Characterization of a chromosomally encoded glycylglycine endopeptidase of Staphylococcus aureus

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The authors previously reported the cloning of a lytic-enzyme-encoding gene, lytM, from an autolysis-defective mutant of Staphylococcus aureus. In the present work, the lytM gene was overexpressed in Escherichia coli and the product was purified to homogeneity by affinity chromatography and HPLC. Biochemical analysis of LytM-cleaved peptidoglycan fragments indicated that LytM is a glycylglycine endopeptidase. Immunoelectron microscopic studies with anti-LytM rabbit IgG showed that LytM is expressed during the early exponential phase and is overexpressed in an autolysis-defective mutant compared with the parent strain. Also, a uniform distribution of gold particles on the surface of actively growing bacterial cells indicates that LytM plays a role in cell growth. Northern blot analyses of lytM expression in two global regulatory mutants, agr and sar, showed that expression of lytM is increased about twofold in these mutants as compared with the parents. Protein homology searches revealed that LytM could be a member of the zinc protease family, as it contained a homologous 38-amino-acid motif, Tyr-X-His-X,-Val-X,-Gly-X,-His. Atomic absorption spectrometric analysis of LytM revealed the presence of 0.9 mol zinc (mol LytM)-1.

Abbreviations: DEPC, diethyl pyrocarbonate; DNP, 2,4-dinitrophenyl; PCW, purified cell wall.

INTRODUCTION

Peptidoglycan hydrolases are enzymes that are ubiquitous in bacteria and which hydrolyse the peptidoglycan of the bacterial cell envelope (Ghuysen et al., 1966). There are five main classes of peptidoglycan hydrolase, the N-acetylmuramidases, N-acetylmuramoyl-L-alanine amidases, endopeptidases, glucosaminidase and transglycosylases (Actor et al., 1988; Hölting & Tuomanen, 1991; Rogers et al., 1980; Tomasz, 1984). Several roles have been proposed for these enzymes in cell wall growth, cell separation, cell wall turnover, lysis initiated by antibiotics affecting the cell wall, competence for genetic transformation, flagellum formation, sporulation and pathogenicity (Actor et al., 1988; Berry et al., 1989; Rogers et al., 1980; Shockman & Barrett, 1983; Shockman & Hölting, 1994; Ward & Williamson, 1984).

Since the first report on Staphylococcus aureus autolysins (Welsch & Salmon, 1950), a variety of intracellular and extracellular lytic enzymes have been reported and characterized in this bacterium (Sugai, 1997). Tipper (1969) demonstrated the presence of amidase, glucosaminidase and endopeptidase activities in isolated cell walls of S. aureus. A bifunctional structural lytic gene encoding the S. aureus N-acetylglucosaminidase and N-acetylmuramidase was later characterized (Foster, 1995; Oshida et al., 1995; Oshida & Tomasz, 1992). Recently, we identified a lytic gene, lytM, from an autolysis-defective mutant (Lyt-) of S. aureus (Ramadurai & Jayaswal, 1997). The deduced amino acid sequence of LytM showed significant homology to lysostaphin, encoded by plasmids of Staphylococcus simulans (Heinrich et al., 1987; Recsei et al., 1987), and ALE-1, encoded by Staphylococcus capitis (Sugai et al., 1997). On the basis of amino acid sequence homology, it was proposed that LytM could be an endopeptidase (Ramadurai & Jayaswal, 1997). Here, we report the overexpression,
purification and biochemical characterization of the \textit{lytM} gene product. This is the first report on a chromosomally encoded, probable glycyglycine endopeptidase in \textit{S. aureus}.

**METHODS**

**Culture conditions.** LB broth was used for the cultivation of \textit{Escherichia coli} cells; tryptic soy broth (Difco) and PYK (Novick, 1967) were used for the cultivation of \textit{S. aureus} strains. Cells were cultured at 37 °C with shaking (200 r.p.m.). When required, the antibiotics ampicillin (25–50 µg ml⁻¹), tetracycline (10–20 µg ml⁻¹), chloramphenicol (30 µg ml⁻¹), and erythromycin (20 µg ml⁻¹) (Sigma) and IPTG (3 mM) (Fisher Scientific) were added at the concentrations indicated.

