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Biochemistry

Cloning, sequence, and expression of the lysostaphin gene from *Staphylococcus simulans*

(preproenzyme/tandem repeats/extracellular processing/bacteriocin/plasmid-encoded)

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ABSTRACT **A 1.5-kilobase-pair fragment of DNA that contains the lysostaphin gene from *Staphylococcus simulans* and its flanking sequences has been cloned and completely sequenced. The gene encodes a preproenzyme of M_r 42,000. The NH_2-terminal sequence of the preproenzyme is composed of a signal peptide followed by seven tandem repeats of a 13-amino acid sequence. Conversion of prolysostaphin to the mature enzyme occurs extracellularly in cultures of *S. simulans* and involves removal of the NH_2-terminal portion of the proenzyme that contains the tandem repeats. The high degree of homology of the repeats suggests that they have arisen by duplication of a 39-base-pair sequence of DNA. In *S. simulans*, the lysostaphin gene is present on a large β-lactamase plasmid.**

Lysostaphin is a cell wall-degrading enzyme secreted by a single known strain of *Staphylococcus simulans* (NRRL B-2628) isolated by Schindler and Schuhardt (1, 2). The enzyme lyses practically all known staphylococcal species but it is inactive against bacteria of all other genera (1, 3, 4). Although its catalytic properties are not well-characterized, lysostaphin apparently hydrolyzes polyglycine cross-links present in the peptidoglycan of the staphylococcal cell wall (5). The enzyme is a monomer of $M_r \approx 25,000$ and is reported to contain zinc (6). Lysostaphin production occurs in stationary-phase cultures grown under certain conditions and appears to be coordinated with production of other extracellular enzymes, including a protease and a hexosaminidase (7). Producing cultures are resistant to the enzyme, while cultures grown under at least some nonproducing conditions are sensitive (8). It is not clear whether resistance to lysostaphin in *S. simulans* results from the action of an immunity product(s) or whether it occurs naturally under certain conditions. Alterations in sensitivity to the enzyme may be due to changes in the amino acid composition of the peptidoglycan (8, 9).

In this paper, we show that the lysostaphin gene is present on a large penicillinase plasmid and encodes a preproenzyme of $M_r \approx 42,000$. Conversion of prolysostaphin to the mature enzyme occurs extracellularly in cultures of *S. simulans* and involves removal of the NH_2-terminal portion of the proenzyme, which contains seven tandem repeats of a 13-amino acid sequence.

MATERIALS AND METHODS

Materials. Restriction enzymes, T4 DNA ligase, *Escherichia coli* DNA polymerase, and ribonuclease were from Boehringer Mannheim; M13 pentadecamer primer was from New England Biolabs; goat antibodies to rabbit IgG were from Miles–Yeda (Rehovot, Israel); calf intestine alkaline phosphatase and 5-bromo-4-chloroindolyl phosphate were from Sigma; and lysostaphin was from Mead Johnson.

Preparation of DNA. *S. simulans* grown to midlogarithmic phase on 0.5 liter of CAA medium (8) was harvested by centrifugation, washed with 50 mM Tris·HCl/50 mM EDTA, pH 7.8, and suspended in 100 ml of this buffer containing lysostaphin (50 μg/ml) and lysozyme (0.5 mg/ml). After 2 hr at 37°C, Pronase (1 mg/ml) and $NaDodSO_4$ (0.6%) were added and the suspension was incubated for 2 hr at 37°C. The lysate was then extracted twice with an equal volume of phenol. Nucleic acid was precipitated by addition of 2 vol of ethanol, collected by centrifugation, dissolved in 10 ml of TE (10 mM Tris·HCl/1 mM EDTA, pH 8.0), and digested with pancreatic RNase (30 μg/ml) and T1 RNase (2 units/ml) for 2 hr at 37°C. The DNA was precipitated with ethanol and dissolved in TE. The yield was 1.5 mg. Chromosomal and plasmid DNA were obtained by CsCl density-gradient centrifugation. Plasmid DNA was isolated from *E. coli* by alkaline $NaDodSO_4$ extraction of cell lysates (10).

