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Sequence Determination and Comparison of the Exfoliative Toxin A and Toxin B Genes from *Staphylococcus aureus*†

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The DNA encoding the exfoliative toxin A gene (*eta*) of *Staphylococcus aureus* was cloned into bacteriophage λgt11 and subsequently into plasmid pLI50 on a 1,391-base-pair DNA fragment of the chromosome. Exfoliative toxin A is expressed in the *Escherichia coli* genetic background, is similar in length to the toxin purified from culture medium, and is biologically active in an animal assay. The nucleotide sequence of the DNA fragment containing the gene was determined. The protein deduced from the nucleotide sequence is a polypeptide of 280 amino acids. The mature protein is 242 amino acids. The DNA sequence of the exfoliative toxin B gene was also determined. Corrections indicate that the amino acid sequence of exfoliative toxin B is in accord with chemical sequence data.

The exfoliative toxins A and B (ETA and ETB) of *Staphylococcus aureus* are the causative agents of staphylococcal scalded-skin syndrome (11). They possess the same biological activity (12), but they are immunologically distinct proteins (11, 12). The two forms also differ in amino acid composition, amino acid sequence, and heat resistance (13). In addition, *eta* is expressed from the chromosome, whereas *etb* is of plasmid origin (14, 21, 29).

To study the mode of action and the molecular properties of these two proteins, we set out to clone and sequence both genes. Recently, we reported the cloning and sequencing of the *etb* gene (9, 10). In this communication, we report the cloning and DNA sequence of *eta*. O'Toole and Foster (17) also reported the cloning of *eta* and in the accompanying paper (18) report their sequence of the gene. Our data and their report contain identical DNA sequence determinations as well as general conclusions regarding the expression of the *eta* gene. *eta* is expressed in the *Escherichia coli* background and is biologically active in the neonatal mouse assay. Furthermore, the sequence of the protein deduced from the DNA sequence is identical to the published sequence of the N-terminal region of the ETA molecule (12).

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. aureus* UT0002, a phage group II staphylococcal scalded-skin syndrome clinical isolate (20), was used as the source of library DNA. *E. coli* Y1090 was used in the Protoclone system (Promega Biotec Co., Madison, Wis.) as a recipient for bacteriophage λgt11 packaging carried out according to the manufacturer's instructions. *E. coli* LE392 was used for transformation and propagation of plasmids and cloned DNA (16).

Media and chemicals. Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) was used for routine cultivation of *S. aureus* strains. L broth was used for cultivation of *E. coli*. Solid medium consisted of broth supplemented with 1.5% agar (Difco Laboratories, Detroit, Mich.). Chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Restriction enzymes, bacteriophage T4 DNA ligase, BAL 31 exonuclease, *Eco*RI linker, and *Eco*RI methylase were obtained from New England BioLabs Inc., Beverly, Mass., and Bethesda Research Laboratories, Inc., Gaithersburg, Md. The large fragment of DNA polymerase (Klenow) was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Reagents for packaging were purchased from Promega Biotec. Adenosine 5′-(α-[^{35}S]thio)triphosphate (800 Ci/mmol) was from New England Nuclear Corp., Boston, Mass. Na^{125}I (13 to 17 Ci/g of I) for protein iodination was from Amersham Corp., Arlington Heights, Ill.

Plasmid and bulk chromosomal DNA purification. Plasmid DNA purification was performed by the procedure of Birnboim (2) and further purified by CsCl-ethidium bromide density gradient centrifugation. Bulk chromosomal DNA was purified by the method of Dyer and Iandolo (3).

Western blot. Cell lysates of *E. coli* containing the cloned *eta* gene were generated by sonication and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing and reducing conditions (27). Detection of proteins blotted to nitrocellulose paper with antiserum and ^{125}I-protein A was as described previously (27). Antiserum to highly purified ETA was prepared in rabbits (12).