**Preparation of autolysin crude extract.** Autolysins from \textit{S. aureus} were extracted by the freeze–thaw method as described previously (Potvin et al., 1988). Sonication was used to prepare the autolysin extract from \textit{E. coli} BLRDE3(pLysS) (Novagen) containing plasmid pLR-05 (Ramadurai & Jayaswal, 1997). Mid-exponential-phase cultures of \textit{E. coli} harbouring recombinant plasmid pLR-05 (lytM under the control of the tac promoter) were grown to an OD₆₀₀ of 0.5 (measured with a Beckman DU-64 spectrophotometer) in LB broth containing the appropriate antibiotics. Cells were induced with IPTG at a final concentration of 3 mM and incubated for an additional 4 h. Cells were then harvested by centrifugation and resuspended in a minimal volume of 50 mM potassium phosphate buffer (pH 7.2). Cell suspensions were subjected to sonication (seven pulses of 30 s spaced 30 s apart, with settings at an output control of 5 and 50% duty cycle) with a Sonifer cell disruptor (Branson Sonic Power). Unbroken cells were removed by centrifugation (10 000 g for 15 min at 4 °C). The supernatant was centrifuged several times at 10 000 g for 15 min at 4 °C until a clarified supernatant was obtained. This supernatant was used for the determination of lytic activity and the subsequent purification of LytM.

**Zymographic detection of lytic activity.** The lytic activity in the sonicated extracts was determined on an SDS-polyacrylamide gel (15%) containing 0.2 % (w/v) lyophilized and autoclaved crude cell walls from \textit{S. aureus} as described previously (Mani et al., 1993). Crude cell walls were prepared from exponential-phase cultures of the strains grown in PYK medium as described earlier (Jayaswal et al., 1990). The gels were subsequently renatured in renaturation buffer to allow development of lytic bands. The gels were stained with 1 % methylene blue for better visualization of the activity bands.

**Purification of LytM.** Sonicated extracts, isolated after induction of LytM in \textit{E. coli} as described above, were applied to a nickel-binding affinity column (Xpress purification system; Invitrogen) and eluted in a step-wise imidazole gradient (50–200 mM) as outlined by the manufacturer. The fractions containing active enzyme, as determined by zymogram, were pooled and concentrated using a Centricon-30 filtration unit (Amicon) at 3000 g for 2 h at 4 °C. The concentrated fractions were further subjected to buffer exchange to 100 mM Tris/HCl (pH 8.0) in the Centricon units. The concentrated extract was applied to an anion-exchange Resource Q column (Pharmacia Biotech) pre-equilibrated with elution buffer (100 mM Tris/HCl, pH 8.0). After washing with two column volumes of elution buffer, the bound proteins were eluted with a linear gradient from 0 to 1 M NaCl in elution buffer (room temperature, 1 ml min⁻¹, 5 MPa) on an HPLC/FPLC apparatus (AKTA Explorer 10; Pharmacia Biotech). Protein concentrations were determined with Bio-Rad reagent using BSA as a standard.