Cloning and DNA Sequencing. Cloning was carried out using pUC8 as the vector and *E. coli* JM105 as the host (11). *S. simulans* DNA was partially digested with *Mbo* I and fractionated by centrifugation through a 12-ml 10–30% sucrose gradient at 35,000 rpm for 20 hr. Fragments (10 μg) from 5 to 15 kilobase pairs (kbp) were pooled and ligated to *Bam*HI-digested pUC8 (2 μg). About 80% of the transformants obtained using the ligated DNA contained recombinant plasmids, as indicated by inactivation of *lacZ'*, the truncated β-galactosidase gene from *E. coli* present on the pUC plasmids. DNA sequences were determined by the dideoxy-chain-termination method (12) using the phage vectors M13mp10 and M13mp11 (13).

Lysostaphin Assays. *Staphylococcus aureus* RN492 (14), a constitutive β-lactamase producer that is relatively resistant to ampicillin, was used as the indicator strain. *E. coli* colonies grown on L agar containing ampicillin (50 μg/ml) were exposed to chloroform vapor for 30 min and overlaid with GL top agar (15) containing a 0.1% (vol/vol) suspension of *S. aureus* RN492 that had been grown to stationary phase on CY medium (15). Liquid samples (5 μl) were added to wells in 1% agarose containing 0.1 M NaCl and 0.05 M potassium phosphate (pH 7.2) and were overlaid in the same way. The area of cell lysis was proportional to the amount of lysostaphin in the range from 1 to 500 ng.

Immunoblots. Rabbit antibodies to lysostaphin were prepared and purified by affinity chromatography as described (16). Goat antibodies to rabbit IgG were cross-linked to alkaline phosphatase with glutaraldehyde (17). *E. coli* JM105 (pRG5) cells grown to late-logarithmic phase in 20 ml of LB medium (18) containing ampicillin (50 μg/ml) were harvested by centrifugation, washed with 10 mM Tris·HCl/30 mM

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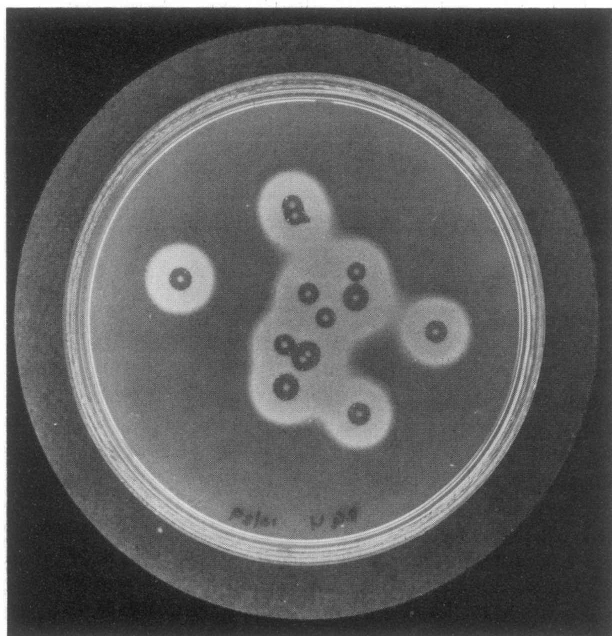

FIG. 1. Lysis of *S. aureus* by *E. coli* JM105 (pRG5) grown on ampicillin plates. *E. coli* colonies degrade ampicillin and permit staphylococcal cells to grow around the colonies. Clear zones at the center of the rings of staphylococcal cells are due to the production of lysostaphin by the *E. coli* colonies.