Construction of bacteriophage λgt11 libraries. Bulk chromosomal DNA prepared from *S. aureus* UT0002 was used to construct a λgt11 library (31). The library was screened with a rabbit antiserum to ETA that had been absorbed repeatedly with lysates of the *E. coli* host strain. The plaques were blotted to nitrocellulose membranes and incubated with antibody. Those plaques binding antibody were identified by reaction with ^{125}I-labeled protein A, and ETA production was confirmed by Western blotting of lysates of small cultures. DNA containing the *eta* gene was recloned into the

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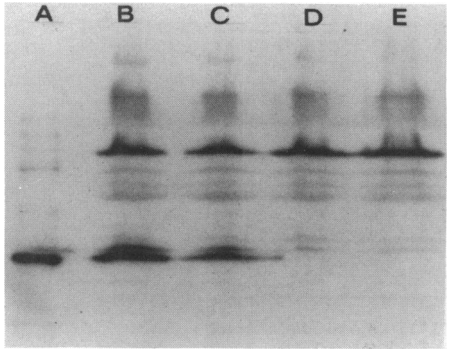

FIG. 1. Immunoblot analysis of cellular extracts of *E. coli* containing cloned staphylococcal DNA fragments expressing ETA. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel was blotted to nitrocellulose and probed with rabbit antiserum to ETA. Lanes: A, ETA purified from *S. aureus*: B, extract of *E. coli* LE392 (pLI50 + 3.2-kilobase insert); C, extract of *E. coli* LE392(pLI50 + 1,391-bp insert); D; extract of *E. coli* LE392(pLI50); E, extract of *E. coli* LE392.

shuttle vector pL150 (16). The recombinant plasmid was subsequently subjected to deletion analysis by digestion at unique restriction sites flanking the insert DNA. The linear molecules were then further digested with BAL 31 exonuclease to obtain deletions of various length from either end of the DNA fragment containing the *eta* gene (16). The digests were then tailed with *Bam*HI linkers, ligated with T4 DNA ligase, and transformed into competent LE392 (16). The plasmid was also digested with restriction enzymes to delete specific sections of DNA and then ligated.

DNA sequence analysis. Various restriction endonuclease fragments of the *eta* gene were subcloned into the M13 bacteriophage derivatives mp18 and mp19 (16) and propagated in *E. coli* JM103. DNA sequencing was carried out by the dideoxy chain termination method of Sanger et al. (23).

Assay for biological activity of ETA. ETA-positive clones of *E. coli* were grown for 24 h in 100 ml of L broth, and the cells were suspended in 2 ml of 0.9% NaCl. After sonic disruption of the cells, debris was removed by centrifugation. These lysates were then assayed for exfoliating activity in neonatal mice (9).

Chemical sequence of toxins. Lyophilized salt-free toxins were dissolved in 70% (vol/vol) formic acid and reacted with cyanogen bromide (5) for 18 to 22 h at room temperature. After removal of excess reagent by repeated lyophilization, the products were dissolved in a small volume of 70% formic acid and chromatographed on columns of Sephadex G-75 (superfine grade; Pharmacia Fine Chemicals, Piscataway, N.J.). Appropriate fractions were pooled and rechromatographed. Amino acid sequences were obtained with a Beckman model 890C automatic sequencer (Beckman Instruments, Inc., Berkeley, Calif.). Run conditions and identification procedures have been described previously (24).

RESULTS

Cloning of *eta*. We cloned the *eta* gene in the λgt11 expression vector (31). The λgt11 library of more than 4×10^4 recombinant phage was screened for production of ETA. Five clones were found that reacted positively with rabbit antiserum to ETA. One recombinant phage was arbitrarily selected for further manipulation. DNA isolated from this phage contained a 3.2-kilobase *Eco*RI restriction enzyme insert of staphylococcal DNA. The 3.2-kilobase *Eco*RI insert was recloned into the shuttle vector pL150 and transformed into *E. coli* LE392.

Immunoblots confirmed that this plasmid contained the *eta* structural gene. ETA produced by *E. coli* LE392 was identical in size to the ETA from *S. aureus* (Fig. 1, lane B), indicating that the plasmid contained the *eta* structural gene and not a fusion product. The upper band in this gel lane is an *E. coli* cross-reactive protein unrelated to ETA. Toxin from lysates of this clone were injected subdermally into neonatal mice (9), and a positive Nikolsky sign was detected after overnight incubation (data not shown).