**Determination of substrate and bond specificity of LytM.** Heat-killed cells of \textit{S. aureus}, the \textit{S. aureus} Lyt mutant (Mani et al., 1993), \textit{Micrococcus luteus} and \textit{Staphylococcus carnosus} were used to determine the substrate specificity of the LytM enzyme. NaOH-treated purified cell walls (PCW) of these strains were used as the substrates to determine the bond specificity. Heat-killed cells were suspended (1 mg ml⁻¹) in 0.1 M Tris/HCl (pH 8.0). An appropriate concentration of the LytM enzyme was added to a 2 ml cell suspension. The mixture was incubated at 37 °C and the rate of decrease in turbidity was measured by following the OD₆₅₀. One unit of enzyme activity was defined as the amount of protein that decreased the turbidity as measured by OD₆₅₀ from 1.0 to 0.5 in 5 h. The PCWs were prepared by treating cell walls with 0.1 M NaOH at 37 °C for 10 min to remove ester-linked D-alanine from teichoic acids, washed five times with cold, double-distilled water and finally lyophilized. The PCWs were suspended (5 mg ml⁻¹) in 0.1 M Tris/HCl buffer (pH 8.0) and incubated with the appropriate amount of purified enzyme at 37 °C in a total volume of 2 ml. Samples were removed at intervals to monitor turbidity and to determine the concentration of reducing and amino groups released. The bond specificity of LytM was determined by analysing the reaction products. The appearance of N-terminal groups was determined according to Ghysen et al. (1966) using 2,4-dinitrofluorobenzene and the reducing sugars were determined by a modification of the Park-Johnson method (Thompson & Shockman, 1969). Hydrolysis was carried out by incubating dried test samples with concentrated HCl for 6 h at 105 °C. 2,4-Dinitrophenyl (DNP)-amino acids were analysed by silica gel TLC. The plate was developed for 4 h with n-butanol/1 % ammonia at room temperature and, after drying, with chloroform/methanol/acetic acid (85:14:1) for 2 h at 4 °C.

**Effect of physical and chemical factors on LytM activity.** The effects of a number of compounds on the autolytic profiles of LytM were determined. The compounds tested, each added separately in renaturation buffer, were: 10 mM each of ampicillin, BaCl₂, CaCl₂, CuCl₂, DTT, EDTA, FeCl₃, glucosamine, HgCl₂, KCl, MgCl₂, MnCl₂, (NH₄)₂SO₄ and ZnCl₂; 200 mM LiCl, 100 mM NaCl, 1 mM PMSF and 5 mM diethyl pyrocarbonate (DEPC). The effect of pH on the LytM lytic band was determined by incubating the gels in Tris/maleate and Tris/HCl buffers in the pH range 4–10. Thermolability was measured by incubating the samples at 100 °C for 5, 10, 15, 20, 25 and 30 min before zymogram analysis.

**Northern blot analysis.** Total RNA was isolated from the parent strain and the agr (Recsei et al., 1986) and sar (Cheung et al., 1992) mutants using a Promega RNA isolation kit with minor modifications. Ten micrograms of total RNA, electrophoresed under denaturing conditions in a 1:4 % agarose gel, was transferred to a nitrocellulose membrane as described by Sambrook et al. (1989). Hybridization was carried out at 42 °C for 20 h in hybridization buffer [50 % formamide, 5 × SSC, 1 × Denhardt’s solution, 50 mM Na₂HPO₄ (pH 6.5), 250 µg denatured herring sperm DNA ml⁻¹]. The membrane was hybridized with an [α-³²P]dCTP (ICN Biochemicals)-labelled \textit{lytM} probe, washed with 2 × SSC buffer at room temperature for 5 min and washed twice in 1 × SSC, 0.1 % SDS, 1 mM EDTA buffer at 50 °C for 10 min or until background levels of radioactivity were undetectable. The bands hybridizing to \textit{lytM} were visualized by autoradiography. Transcript sizes were determined by running standard RNA markers (Promega). To determine the levels of \textit{lytM} gene expression during the growth cycle, total RNA was isolated from cells at different stages of growth (at OD₆₅₀ of 0.2, 0.4, 0.8, 1.2 and 2.0) and probed with \textit{lytM}.
Determination of zinc content in LytM. To determine the zinc content, purified LytM was hydrolysed with concentrated HCl at 105 °C for 6 h. Zinc ions released after hydrolysis were detected with an atomic absorption spectrophotometer (Thermo Jarrell Ash). The data are means of three determinations of different dilutions of the sample.