NaCl, pH 8.0, suspended in 1 ml of this buffer, and sonicated for 2 min at 0°C using a Branson S 125 sonicator. The culture supernatant was concentrated 20-fold by ultrafiltration using an Amicon YM-10 membrane. Samples were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis (19) and transferred to nitrocellulose at 1.5 A for 2 hr. Immunoreactive proteins were detected as described by Blake *et al.* (20).

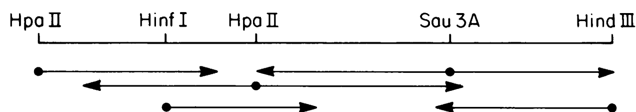

FIG. 2. Strategy for sequencing the 1.5-kbp *Hpa* II/*Hin*dIII fragment containing the lysostaphin gene from *S. simulans*. Only the restriction sites used for sequencing are shown. Arrows indicate the direction and length of determined sequences. Greater than 95% of the sequence was determined from both strands.

Southern Blots. DNA was fractionated by electrophoresis on 1% agarose in 50 mM Tris·HCl/50 mM boric acid/1 mM EDTA, pH 8.0, at 80 V for 4 hr and transferred to nitrocellulose (21). Filters were prehybridized at 65°C for 2 hr in a solution containing 0.8 M NaCl, 0.08 M sodium citrate, 0.5% $NaDodSO_4$, denatured salmon sperm DNA (50 μg/ml), and 5× Denhardt's solution (22). Filters were hybridized at 65°C for 16 hr with 2×10^6 cpm of ^{32}P-labeled probe (23), washed as described (24), and exposed to Fuji RX film for 24 hr at −70°C.

Protein Sequencing. Automated Edman degradation was performed on a Beckman 890C liquid-phase sequencer. Phenylthiohydantoin-derivatized amino acids were identified by high-pressure liquid chromatography as described by Tarr (25).

