatory genes (Brunskill & Bayles, 1996a). However,

Fig. 5. Muropeptide profiles of *S. aureus* strains. Peptidoglycan was obtained from *S. aureus* strains NCTC 8325-4 (a) and KB323 (b) and digested with muramidase; the resulting muropeptides were separated by reverse-phase HPLC.

this reduced autolysis rate did not appear to be the result of altered murein hydrolase activity since no difference in the zymographic profiles of NCTC 8325-4 and KB323 could be detected (data not shown). Therefore, it is possible that the decreased autolysis rate exhibited by KB323 arose as a result of structural changes within the peptidoglycan.

To examine KB323 cells more closely, transmission electron microscopy was utilized; it revealed that the *scdA* mutation brought about dramatic defects in cellular morphology (Fig. 4). These defects appeared to be caused by the aberrant placement of septa resulting in misplaced, multiple and/or aborted cross-wall formation. In addition, the cell wall of KB323 was rough and diffuse compared to the parental strain. Interestingly, the aberrant cellular morphology of KB323 was strikingly similar to *S. aureus* cells carrying *femA* or *femB* mutations (Henze *et al.*, 1993). These mutants also exhibited reduced autolysis, which is probably due to the reduced glycine content of their pentapeptide cross-bridges (de Jonge *et al.*, 1993; Henze *et al.*, 1993; Maidhof *et al.*, 1991).

Cell wall analysis

To determine the composition of NCTC 8325-4 and KB323 peptidoglycan, cell walls were prepared and hydrolysed, and the resulting muropeptides were analysed using reverse-phase HPLC as described by de

Jonge *et al.* (1992). Although the composition of the cross-bridges appeared to be unaffected by the *scdA* mutation, the total amount of peptidoglycan cross-linking in KB323 was higher than in NCTC 8325-4 (Fig. 5). In KB323 cells, 44% of the muropeptides (expressed as a percentage of total UV absorption) were found in highly cross-linked form (> octamers), compared to 37% in strain NCTC 8325-4. Whether this increase in cross-linking is responsible for the decreased autolysis rate of this mutant is not known. Since *femA* and *femB* mutant cells have similar morphological abnormalities to KB323, but their peptidoglycan exhibited decreased cross-linkage and a reduced glycine content (de Jonge *et al.*, 1993; Henze *et al.*, 1993; Maidhof *et al.*, 1991), these morphological abnormalities may be the result of a factor(s) unrelated to the physical state of the cell wall.

Although the precise function of *scdA* is unknown, the data generated by this study demonstrate that it can be added to the growing list of cell-wall-related genes in this gene cluster. In spite of the fact that the transcription of *scdA* was unaffected by *LytS* and *LytR*, *scdA* was clearly required for normal peptidoglycan metabolism and cell development, indicating that this region of the chromosome is involved in different aspects of cell wall physiology. Continued analysis of these, and additional genes in this region are ongoing in our laboratory and will undoubtedly lead to further insight into the regulation of the components necessary for peptidoglycan assembly and processing in *S. aureus*.

ACKNOWLEDGEMENTS

We thank Ron Yasbin and Hal Schreier for critical reading and insightful comments on this manuscript. This work was supported by USDA grant no. 9304112.

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Received 26 February 1997; revised 7 May 1997; accepted 6 June 1997.