To localize the *eta* gene, various plasmids containing deletions at either end of the 3.2-kilobase fragment were generated. The deleted plasmids contained in *E. coli* LE392 were tested for their ability to produce ETA; one (Fig. 1, lane C) localized the *eta* gene to within a 1,391-base-pair (bp) DNA fragment.

The nucleotide sequence of *eta* is shown in Fig. 2. The entire *eta* reading frame was sequenced on both strands (Fig. 2) and is shown relative to the entire 1,391-bp fragment. There was only one large open reading frame, extending from residues 313 to 1153, that could code for a polypeptide similar in length to that reported for ETA (11, 12).

The G+C content of *eta* was 31%, which is typical of the *S. aureus* genome (16). However, the G+C content of the 150-bp sequence upstream from the methionine start codon (nucleotide 313) was even lower in G+C content (19%), suggesting that this region could serve as the potential binding sites for RNA polymerase to initiate transcription. A potential −35 sequence and a −10 sequence that could serve as promoter regions (6) were identified (Fig. 2). Furthermore, the probable ATG methionine initiation codon (26a) is preceded 6 bp upstream by the sequence GGATGA, which qualifies as a potential ribosome binding site (25).

A potential stem-loop structure can be formed from nucleotides 1232 to 1259 (Fig. 2). This stem-loop structure was 79 bp distal to the stop codon of the *eta* gene and was followed by a potential mRNA stop sequence (7). Whether any of the proposed regulatory sequences is functional either in vivo or in vitro remains to be determined. Codon usage of the ETA gene was compared with many highly expressed *E. coli* genes (data not shown). In general, the preferential codon usage for the ETA gene differed from the genes that are highly expressed in *E. coli* (4). This difference has also been observed in genes for other staphylococcal proteins, such as ETB and glycerol ester hydrolase (*geh*) (16). The difference in G+C content between *S. aureus* and *E. coli* may account for these discrepancies.

Translation of the open reading frame identified from the DNA sequence yielded a 280-amino-acid polypeptide that corresponded to published properties of ETA (12). A 38-amino-acid signal peptide precedes the N terminus of the mature ETA protein. The peptidase cleavage site occurs immediately after the sequence Ala-Lys-Ala of residues 36 to 38. This cleavage site agrees with the accepted cleavage sites of extracellular proteins with known signal peptides (28). Removal of the signal peptide results in a mature ETA protein containing 242 amino acid residues with a molecular weight of 26,950. This value compares favorably with the molecular weight determined by chemical and physical methods (12) and is identical to that reported by O'Toole and Foster (18).

The amino acid sequence deduced from the DNA was compared with that determined by chemical methods (12). The first 44 residues of mature ETA were determined by