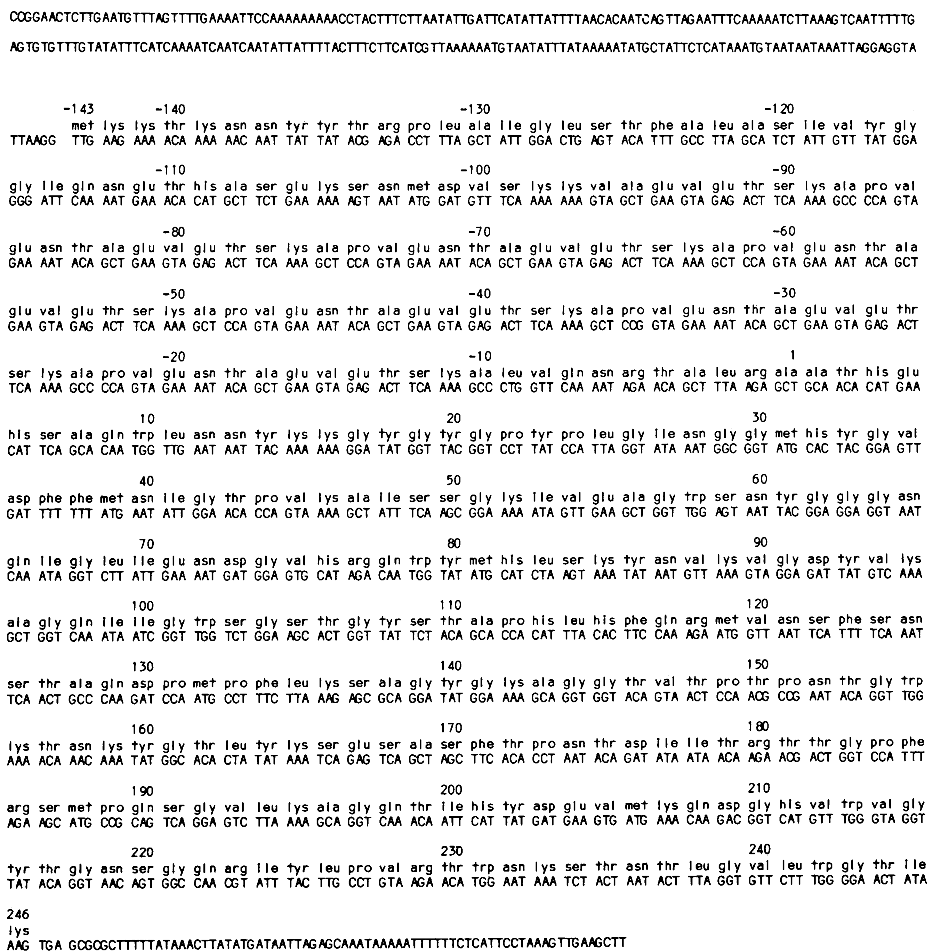

FIG. 3. Nucleotide sequence of the lysostaphin gene from *S. simulans* and amino acid sequence of the encoded gene product. The NH_2-terminal formylmethionine of preprolysostaphin is designated as residue −143 and the NH_2-terminal alanine of mature lysostaphin is designated as residue +1.

RESULTS

Isolation of Lysostaphin-Producing Clones of *E. coli*. *S. simulans* DNA was partially digested with *Mbo* I and fragments with an average size of 10 kbp were isolated by sucrose gradient centrifugation and ligated to *Bam*HI-digested pUC8 (11). The ligated DNA was used to transform *E. coli* JM105 and ampicillin-resistant colonies were overlaid with a suspension of *S. aureus* RN492 to screen for lysostaphin production. Nine of ≈1000 *E. coli* clones containing recombinant plasmids (amp^+, $lacZ'^-$) were lysostaphin producers (Fig. 1). Assuming that the *S. simulans* chromosome is ≈2000 kbp (26), the probability of cloning a chromosomal gene at this frequency is ≈0.4 (27).

Restriction Analysis, Subcloning, and Sequencing of the Lysostaphin Gene. Lysostaphin-producing transformants contained recombinant plasmids with inserts of either 6.0, 6.5, or 8.0 kbp. Restriction analysis showed that these inserts were present in either orientation with respect to the vector and contained a 4.3-kbp fragment in common. The lysostaphin gene was localized within a 1.5-kbp *Hpa* II/*Hin*dIII fragment, which appeared to contain a promoter that was functional in *E. coli*, since clones containing the insert in either orientation with respect to the *lacZ'* gene (i.e., in either pUC8 or pUC9) produced similar amounts of enzyme. The nucleotide sequence of the cloned DNA was determined as shown in Fig. 2 and is given in Fig. 3. A 1167-nucleotide open reading frame extends from a UUG initiation codon (nucleotides 245–247) to a UGA termination codon (nucleotides 1412–1414). The inferred ribosome-binding site AGGAGGU (nucleotides 231–237) is separated from the initiation codon by 7 bp and shows complete complementarity to the postulated mRNA binding site of the 16S ribosomal RNA (28). A presumed promoter with −35 and −10 regions at nucleotides 89–95 and 110–119, respectively, is highly homologous to σ^{37} promoters from *Bacillus subtilis* (29). No additional open reading frames are present in the cloned fragment.

The Lysostaphin Gene Encodes a Preproenzyme. The lysostaphin gene encodes a protein composed of 389 amino acids with M_r 42,205 (Table 1). The NH_2-terminal sequence of the encoded product contains a cluster of four positively charged residues followed by an uncharged largely hydrophobic sequence and, therefore, has the properties characteristic of a signal peptide (30). The Ala-Ser bond at position −121 and −120 or that at position −108 and −107 (Fig. 4) is the likely signal cleavage site. The sequence from alanine −95 through arginine −5 is composed of seven tandem repeats of a 13-amino acid sequence (Fig. 4). The first six of these repeats are identical, while the seventh contains 3 amino acid substitutions. This portion of the molecule is highly ionic (31% of the residues are acidic or basic) and has a net negative charge. The 39-bp nucleotide sequences that encode these repeats are also highly homologous (Fig. 4). Repeats two,

