Analysis of a Peptidoglycan Hydrolase Gene from *Staphylococcus aureus* NCTC 8325

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We have investigated the expression of the peptidoglycan hydrolase gene (*lytA*) of *Staphylococcus aureus* NCTC 8325. Results from in vitro transcription-translation analysis, maxicell experiments, and Northern (RNA) blot analysis suggest that the *lytA* gene encodes a polypeptide of M_r ~50,000. Physical mapping data indicate that the *lytA* gene originated from prophage 11 in the NCTC 8325 strain.

A variety of peptidoglycan hydrolase activities have been reported to exist in *Staphylococcus aureus* (16, 17). Roles have been proposed for these enzymes in cell wall growth and turnover, cell division, irreversible effects caused by treatment with antibiotics, competence for genetic transformation, liberation of phage progeny, flagellar extrusion, and pathogenicity (1, 4, 12, 18). However, their precise roles are still speculative because of the difficulty in obtaining mutants for the genes determining these enzymes. Recently, we reported the cloning of a gene encoding a peptidoglycan hydrolase activity from *S. aureus* NCTC 8325 that was tentatively identified as an *N*-acetylmuramyl-L-alanine amidase (6). Enzymographic studies showed that recombinant *Escherichia coli* produces a lytic enzyme with an apparent M_r of 23,000 which corresponds to one of the lytic protein bands from *S. aureus* (6). The nucleotide sequence of an open reading frame believed to correspond to the amidase gene (*lytA*) was determined (19). The deduced primary amino acid sequence revealed a putative protein of 481 amino acid residues with an M_r of 53,815. Hence, it was necessary to determine whether the failure to see a band of activity at an M_r of ~54,000 in enzymographic studies was due to low concentrations of the enzyme in our previous preparations or whether it was due to protein processing that converted a preamidase (M_r of ~54,000) into a mature amidase (M_r of ~23,000). The transcript mapping and in vivo and in vitro analyses of the *lytA* gene product reported here were undertaken to resolve this discrepancy.

The bacterial strains and plasmids and their relevant characteristics are listed in Table 1. Luria-Bertani (14) medium was used for the maintenance and growth of *E. coli* strains. PYK medium (6) was used for the growth of *S. aureus* NCTC 8325. K medium (20) was used for culturing irradiated *E. coli* IT2761. Ampicillin (50 μg/ml), tetracycline (25 μg/ml), and chloramphenicol (25 μg/ml) were added to the growth media as necessary. The bacteria were grown at 37°C with shaking.

Restriction enzymes, T4 DNA ligase, exonuclease III, and molecular weight markers were purchased from BRL Life Technologies, Inc. (Gaithersburg, Md.). The oligonucleotide-labeling kit was purchased from NEN Research Prod-

ucts, Biotech Systems (Boston, Mass.). L-[^{35}S]methionine, L-[^{35}S]cysteine, and ^{14}C-labeled protein markers were obtained from DuPont Co. Biotech Systems (Wilmington, Del.). α-^{32}P-labeled dCTP was obtained from ICN Biochemicals, Inc. (Costa Mesa, Calif.). Nylon membranes were obtained from Micro Separations, Inc. (Westboro, Mass.). The DNA-directed translation kit was obtained from Amersham Co. (Arlington Heights, Ill.). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Bio-Rad Laboratories (Richmond, Calif.).

Total cellular RNA was isolated from desired clones by using the hot phenol method (5, 8). The isolated RNA was blotted onto a nylon membrane, hybridized to the radiolabeled DNA probe, and washed under standard conditions (14).

The in vitro transcription-translation experiments were carried out by using the Amersham prokaryotic DNA-directed translation system as instructed by the manufacturer. The in vivo maxicell experiments were carried out by using a standard method (15). The protein samples from in vivo and in vitro experiments were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% polyacrylamide gel) (7), followed by staining with Coomassie brilliant blue or autoradiography of the gel.

The lytic activity of the clones was checked by exposing the colonies to chloroform and overlaying them with soft agar containing 0.2% heat-killed, lyophilized cells of *Micrococcus luteus* as described previously (6). Colonies showing a clearing halo were scored as positive for lytic activity (Lyt$^+$).

