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Molecular Characterization and Functional Analysis of the Major Autolysin of *Staphylococcus aureus* 8325/4

SIMON J. FOSTER*

Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, United Kingdom

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The gene encoding the major autolysin of *Staphylococcus aureus* 8325/4 has been cloned, sequenced, and insertionally inactivated. The three-domain, 137,384-Da protein has a C-terminal glucosaminidase active site and is involved in cell separation, generalized cell lysis, and release of wall material at the cell surface. Expression occurs throughout growth and is stimulated by low temperatures and in the presence of 1 M NaCl.

Bacterial autolysins are potentially lethal enzymes capable of hydrolyzing the peptidoglycan component of the cell wall (6). Zymogram analysis using renaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) has visualized at least 20 distinct bands of lytic enzyme activity in *Staphylococcus aureus* (16). Various enzymes with amidase or glucosaminidase activity have been extracted from cells or isolated from the culture supernatant (1, 7, 17, 19, 21), and two peptidoglycan hydrolase structural genes have been cloned (2, 8). This paper describes the characterization of the structural gene encoding the major autolysin of *S. aureus* 8325/4 and analysis of the role of the enzyme.

Identification of an autolysin structural gene. A λ ZAP Express library of a partial *Sau*3A digest (2 to 10 kb) of *S. aureus* 8325/4 genomic DNA was created by using the protocol of the manufacturer (Stratagene, San Diego, Calif.), and the clones were screened for the ability to lyse Procion red-stained *Micrococcus luteus* cell walls (3). A number of isolates containing overlapping inserts were excised from λ ZAP Express to give stable, lytic enzyme-expressing phagemid clones in *Escherichia coli* XLOLR (Stratagene). Renaturing SDS-PAGE of an SDS extract of *E. coli* XLOLR(pSA1) revealed the presence of multiple lytic enzyme bands (Fig. 1, lane 2; Fig. 2), whereas *E. coli* XLOLR(pBKCMV) had no activity (phagemid without insert; results not shown). *E. coli* XLOLR(pSA2) showed the largest lytic enzyme bands (Fig. 1, lane 1; Fig. 2).

Sequencing of the autolysin structural gene. The insert in pSA2 was subcloned by nested deletion (Erase-A-Base; Promega) and sequenced. Appropriate rescreening of the library by hybridization, sequencing, and analysis using established methods (15) revealed the physical and genetic map of the region (Fig. 2). Four open reading frames (ORFs) were identified. ORF4 encodes a 1,256-amino-acid, 137,384-Da autolysin. There are three imperfect direct repeats in the central region of the autolysin, each between 140 and 164 residues long, showing 31% identity between the three repeats. During the preparation of this report, the cloning and sequencing of the autolysin gene was published by Oshida et al. (14); the homologies and sequence features are discussed in that report, and the gene is designated *atl.*

Analysis of the recombinant autolysin. Subclone activity mapping showed the active site of the enzyme to within the final C-terminal 187 residues (Fig. 2). The smallest nested deletion subclone giving high levels of expression was pSAF14. The autolysin profile of this clone is shown in Fig. 1 (lane 3), and this clone was chosen for recombinant enzyme studies. Extracts were prepared from 51 cultures (A_{600} of 1.5) grown at 37°C in Luria-Bertani medium. Cells were harvested by centrifugation (10,000 × *g*, 4°C, 5 min) and broken in the French press in 40 ml of 50 mM Tris-HCl (pH 7.6)–200 mM NaCl–0.5 mM phenylmethylsulfonyl fluoride at 4°C. The supernatant, after removal of debris (100,000 × *g*, 4°C, 2 h), was dialyzed against a 100× volume of 20 mM potassium phosphate buffer (pH 7.4) overnight at 4°C. The dialysate was then centrifuged (100,000 × *g*, 4°C, 2 h), and the supernatant was used as the source of enzyme. The enzyme has a pH optimum of 7.4 in 20 mM potassium phosphate buffer. Activity was enhanced 2.5-fold by the addition of 2 mM $MgCl_2$ or 2 mM $CaCl_2$, whereas 1 mM $HgCl_2$ or $ZnCl_2$ completely inhibited the enzyme. Even low salt concentrations had a detrimental effect on activity; 200 mM NaCl, KCl, or LiCl inhibited the enzyme by >95%.