Table 1. Predicted and observed amino acid composition of preprolysostaphin and lysostaphin

Amino acid	Preprolysostaphin predicted	Lysostaphin	
		Predicted	Observed*
Ala	31	12	12
Arg	9	6	6
Asn	27	16	
(Asx)	(35)	(23)	(24)
Asp	8	7	
Cys	—	—	—
Gln	12	10	
(Glx)	(39)	(15)	(15)
Glu	27	5	
Gly	38	35	33
His	10	9	9
Ile	16	13	12
Leu	16	11	11
Lys	29	16	16
Met	9	7	7
Phe	8	7	7
Pro	19	12	13
Ser	31	19	21
Thr	40	22	22
Trp	8	8	5
Tyr	19	16	15
Val	32	15	15
Total residues	389	246	
M_r	42,205	26,921	

Values are expressed as mol per mol of enzyme.
*Data from Trayer and Buckley (6).

					f-MET$_{-143}$	LYS	LYS	THR	LYS	ASN	ASN	TYR	TYR
A	THR	ARG	PRO	LEU	ALA	ILE	GLY	LEU	SER	THR	PHE	ALA	LEU
	ALA	SER	ILE	VAL	TYR	GLY	GLY	ILE	GLN	ASN	GLU	THR	HIS
	ALA	SER	GLU	LYS	SER	ASN	MET	ASP	VAL	SER	LYS	LYS	VAL
1	ALA$_{-95}$	GLU	VAL	GLU	THR	SER	LYS	ALA	PRO	VAL	GLU	ASN	THR
2	ALA	GLU	VAL	GLU	THR	SER	LYS	ALA	PRO	VAL	GLU	ASN	THR
3	ALA	GLU	VAL	GLU	THR	SER	LYS	ALA	PRO	VAL	GLU	ASN	THR
4	ALA	GLU	VAL	GLU	THR	SER	LYS	ALA	PRO	VAL	GLU	ASN	THR
5	ALA	GLU	VAL	GLU	THR	SER	LYS	ALA	PRO	VAL	GLU	ASN	THR
6	ALA	GLU	VAL	GLU	THR	SER	LYS	ALA	PRO	VAL	GLU	ASN	THR
7	ALA	GLU	VAL	GLU	THR	SER	LYS	ALA	LEU	VAL	GLN	ASN	ARG$_{-5}$
	THR	ALA	LEU	ARG$_{-1}$	ALA$_1$	ALA	THR	HIS	GLU	HIS	SER	ALA	GLN

B 1	GCT	GAA	GTA	GAG	ACT	TCA	AAA	GCC	CCA	GTA	GAA	AAT	ACA
2	GCT	GAA	GTA	GAG	ACT	TCA	AAA	GCT	CCA	GTA	GAA	AAT	ACA
3	GCT	GAA	GTA	GAG	ACT	TCA	AAA	GCT	CCA	GTA	GAA	AAT	ACA
4	GCT	GAA	GTA	GAG	ACT	TCA	AAA	GCT	CCA	GTA	GAA	AAT	ACA
5	GCT	GAA	GTA	GAG	ACT	TCA	AAA	GCT	CCG	GTA	GAA	AAT	ACA
6	GCT	GAA	GTA	GAG	ACT	TCA	AAA	GCC	CCA	GTA	GAA	AAT	ACA
7	GCT	GAA	GTA	GAG	ACT	TCA	AAA	GCC	CTG	GTT	CAA	AAT	AGA$_{661}$

FIG. 4. Repeated sequences of preprolysostaphin (*A*) and of the lysostaphin gene (*B*). The NH_2-terminal sequence of preprolysostaphin is shown from formylmethionine −143 through glutamine +9. The proenzyme cleavage site is the bond between arginine −1 and alanine +1. The repeated nucleotide sequence extends from bp 389 to 661 of the sequence shown in Fig. 3. Repeat numbers are given on the left.

three, and four are identical; repeats one and six (which are identical) and five contain a single substitution in the third base of a codon with no effect on the amino acid coded; and repeat seven contains six substitutions.