Lytic activity of deletion clones. To locate the functional region of the gene, various deleted clones were constructed either by deletion of unique endonuclease restriction fragments or by unidirectional exonuclease III deletion. Table 1 shows the insert sizes and lytic activities of various clones. Our sequencing data suggest that the coding region initiates with ATG at nucleotide 290 from the *Nhe*I site and extends to nucleotide 1732 (Fig. 1). A deletion of ~300 bp from the *Xba*I site toward the *Acc*I site (pXW59) resulted in the loss of lytic activity. Deletion of the *Nhe*I fragment (pXW95) up to nucleotide 278 from the 5' direction (pXW127) had no effect on *lytA* gene expression. However, further deletion (pXW126, deleted up to nucleotide 302) resulted in the loss of lytic activity. Whether the loss of activity in pXW126 is due to the absence of the Shine-Dalgarno sequence and the start codon or whether it is due to the absence of four amino

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
E. coli		
JM109	*recA supE44 endA1 hsdR gyrA96 relA1 thi-1*, Δ(*lac, pro*), F' (*traD36 proA$^+$B$^+$ lacI^{q}ZΔM12*)	21
IT2761	K-12 derivative, Δ*lacU169*, Δ*recA*	13
S. aureus		9
NCTC 8325		
Plasmids		
pRJ2-19	Lyt$^+$, 7.4-kbp insert in pBR322	6
pRJ2-19E1	Lyt$^+$, 3.2-kbp insert in pBR322	6
pRJ19E1	Lyt$^+$, 3.2-kbp insert in pACYC184	6
pRJ19H-X	Lyt$^+$, 2.7-kbp insert in pUC13	6
pXW56	Lyt$^+$, 3.2-kbp insert in pTZ19R (N)	This study
pXW57	Lyt$^+$, 3.2-kbp insert in pTZ19R (X)	This study
pXW58	Lyt$^+$, 2.5-kbp insert (X)	This study
pXW59	Lyt$^-$, 2.1-kbp insert (X)	This study
pXW93	Lyt$^-$, 2.1-kbp insert (N)	This study
pXW95	Lyt$^+$, 2.65-kbp insert (N)	This study
pXW96	Lyt$^+$, 2.65-kbp insert (N)	This study
pXW106	Lyt$^-$, 2.3-kbp insert (N)	This study
pXW126	Lyt$^-$, 2.4-kbp insert (N)	This study
pXW127	Lyt$^+$, 2.4-kbp insert (N)	This study

[a] N, N orientation (a clone in which a deletion of the *lytA* gene was carried out in the 5' → 3' direction); X, X orientation (a clone in which a deletion of the *lytA* gene was carried out in the 3' → 5' direction).

acids from the N terminus remains to be determined. In clone pXW59, deletion of the C-terminal amino acids resulted in the loss of lytic activity, possibly as a result of deletion of the cell wall-binding domain. From this study, we conclude that nucleotides 278 to 1732 are essential for the enzyme activity.

Northern (RNA) hybridization analysis. The primary amino acid sequence deduced from the nucleotide sequence indicated a putative polypeptide of 481 amino acid residues with an M_r of 53,814 (19). Although we were unable to detect the insert encoded polypeptide by SDS-PAGE (6), a polypeptide
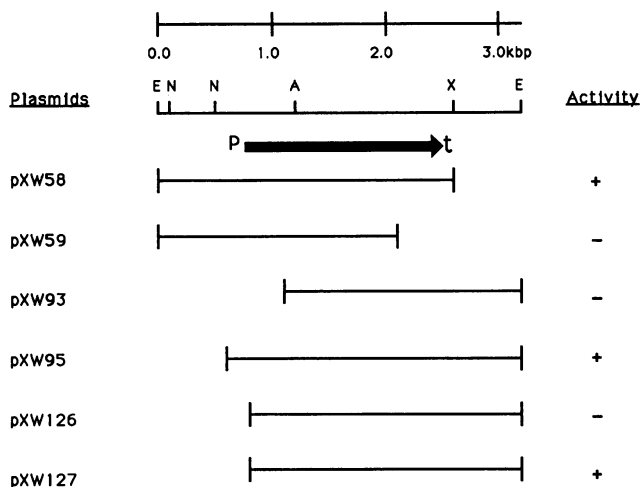