One milliliter of purified cell walls of *M. luteus* (5 mg [dry weight]/ml) in 10 mM potassium phosphate buffer (pH 7.4)–2 mM $MgCl_2$ was digested for 22 h with 20 U of recombinant enzyme at 37°C (3). No increase in amino termini appeared, whereas 50 nmol of new reducing termini per mg compared with the control was found (3). The method of Sugai et al. (17) was used to show that the enzyme has glucosaminidase activity.

Functional analysis of the *S. aureus* glucosaminidase. To determine the function of the cloned enzyme, a mutant insertionally inactivated in the structural gene was created. A 1.7-kb *Hin*dIII fragment from pSA104 was end filled and cloned into *Sma*I-digested, phosphatase-treated suicide vector pAZ106 (9) to give plasmid pSA407, which is stable in *E. coli* (Fig. 2). Electroporation (13) of this plasmid into *S. aureus* RN4220 (restriction minus, modification plus) allowed the selection of an erythromycin-resistant transformant (strain SH105). As this vector does not contain a replicon functional in *S. aureus*, all erythromycin-resistant clones occurred as a result of stable integration of the vector into the chromosome by homologous recombination at the autolysin locus by a single crossover event. The 1.7-kb *Hin*dIII fragment is internal to the autolysin coding sequence (Fig. 2), and so insertional inactivation of the gene occurs. Plasmid pAZ106 also contains a promoterless copy of *lacZ*, and the orientation of the insert in pSA407 was such that upon integration, a transcriptional fusion between the autolysin gene and β-galactosidase was produced for expression analysis. Phage transduction (13), using φ85, transferred the mutated gene to the *S. aureus* 8325/4 background to

* Mailing address: Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, United Kingdom. Phone: 44 114 282 4411. Fax: 44 114 272 8697. Electronic mail address: s.foster@sheffield.ac.uk.

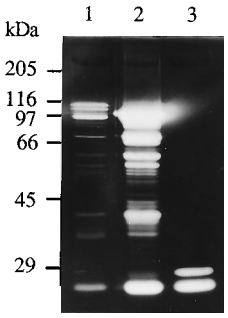

FIG. 1. Lytic activity of recombinant clones. Samples were prepared, separated by SDS-PAGE, and renatured as described previously (3, 4). The 10% (wt/vol) acrylamide gel contained 0.1% (wt/vol) purified *M. luteus* 4698 cell walls as the substrate (4). Each lane contains the extract from the equivalent of 150 μl of original culture. Lanes: 1, *E. coli* XLOLR(pSA2); 2, *E. coli* XLOLR(pSA1); 3, *E. coli* XLOLR(pSAF14). Molecular masses of standards are indicated.

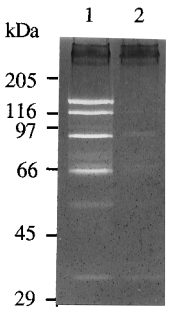

FIG. 3. Autolysin profile of *S. aureus* strains. Cultures were grown in tryptic soy broth to an A_{600} of 2.0 at 37°C prior to harvesting, SDS extraction, separation by SDS-PAGE, and renaturation (3, 4). The 9% (wt/vol) acrylamide gel contained 0.05% (wt/vol) purified *S. aureus* 8325/4 cell walls (4) as the substrate. Each lane contains the extract from the equivalent of 1 ml of original culture. Lanes: 1, 8325/4; 2, SH108. Molecular masses of standards are indicated.

create strain SH108. The correct integrational event was confirmed by Southern blotting. Pulsed-field gel electrophoresis, Southern blotting, and probing with pSA407 revealed the insertion to have occurred in *Sma*I fragment B of the *S. aureus* 8325/4 chromosome.