Generation of polyclonal antibody. The affinity-purified LytM protein was used to raise antibodies. The active protein (1 mg protein ml⁻¹) dissolved in PBS (137 mM NaCl, 3.7 mM KCl, 43 mM Na₂HPO₄, 1.4 mM KH₂PO₄) was emulsified with an equal volume of Freund's complete adjuvant (Difco). The mixture was injected intraperitoneally into a rabbit (weighing 2 kg and 3 months old). Three booster doses of the same amount of protein emulsified in Freund's incomplete adjuvant were subsequently injected intraperitoneally into the rabbit after 2, 4 and 6 weeks. The rabbit was bled following each injection and the antibody titre against LytM was determined by ELISA. Western blot analyses were performed as described by Sambrook et al. (1989).

Electron microscopic studies. For scanning electron microscopy, bacterial cells were grown in PYK at 37 °C with continuous shaking. Samples were withdrawn at OD₆₀₀ of 0.7 (exponential phase) and 1.3 (early stationary phase). Cells were harvested, washed twice in PBS and finally resuspended in PBS. This cell mixture was incubated with an appropriate dilution of anti-LytM IgG for 1 h at 37 °C with gentle shaking. The localization of LytM on the cell surface was examined by the protein A–gold labelling method (Yamada et al., 1996). The cell suspension was centrifuged and washed several times in PBS to remove unbound anti-LytM IgG. The cells were resuspended in PBS and incubated in the presence of a protein A–gold colloidal particle suspension (particle diameter 20 nm; E-Y Laboratories) at room temperature for 1 h. The cell suspension was washed several times with PBS. Samples were fixed with 2.5% glutaraldehyde followed by 1% OsO₄. Cells were then treated with 1% tannic acid and dehydrated in an acetone series. Samples were mounted on stubs, coated with gold and examined with a Cambridge Stereoscan 240 scanning electron microscope at 20 kV. For transmission electron microscopy, the cell suspension was mixed with agar, dehydrated in an ethanol series and then embedded in Epon as described by Vijarankul et al. (1995). Ultra-thin sections were cut using a diamond knife and examined in a Zeiss 10C electron microscope operating at 60 kV.

RESULTS AND DISCUSSION

Protein purification and characterization of bond specificity

To purify the LytM enzyme, the lytM gene was cloned into an overexpression vector, pRSETa, under the control of the T7 promoter as described previously (Ramadurai & Jayaswal, 1997). In this system, LytM was expressed as a fusion protein with a six-histidine-residue tag at its N-terminus. E. coli cells carrying the above recombinant plasmid, pLR-0.5, were grown at 37 °C to an OD₆₀₀ of 0.5 and then induced for LytM overexpression with 3 mM IPTG. Cells were further incubated for 4 h and subjected to sonication as described in Methods. This sonicated extract was used to purify the LytM fusion protein by nickel-binding affinity column chromatography (Invitrogen). The fusion protein was purified to about 80% homogeneity in the first step of purification. The concentrated active fractions, as determined by zymograms, were subjected to ion-exchange HPLC. The lytic activity was eluted in a single peak. The purified enzyme migrated as a single band on SDS-PAGE gels with an apparent molecular mass of 40 kDa. A lower molecular mass band of less than 40 kDa was also observed occasionally. This second band appears to be a partially degraded by-product of LytM that originated during the purification process. N-terminal sequencing of the amino acid residues of the purified proteins matched the deduced amino acid sequence (Ramadurai & Jayaswal, 1997). A summary of the purification of the LytM fusion protein is shown in Table 1. The increase in the yield observed after the third step of purification could be due to the removal of impurities that were inhibiting the activity of the enzyme in the initial steps.