FIG. 1. Physical map of insert DNA containing the *S. aureus* amidase gene. The arrow indicates the orientation and size of the *lytA* gene. P, promoter; t, terminator; E, *Eco*RI; N, *Nhe*I; X, *Xba*I; A, *Acc*I.
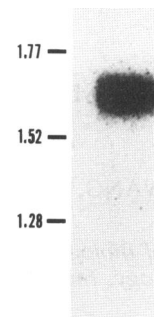

FIG. 2. Northern blot analysis of the *lytA* transcript. A 10-μg sample of total RNA from *E. coli* was electrophoresed on a 1.5% agarose formaldehyde gel. After transfer of the RNA, the membrane was probed with a ^{32}P-labeled *Hind*III-*Xba*I fragment from pXW127. The positions (Kilobases) of RNA standards are shown on the left.

of M_r 22,000 to 23,000 with lytic activity was observed in enzymographic studies. Therefore, we hypothesized that protein processing is responsible for converting the preamidase of M_r ~54,000 into a mature amidase of M_r ~23,000.

Before testing this hypothesis, we set up an experiment to determine the size of the transcript. Total RNA from an *E. coli* strain harboring pXW57 was isolated and analyzed as described above. Northern hybridization was performed with a 1.6-kbp *Hind*III-*Xba*I probe (*lytA*-specific clone). A band of ~1.6 kbp hybridized with the *lytA* probe (Fig. 2). RNA isolated from a strain containing vector pTZ19R (without the *lytA* gene) did not show any hybridization with the probe (data not shown). These data suggest that the 1.6-kbp band represents the transcript from the *lytA* gene.

In vitro transcription-translation analysis. Analysis of total protein from the recombinant *E. coli* did not show any insert-specific polypeptide either by Coomassie brilliant blue staining or by in vivo labeling with [^{35}S]methionine (6). An in vitro coupled transcription-translation system from *E. coli* was used to analyze the *lytA* gene product. A polypeptide of apparent M_r 50,000 was synthesized by all LytA$^+$ plasmids: pRJ-19, pRJ2-19, pRJ19E, and pRJ2-19H-X (Fig. 3, lanes 2, 4, 6, and 8). This polypeptide was absent from vectors
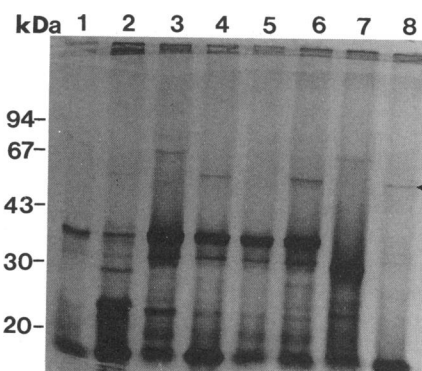

FIG. 3. In vitro transcription-translation of the various *lytA* plasmids, performed in the presence of ^{35}S-labeled methionine and cysteine. Two micrograms of purified plasmid was used for each reaction, and the product was analyzed by SDS-PAGE (15% polyacrylamide gel). Lanes: 1, pBR322; 2, pRJ2-19; 3, pTZ19R; 4, pRJ2-19E1, 5, pUC13; 6, pRJ2-19H-X, 7, pACYC184; 8, pRJ19E1. Molecular size markers are indicated on the left.
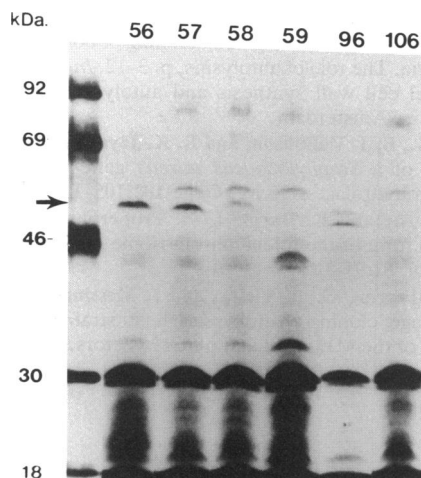
FIG. 4. Maxicell analysis of the *lytA* gene. Maxicells containing plasmids pXW56, pXW57, pXW58, pXW59, pXW96, and pXW106 were irradiated by UV light, and proteins were labeled with [^{35}S]methionine and [^{35}S]cysteine. Labeled proteins were fractionated on an SDS–15% polyacrylamide gel and exposed to X-ray film after drying. The arrow indicates *lytA*-encoded protein. The sizes of the protein markers are indicated on the left.