The autolysin profiles of the parent and insertionally inactivated mutant are shown in Fig. 3. The parent has six major lytic bands ranging from approximately 130 to 36 kDa (Fig. 3, lane 1). In contrast, SH108 has only one major enzyme of 36 kDa and a few minor bands (Fig. 3, lane 2). This finding implies that most of the autolysins of *S. aureus* 8325/4 are the product of a single gene, and proform processing results in the multiple bands visualized by renaturing gel electrophoresis.

The role of the autolysin was determined by a comparison of the phenotype of SH108 with that of its parent. The mutant grew as well as the parent; however, light microscopy revealed SH108 to form small clumps of 10 to 20 cells rather than the doublets formed by 8325/4. Ultrastructural analysis by electron microscopy showed that the mutant cells had a somewhat rough outer surface compared with the parent (Fig. 4), which is indicative of a lack of peptidoglycan release at the cell surface. The MIC of penicillin was unchanged (0.02 μg/ml). Penicillin-induced lysis was greatly retarded in the mutant; however, the bacteriocidal effect was unaltered. Triton X-100-induced lysis was also inhibited; the A_{600} of the SH108 culture dropped by half that of the lysed control after 6 h. Four molar LiCl extraction (4) of SH108 cells resulted in an enzyme-containing sample having only 3% of the activity of the parent when assayed spectrophotometrically (3).

Expression analysis of the major autolysin. Reporter gene analysis using the *lacZ* fusion (12) created as described above was used to monitor the expression of the autolysin gene. The level of expression of *lacZ* did not change during growth and into early stationary phase, which correlates with the autolysin profile (results not shown) (20). Availability of oxygen, growth medium, or KCl concentration had no significant effect on expression. Autolysin gene expression was temperature dependent, being threefold higher when the culture was grown at 25°C compared with 37°C (Fig. 5). NaCl also enhanced expression twofold, having an optimum of 1 M.

Concluding remarks. By using a direct cloning procedure, the structural gene (*atl*) encoding the major autolysin of *S. aureus* has been isolated and studied at the molecular level. The gene encodes a protein of 137 kDa which is exported and processed into the multiple autolysin bands seen by renaturing

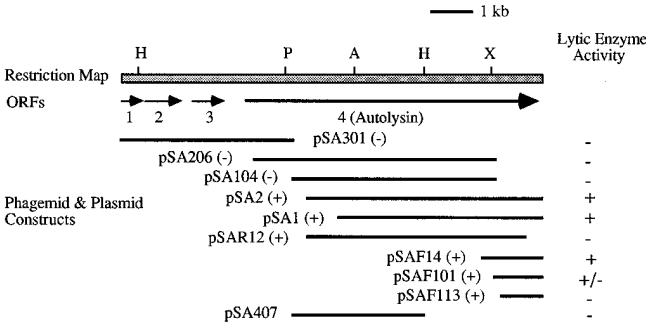

FIG. 2. Physical and genetic map of the autolysin gene region and lytic activity of recombinant plasmid constructs. The restriction map of the sequenced region shows all cut sites for the enzymes mentioned: H, *Hin*dIII; P, *Pst*I; A, *Acc*I; X, *Xho*II. The map is bounded by *Sau*3A sites, with 10 others within the region. The positions and directions of the ORFs is shown by the arrows. The lytic activity of *E. coli* XLOLR carrying each of the phagemid and plasmid constructs is indicated as measured by the Procion red wall plate test (3): +, zone of clearing in the red wall background around the colony; +/−, much reduced zone merely under the colony; −, no zone of clearing. The + or − in parentheses after the phagemid name refers to the orientation of the autolysin gene in respect to the *lac* promoter in pBKCMV: +, in the orientation to allow transcription of the autolysin gene; −, in the opposite orientation.

