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

Eric W. Brunskill,† Boudewijn L. M. de Jonge‡ and Kenneth W. Bayles§

Author for correspondence: Kenneth W. Bayles. Tel: +1 208 885 7966. Fax: +1 208 885 6518.
E-mail: kbayles@uidaho.edu

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 DH5α. 1-broth and Trypticase-soy broth (TSB) were used for cultivation of *E. coli* and *S. aureus*, respectively. Ampicillin (50 μg ml⁻¹) and ery-
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 [γ³²P]ATP (Sambrook *et al.*, 1989). Plasmid transformations of *E. coli* were carried out using the 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 (Iandolo, 1990). Transduction in *S. aureus* strains was performed using φ11 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 φ11, 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 dyeoxy 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 (OD₆₆₀ 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 OD₆₆₀ 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).
The scdA locus of Staphylococcus aureus

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 25369 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 the greatest amino acid sequence similarity (26.9% and 27.2% identical residues, respectively) with the hypothetical E. coli and Haemophilus influenzae YfE 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 σA promoter region (TTTATT-N18-TATACA), which matches 7 out of 12 bases of the prototypical σA 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.
E. W. BRUNSKILL, B. L. M. DE JONGE and K. W. BAYLES

Fig. 4. Transmission electron micrographs of cells of S. aureus NCTC 8325-4 (a) and KB323 (b-d). Bar, 0.2 µm (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,
Maidhof exhibited reduced autolysis, which is probably due to the reduced glycine content of the pentapeptide mutations (Henze et al., 1993). Interestingly, the aberrant cellular morphology of strain KB323 was rough and diffuse compared to the parental strain. Interestingly, these mutants also exhibited reduced autolysis, which is probably due to the reduced glycine content of the 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.

REFERENCES


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