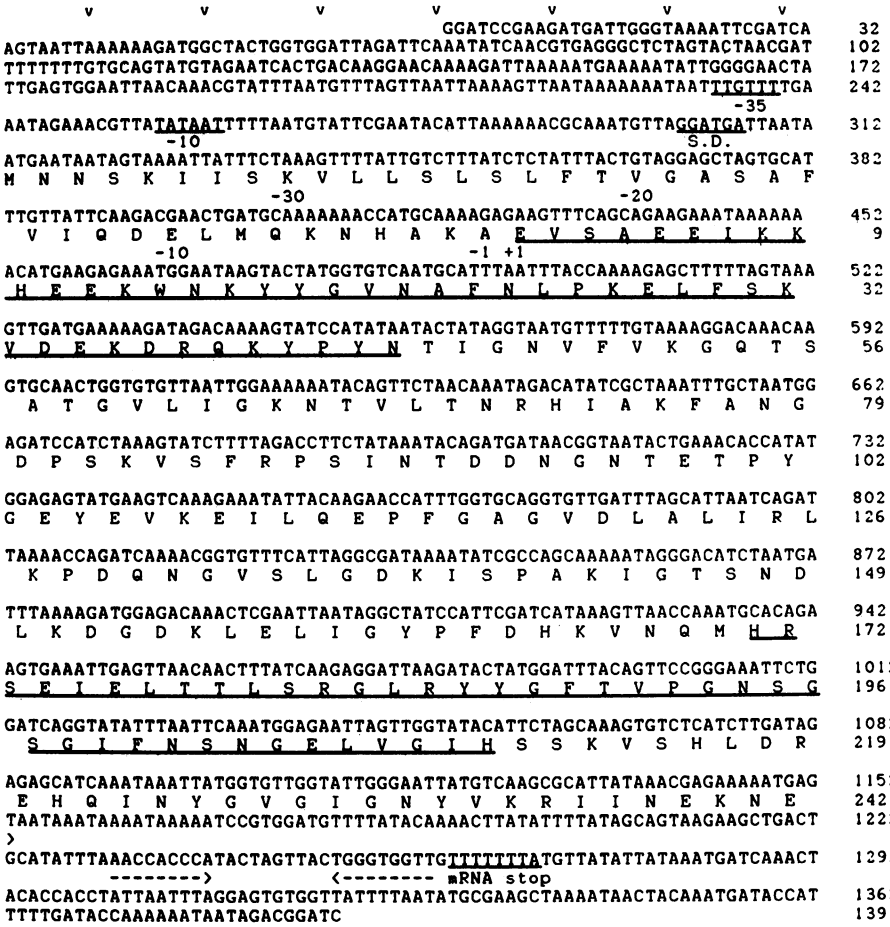

FIG. 2. Sequence of the 1,391-bp DNA fragment containing the *eta* gene and sequence of ETA derived from it. The locations of the presumptive −35, −10, Shine-Dalgarno ribosome binding site (SD), −1, +1 protease processing site, stem-loop termination structure (facing arrows), the stop codon (>), the mRNA transcription stop site, and the chemically derived peptide sequence (underlined) are indicated.

automated Edman degradation and agreed completely with the DNA-derived protein sequence (Fig. 2, underlined residues +1 to 40). A cyanogen bromide peptide that contained the original C-terminus of ETA was also purified and sequenced by automated Edman degradation. The first 40 residues of this peptide were identified, and an exact match with the corresponding region of the DNA-derived structure was obtained (Fig. 2, underlined residues 171 to 210). A contrasting finding was that the C-terminal residue of ETA is glutamic acid rather than lysine. This result was also noted by O'Toole and Foster (18) and may be due to limited proteolysis during protein purification. In particular, our data also confirm the presence of one tryptophan residue at position 14, one internal methionine residue at position 170, and the absence of cysteine.

The amino acid composition of the DNA-derived ETA protein sequence minus the signal peptide (data not shown) agreed well with the composition determined by chemical methods (12). Comparison of the amino acid composition of both ETA and ETB indicates that they are reasonably similar proteins that are rich in polar amino acids.

The DNA sequence and hence the protein sequence of ETB published earlier (10) was not consistent with a partial sequence of ETB determined by chemical methods. In fact, these data indicated that at least two frame shifts and several single-base insertions and deletions were reported. We analyzed the ETB gene and present the corrected sequence in Fig. 3. The newly derived ETB molecule is composed of 277 amino acids with a signal peptide of 31 amino acids and a mature protein length of 246 amino acids. The amino acid composition of ETB agrees quite well with the chemically derived analysis (12). Although a single tryptophan residue was originally reported (12), our protein sequence verifies later wet chemical analyses which indicated that there is no tryptophan present. Furthermore, there is complete agreement between the DNA-derived protein sequence and the chemically derived sequence of the first 40 amino acid residues (+1 to 40, Fig. 3, underlined) and between the DNA-derived sequence and the direct chemical sequence of the first 48 amino acids from a cyanogen bromide peptide fragment of ETB beginning at residue 172 and continuing through residue 219 to the original C terminus of the molecule (Fig. 3, underlined).