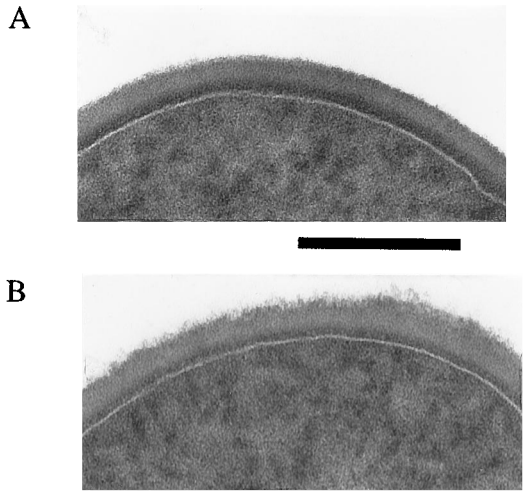

FIG. 4. Electron micrographs of the cell wall of parent strain 8325/4 (A) and strain SH108 (*atl*) (B) of *S. aureus*. Note the rough outer surface of the wall in SH108. Both micrographs are of the same magnification. Bar = 0.1 μm.

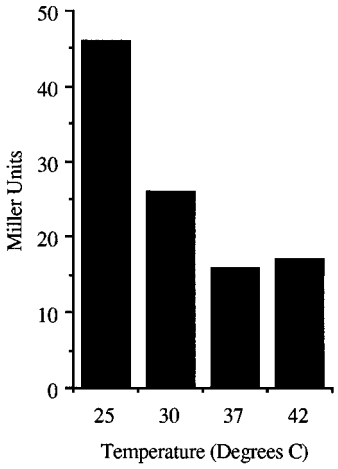

FIG. 5. Effect of temperature on autolysin gene expression. Cells were grown for 18 h in tryptic soy broth at the temperatures indicated, washed, and resuspended in Z buffer (12). β-Galactosidase levels were determined as previously described, using *o*-nitrophenyl-β-D-galactopyranoside as the substrate (12).

SDS-PAGE. The protein has three domains, the central of which contains three imperfect direct repeats probably involved in wall binding (5). The C-terminal domain of the protein has glucosaminidase activity, and the N-terminal domain has been shown independently to have amidase activity (14), and so the enzyme is bifunctional. The insertionally inactivated mutant SH108 is missing almost all of the autolysin bands, as judged by renaturing SDS-PAGE, and thus *atl* encodes the major autolysin of *S. aureus*. The clumping of SH108 is entirely consistent with the synergistic declumping ability of the purified amidase and glucosaminidase forms of Atl (18). Atl has more than one role; it is involved in cell separation, general postmortem lysis, and peptidoglycan release at the cell surface.

A putative regulator of *atl* expression has been identified (11); the *lyt* mutant is missing all of the autolysins apart from the 36-kDa enzyme present in SH108, and the cells have a rough outer surface appearance (10, 11). Both *atl* and *lyt* are expressed to the greatest extent at temperatures encountered in the environment not associated with a mammalian host. Thus, Atl may have an important role in cellular physiology in the terrestrial or aquatic environment.

An 80-kDa glucosaminidase purified from *S. aureus* which corresponds to one of the Atl processed forms acts as an immunosuppressant (22); also, the *lyt* mutant has attenuated virulence in a rat model (10). This finding suggests that in *S. aureus*, Atl has a role in pathogenicity either directly by an effect on the host or indirectly by its involvement in the normal physiological processes of the bacterial cell. The two Atl activities may have quite separate roles. Any attempts to study the biochemical regulation of Atl must take into account its relative complexity with two activities and a number of processed forms.

Nucleotide sequence accession number. The total 5,417-bp sequence reported in this paper has been deposited in the GenBank database under accession number L41499.

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