To determine the substrate specificity, LytM was incubated with heat-killed and autoclaved cells of S. aureus RN450, the S. aureus Lyt⁻ mutant (Mani et al., 1993), M. luteus and S. carnosus. LytM showed activity against S. aureus RN450, the S. aureus Lyt⁻ mutant and S. carnosus (50% decrease in turbidity in 5 h). However, it had no detectable activity against M. luteus cells (data not shown). This indicated that LytM could hydrolyse a bond that is not present in the M. luteus cell wall. Based on previous comparisons of amino acid homology between LytM and other enzymes, it was postulated that LytM could be an endopeptidase (Ramadurai & Jayaswal, 1997). To determine the bond specificity, LytM was incubated with PCWs of S. aureus RN450, the S. aureus Lyt⁻ mutant, S. carnosus and M. luteus. Hydrolysis was observed in the case of S. aureus RN450, S. carnosus (Fig. 1a, c) and the S. aureus Lyt⁻ mutant cell walls (data not shown). However, a large quantity of enzyme was required to bring about the hydrolysis, suggesting that LytM might have a low specific activity or might act very slowly. No hydrolysis was observed on previous comparisons of amino acid homology between LytM and other enzymes. To identify the N-terminal amino acid released during the hydrolysis of the PCWs, the supernatants of the reaction mixture were incubated with 2,4-dinitrofluorobenzene and subjected to further hydrolysis with HCL. TLC analysis of the hydrolysates revealed the presence of DNP-glycine (Fig. 2) and not DNP-alanine, suggesting that LytM is a glycylglycine endopeptidase.

Qualitative effects of physical and chemical factors on the LytM activity

Autolysins are reported to be very stable but their activities can be modulated by environmental factors (Foster, 1995; Mani et al., 1993; Sugai et al., 1990; Tobin et al., 1994). An attempt was made to determine whether the specific activity of LytM could be modified.
Table 1. Summary of LytM purification

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein (µg)</th>
<th>Specific activity (units µg⁻¹) *</th>
<th>Purification (fold)</th>
<th>Yield (units)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induced cell lysate supernatant</td>
<td>112340</td>
<td>0.001</td>
<td>1</td>
<td>11234</td>
</tr>
<tr>
<td>Nickel-affinity-purified LytM</td>
<td>2013</td>
<td>0.020</td>
<td>20</td>
<td>4026</td>
</tr>
<tr>
<td>Resource-Q-column-purified LytM</td>
<td>50</td>
<td>0.400</td>
<td>400</td>
<td>20000</td>
</tr>
</tbody>
</table>

* One unit of enzyme was defined as the amount of protein that decreased the OD₅₈₀ from 0.5 to 0.25 in 5 h.
† Yield is defined as the number of enzyme units recovered.

![Graphs](a) OD₅₈₀ vs Time (h) (b) Amino and reducing groups released vs Time (h)

by various physical and chemical factors, as tested previously by Sugai et al. (1990, 1997) for the lytic enzymes of staphylococci. Among the compounds tested, ampicillin, Ba²⁺, Cu²⁺, DTT, EDTA, Fe²⁺, Mg²⁺, Mn²⁺, PMSF and Zn²⁺ appeared to have some inhibitory effect on lytic activity. Surprisingly, NaCl and KCl appeared to inhibit activity at concentrations as low as 100 mM. DEPC, HgCl₂, (NH₄)₂SO₄ and glucosamine completely inhibited the activity. The pH optimum of LytM activity was in the range 5–8. The activity of LytM was stable for 15 min at 100 °C. Activity was destroyed on heating for 30 min. This indicates that the enzyme is very stable and has a broad pH range of activity.

Zinc ligands in LytM

Protein homology searches had earlier revealed that LytM has significant identity to lysostaphin and ALE-1. Studies with the two peptidases, lysostaphin originating from S. simulans (Heinrich et al., 1987; Recsei et al.,
We reported previously that lytM appears to be highly conserved within S. aureus strains at the nucleotide level (Ramadurai & Jayaswal, 1997). Freeze–thaw extracts from 10 different species of staphylococci were analysed in an attempt to identify cross-reactivity with anti-LytM rabbit IgG. No significant cross-reactivity was observed in the species tested, except with the lytic enzymes of S. carnosus (data not shown). This suggests that there is some homology between S. carnosus and S. aureus autolysins.