Direct comparison of the protein sequences of ETA and ETB is shown in Fig. 4. Three prominent regions of homology are evident, in which the match was extensive. The first occurred in the N-terminal portion of the molecule at positions 46 through 70 (20 of 25 residues match, 80%), the second near the middle at positions 106 through 134 (17 of 29 residues match, 58%), and the third near the C terminus at positions 201 through 221 (17 of 21 residues match, 81%). No other regions of homology were present. The total number of

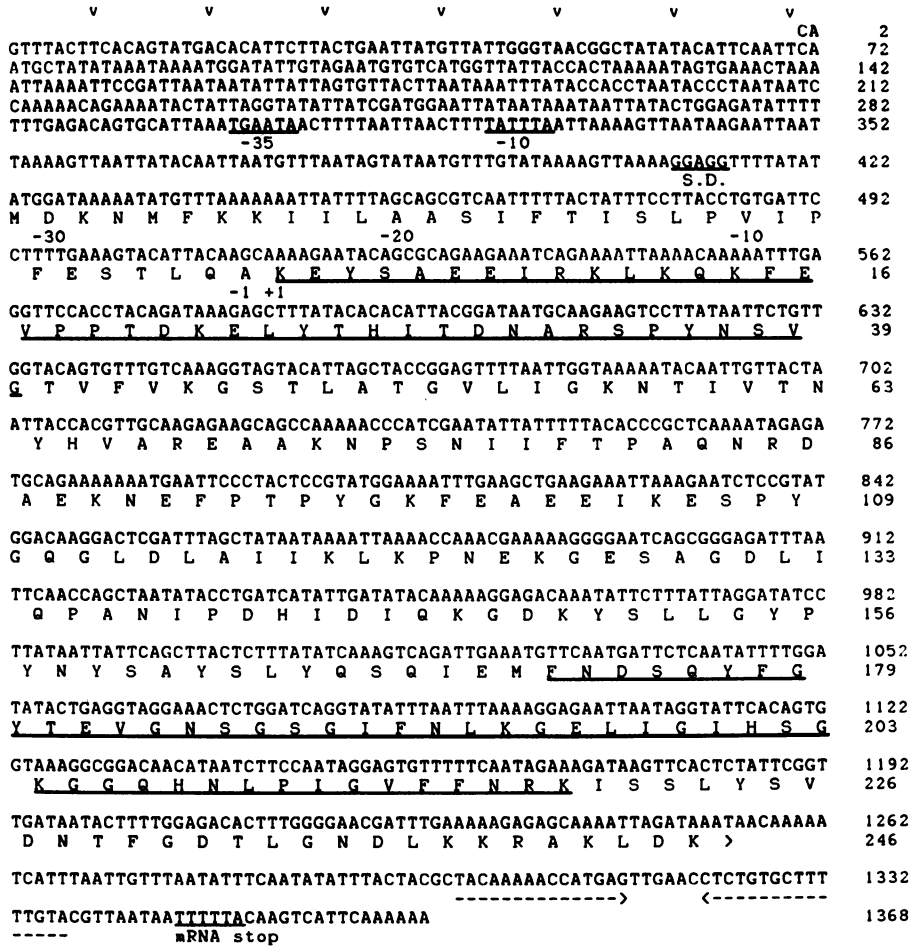

FIG. 3. Sequence of the 1,368-bp DNA fragment containing the *etb* gene and sequence of ETB derived from it. The locations of the presumptive −35, −10, Shine-Dalgarno ribosome binding site (SD), −1, +1 protease processing site, stem-loop termination structure (facing arrows), the stop codon (>), the mRNA transcription stop site, and the chemically derived peptide sequence (underlined) are indicated.

amino acids matched by computer alignment was 110 (45%) out of an average of 245 total residues.

DISCUSSION

The nucleotide sequence of *eta* presented in Fig. 2 has only a single large open reading frame which, based on the following observations, was concluded to code for ETA: (i) the amino acid composition of the deduced toxin agrees closely with that determined by chemical methods, (ii) a peptide comprising the first 44 amino acids of the amino-terminal end and a peptide spanning residues 171 through 242 agree perfectly with that determined from purified toxin by Edman degradation, (iii) the cloned DNA fragment produces exfoliating activity in the *E. coli* background, (iv) deletion analysis identified this region as being required for ETA production, and (v) a ribosome binding site is located 6 nucleotides upstream of the ATG initiation codon.