pBR322, pTZ18R, pUC13, and pACYC184 (lanes 1, 3, 5, and 7). It seems likely that the *lytA* gene product migrates anomalously on SDS-polyacrylamide gels, displaying an apparent M_r of ~50,000 rather than the M_r of ~53,000 calculated from the nucleotide sequence (19). This difference in mobility may be due to the hydrophilic nature of the polypeptide (19).

In vivo maxicell analysis. To further confirm the hypothesis that the in vitro transcription-translation product is the *lytA* gene product, maxicell analyses were performed. Various plasmids (pXW56, pXW57, pXW58, pXW96, and pXW106) were introduced into maxicells of *E. coli* IT2761 by transformation. Transformed cells were irradiated with UV light, and plasmid-coded proteins were labeled selectively with ^{35}S-labeled methionine and cysteine (15).

The total protein was isolated and analyzed by SDS-PAGE. All lytic clones (pXW56, pXW57, pXW58, and pXW96; Fig. 4) synthesized a polypeptide with an apparent M_r of 50,000. This polypeptide band was not detectable in clones pXW59 and pXW106 (Fig. 4) lacking the lytic gene. Also, maxicells containing the vector alone did not show the polypeptide of M_r ~50,000, suggesting that this polypeptide is a product of the *lytA* gene. From these results, it seems that the polypeptide band with an M_r of ~23,000 observed in the earlier study (6) was most probably a degraded product of the *lytA* gene.

Chromosomal localization of the *lytA* gene. In an effort to map the *lytA* gene on the *S. aureus* chromosome, DNA from various derivatives of strain NCTC 8325 was digested with *Sma*I, *Csp*I, *Sgr*AI, and *Asc*I (10), resolved by pulsed-field agarose gel electrophoresis, transferred to a nylon membrane, and probed with biotinylated *lytA* DNA. The probe hybridized with strain NCTC 8325, which carries prophages φ11, φ12, and φ13, and with single lysogens carrying prophages φ11 and φ13 but not with strain 8325 4 (RN-450), which is cured of the prophages (9), or with a single lysogen carrying prophage φ12 when single lysogens were tested. Hybridization of the probe with phage φ13 is probably due to cross-reactivity, as both phages are from serogroup B. The

probe hybridized to the *Sma*I F, *Csp*I A, *Sgr*AI D, and *Asc*I E fragments known to harbor prophage φ11. The probe also hybridized strongly to phage φ11 DNA. These observations strongly indicate that the peptidoglycan hydrolase gene originated from prophage φ11 in strain NCTC 8325.

The nucleotide sequences of a number of *Streptococcus pneumoniae* bacterial and bacteriophage peptidoglycan hydrolases have been determined (2). Significant homologies in the C-terminal regions of genes specifying peptidoglycan hydrolases with different bond specificities have been observed. On the basis of this observation, a modular organization for these genes, with the cell wall-binding domain residing in the C-terminal region and the active site in the N-terminal region, has been proposed. We have noted previously the homology between the C-terminal regions of the *lytA* gene and lysostaphin, which have different peptidoglycan bond specificities (3, 11, 19). By analogy with the *S. pneumoniae* peptidoglycan hydrolases, the cell wall-binding region of the LytA protein also may reside in the C-terminal region.

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