**Transcriptional regulation of LytM**

We reported previously that an agr mutation had no obvious effect on the rate of autolysis and enzyme profiles (Tobin et al., 1994). Fujimoto & Bayles (1998) have recently reported that mutations in two global regulatory loci, agr (Recsei et al., 1986) and sar (Cheung et al., 1992), which control exoprotein production in S. aureus, cause decreased and increased rates of autolysis, respectively. Their zymographic analyses showed that agr mutants produced high levels of higher molecular mass hydrolases, whereas sar mutants appeared to overproduce a 32 kDa murein hydrolase. Fujimoto & Bayles (1998) have proposed that agr probably regulates the expression of a protease that may be involved in proteolytic processing of atl-encoded murein hydrolases and that sar could negatively control the expression of the lytM-encoded murein hydrolase. To test whether the effects of agr and sar are exerted on lytM at the transcriptional level, lytM RNA levels were analysed at various stages of the growth cycle (Fig. 3). Expression of lytM was about twofold higher in agr and sar mutants during the early exponential phase of growth when compared with the parent. The significance of this result is currently under investigation. A putative regulatory locus has been reported that affects autolysis in S. aureus cells (Brunskill & Bayles, 1996a, b). This locus consists of a two-component regulatory system, the components of which, lytS and lytR, are presumed to regulate murein hydrolase genes transcriptionally in S. aureus (Brunskill...
& Bayles, 1996a). Mutations in lytS augment the rate of autolysis. Whether this locus controls the expression of lytM remains to be determined.

**Cell surface localization of LytM**

To deduce the role of LytM in the *S. aureus* cell, we attempted to localize LytM on the cell surface by immunoelectron microscopy. Cells at various stages of the growth cycle were examined by scanning and transmission electron microscopy as described in Methods. In the electron micrographs, the protein A–gold particles were distributed over the cell surface in exponential-phase samples of the parent (Fig. 4a) and the mutant (Fig. 4c). The numbers of gold particles per cell for the parent and the mutant in exponential phase were 4.8 ± 0.04 and 14.2 ± 0.2. In the stationary phase, the amount of LytM label was similar in the parent (Fig. 4b) and the Lyt− mutant (Fig. 4d). The numbers of gold particles per cell for the parent and the mutant in the stationary phase were 4.96 ± 0.04 and 4.8 ± 0.05. No specific pattern of LytM distribution was observed on the cell surface, as reported for ATL by Yamada et al. (1996). The *S. aureus* Lyt− mutant showed a higher level of staining for LytM (Fig. 4c) on the cell surface as compared with the parent (Fig. 4a). The Lyt− mutant was isolated by transposon mutagenesis (Mani et al., 1993). However, later studies showed that the stable Lyt− phenotype is due to a secondary mutation, probably caused by transposon-mediated rearrangement or deletion (A. Xiong & R. K. Jayaswal, unpublished data). In this background, LytM expression is higher than in the parent strain. These results further support an earlier report of RNA data (Ramadurai & Jayaswal, 1997), where it was reported that lytM appears to be overexpressed in the exponential phase of the growth cycle of the Lyt− and atl mutants.

In the present study, we have identified an *S. aureus* autolysin (LytM) as a glycylglycine endopeptidase. This is the first report of a chromosomally encoded endopeptidase in *S. aureus*. We believe that LytM may play a role in actively growing and dividing cells. We reported previously that the Lyt− mutant was normal with respect to growth rate, cell division, cell size and adaptive responses to environmental changes, except that it shows attenuated pathogenicity (Mani et al., 1994; Ramadurai & Jayaswal, 1997). It is hypothesized that...
LytM was being overexpressed in the mutant to compensate for the functions of the missing autolysin and that it was vital to the organism. To determine whether this is true, work is underway to generate a $\text{lytM}$ mutation and transduce it back into the original Lyt$^{-}$ mutant in order to generate a double mutant that will lack all apparent autolysin functions.

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