The NH_2-terminal sequence of lysostaphin as determined by Edman degradation (data not shown) is Ala-Ala-Thr-His-Glu, corresponding to residues 1–5 of the encoded gene product (Fig. 4). Predicted values for the amino acid composition and molecular weight of the enzyme, calculated assuming that the lysostaphin sequence extends from Ala-1 to the COOH-terminal Lys-246 of the encoded product, are in excellent agreement with those determined experimentally (6), as shown in Table 1. The only significant difference is in tryptophan content, which is not unexpected, however, as the reaction used for tryptophan determination is often not quantitative (31). The mature enzyme has M_r 26,921 as calculated from the sequence, contains no cysteine, and has more basic and amidated residues (13% and 11% of the total, respectively) than acidic residues (5% of the total).

Synthesis of Precursor and Mature Forms of Lysostaphin in *E. coli* and *S. simulans*. Late-logarithmic-phase cultures of *E. coli* JM105 containing pRG5 (the recombinant plasmid containing the 1.5-kbp *Hpa* II/*Hin*dIII fragment of *S. simulans* DNA cloned into the *Acc* I/*Hin*dIII sites of pUC8) show lysostaphin activity in the supernatant, periplasmic, and cytoplasmic fractions (65%, 15%, and 20%, respectively, of the total of 3 μg of enzyme per ml). Protein blotting using affinity-purified antibodies to lysostaphin showed the presence of the mature enzyme in the supernatant (Fig. 5, lane 8). The *E. coli* cellular fraction contains smaller amounts of the mature enzyme and larger amounts of a cross-reactive protein with an apparent M_r of ≈64,000 (lane 7). A cross-reactive protein with identical electrophoretic mobility is also present in the supernatant of lysostaphin-producing cultures of *S. simulans* (lane 6). This protein is most likely prolysostaphin since it precedes lysostaphin in appearance and then disappears as lysostaphin accumulates (lanes 1–4). It is not present in nonproducing cultures of *S. simulans* or *E. coli*.

Lysostaphin Is Plasmid-Encoded. *S. simulans* contains several plasmids, including a large plasmid of ≈40 kbp (Fig. 6, lane 2). DNA blot analysis using the cloned lysostaphin gene as probe showed that the gene is present on the large plasmid (lane 3). *S. simulans* is resistant to penicillin (2), a trait that is often associated with large plasmids in *S. aureus* (32). Hybridization with a restriction fragment derived from the β-lactamase gene of the *S. aureus* plasmid pI258 (14) showed that the large *S. simulans* plasmid also carries a β-lactamase determinant.

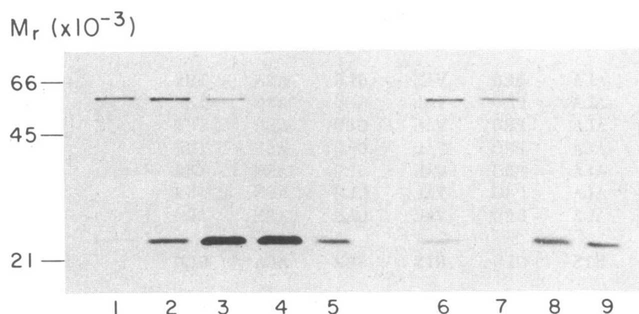

FIG. 5. Immunological detection of lysostaphin and prolysostaphin in *S. simulans* and *E. coli* (pRG5). Samples were fractionated and detected as described. Supernatants from *S. simulans* cultures were taken at late-logarithmic (lanes 1 and 6), early-stationary (lane 2), mid-stationary (lane 3), and late-stationary (lane 4) phase. *E. coli* supernatant (lane 8) and cell extract (lane 7) fractions were prepared from late-logarithmic phase cultures. Lysostaphin was applied to lanes 5 and 9. Positions of molecular weight standards are shown on the left. No reaction was observed when preimmune serum was used in place of antibodies to lysostaphin.