Surprisingly, *eta* was expressed in *E. coli* but *etb* was not. Many other cloned staphylococcal genes such as lipase (16), staphylokinase (22), nuclease (26), and enterotoxins A (1) and D (unpublished results) are expressed in the *E. coli* genetic background when the genes are cloned with intact promoters. However, in addition to *etb*, the gene encoding enterotoxin B (19) is not transcribed from its own promoter in *E. coli* and must be positioned downstream from a strong gram-negative promoter for expression. There is no promoter preceding the cloning site of *eta* in plasmid pLI50; therefore the cloned gene must contain its own promoter which is recognized by *E. coli* RNA polymerase. The promoter region of *eta* highly corresponds to the canonical *E. coli* sequences (6), whereas for *etb* (Fig. 3) it is difficult to pinpoint transcription signals or proper spacing between −10 and −35 sequences. Enterotoxin B, on the other hand, does have fairly distinct transcription signals but nevertheless does not express in *E. coli* (19). The reasons for these differences are unclear; however, the results define at least two classes of promoter sequences that have variable efficiency with *E. coli* RNA polymerase. Hudson and Stewart (8) recently published a detailed examination of the utilization of staphylococcal promoter sequences and named those that function in gram-positive and gram-negative backgrounds class I and those that only work in gram-positive hosts class II. They speculate on possible reasons for differences in the efficiency of expression of certain staphylococcal genes in *E. coli*, but the question remains open.

We sequenced that *etb* gene from the plasmid pRW001. Our earlier report (10) contained several errors in sequence that have been corrected. The protein derived from the new DNA sequence is in complete accord with chemical se-

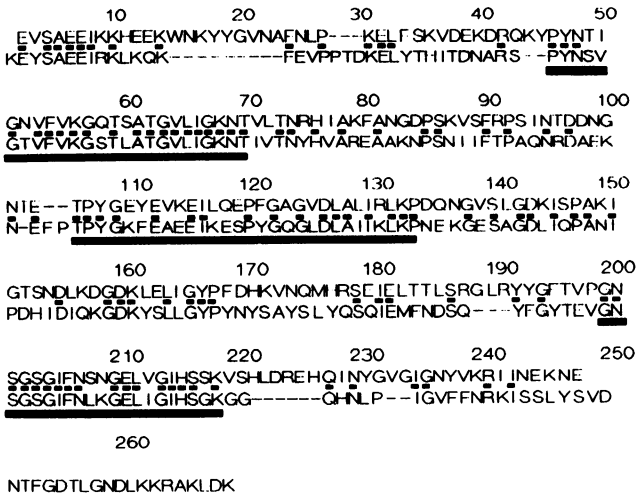

FIG. 4. Comparison of the amino acid sequences of ETA (top row) and ETB (bottom row). Sequence identities are indicated by bars, and dashed lines indicate gaps introduced to produce the optimal alignment. Numbering includes gaps and does not correspond to the residue number obtained from the DNA sequence. Alignment was constructed by computer using the algorithm of Wilbur and Lipman (30) with a K-tuple of 1, window of 20, and gap penalty of 1. Three regions of substantial homology are underlined.

quence data which comprises about 50% of ETB. The mature ETB molecule consists of 246 amino acid residues and has a molecular weight of 27,318, which compares favorably with the value of 26,000 reported previously (13). ETA consists of 242 amino acid residues with a molecular weight of 26,951, which again compares well with the reported value of 26,500 (12). Alignment of the DNA sequences of both toxin genes showed that greater than 50% of the DNA was homologous, but this was not reflected as significantly in the amino acid sequences of the two toxins.

Direct alignment of the protein sequences indicated that significant regions of homology were apparent at the N terminus, the middle, and the C terminus of the toxins. This might not have been predicted because of the lack of antigenic relationship between them (15). Furthermore, comparison of hydropathicity (15) (data not shown) indicates that much of the sequence of each toxin represents highly conserved domains in which amino acid differences are fairly conservative. We interpret this to indicate that folding of the two protein sequences is similar, so that the sites of biological activity, presumably focused at the regions of sequence homology, can be similarly presented to the appropriate substrate.

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