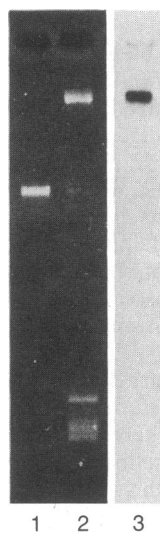

FIG. 6. Agarose gel electrophoresis of chromosomal (lane 1) and plasmid (lane 2) DNA from *S. simulans* photographed after staining with ethidium bromide. The DNA was transferred to nitrocellulose and hybridized with the 1.5-kbp *Hpa* II/*Hin*dIII fragment, which contains the lysostaphin gene (lane 3). A similar result was obtained when the 840-bp *Xba* I/*Hin*dIII fragment from the β-lactamase gene of the *S. aureus* plasmid pI258 (14) was used as the probe.

DISCUSSION

The lysostaphin gene from *S. simulans* encodes a preproenzyme of M_r 42,205 with an NH_2-terminal sequence that is composed of a signal peptide followed by seven tandem repeats of a 13-amino acid sequence. Conversion of the proenzyme to the mature enzyme involves cleavage of the bond between arginine −1 and alanine +1 with removal of the NH_2-terminal portion of the proenzyme, which contains the repeated sequences (Fig. 4). Immunoblot analysis using antibodies to lysostaphin shows that a cross-reactive protein with an apparent M_r of ≈64,000 accumulates in the supernatant of early stationary-phase cultures of *S. simulans* and then disappears as lysostaphin accumulates. Evidence that this cross-reactive protein is in fact prolysostaphin has been obtained by showing that the purified protein is converted to mature lysostaphin *in vitro* (R. Zhou and P.A.R., unpublished results). Overestimation of the molecular weight of prolysostaphin by $NaDodSO_4$/polyacrylamide gel electrophoresis is probably the result of below-average binding of $NaDodSO_4$ to the protein because of the high content of glutamyl residues in the tandem repeats. It is known, for example, that esterification of single glutamyl residues of the *E. coli* chemotaxis proteins results in an increase in migration rate on $NaDodSO_4$/polyacrylamide gels (33). The high degree of homology of the repeats suggests that they have arisen by duplication of a 39-bp sequence of DNA. Their role, if any, remains to be established. It is interesting to note that the mature forms of two proteins associated with the cell envelope of Gram-positive bacteria, protein A from *S. aureus* (34), and M protein from *Streptococcus pyogenes* (35), also contain tandemly repeated peptides. The use of UUG as an initiation codon, described here for the lysostaphin gene, has also been observed for several other staphylococcal genes (36). Alkaline protease and neutral protease from *B. subtilis* (29, 37, 38) and *Bacillus amyloliquefaciens* (39, 40) are also synthesized as preproenzymes. While the *Bacillus* proproteases differ from prolysostaphin in structural organization and do not contain repeated sequences, the proenzyme cleavage sites of these proteins are similar.

Lysostaphin and other bacterial proteins with bactericidal activity are known as bacteriocins (41). Plasmids of various sizes have been associated with bacteriocin production and immunity in *Staphylococcus* (42–44). Additional studies will be required to determine whether the lysostaphin plasmid encodes an immunity product(s) that protects the host against

lysostaphin. Hybridization analyses show that the β-lactamase determinants from *S. aureus* and *S. simulans* are homologous, and it seems likely that the lysostaphin plasmid will show other similarities to the *S. aureus* β-lactamase plasmids. Lysostaphin itself may be related to autolytic enzymes, which appear to be widely distributed in staphylococci (45). Although little is known about their properties, these enzymes are apparently associated with the cell wall of the producing organism and may play an essential role in reshaping the wall during cell growth and division.

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