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Genetic and Molecular Analyses of the Gene Encoding Staphylococcal Enterotoxin D†

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The gene (*entD*) encoding staphylococcal enterotoxin D (SED) has been located on a 27.6-kilobase penicillinase plasmid designated pIB485. This plasmid was present in all SED-producing strains tested. The *entD* gene was cloned on a 2.0-kilobase DNA fragment and was expressed in *Escherichia coli*. Sequence analysis of this fragment revealed an open reading frame that encoded a 258-amino-acid protein that possessed a 30-amino-acid signal peptide. The 228-amino-acid mature polypeptide had a molecular weight of 26,360 and contained a high degree of sequence similarity to the other staphylococcal enterotoxins. S1 nuclease mapping showed that transcription of *entD* was initiated 266 nucleotides upstream from the translation start codon. The *entD* gene was also shown to be activated by the staphylococcal regulatory element known as *agr*.

The recent cloning and sequencing of the genes encoding the staphylococcal enterotoxin serotypes A, B, C_1, and E (5, 8, 13, 23) have allowed a comparison of the primary structures of their gene products and the physical locations and carriage of these genes. The proteins are quite similar (13), but carriage of their genes appears to be very diverse. Betley et al. (4a) have shown that the gene (*entA*) encoding staphylococcal enterotoxin A (SEA) is contained on a bacteriophage having at least two integration sites within the staphylococcal chromosome. The gene (*entB*) encoding staphylococcal enterotoxin B (SEB), which has also been associated with a large genetic element within the chromosome (22), is thought to be a defective bacteriophage or an integrated plasmid (22). A large penicillinase plasmid containing *entB* and the gene (*entC*) encoding staphylococcal enterotoxin C_1 (SEC_1) was also reported (1), but this appears to be a unique isolate. Finally, the gene (*entE*) encoding staphylococcal enterotoxin E (SEE) has recently been analyzed and proposed to be associated with a defective bacteriophage (13).

To study staphylococcal enterotoxin D (SED), we cloned and sequenced its structural gene (*entD*) and the flanking DNA. *entD* was shown to reside on a large penicillinase plasmid designated pIB485. The deduced amino acid sequence of SED shows that it is highly related to the other enterotoxins. In addition, an analysis of the 5′-flanking sequence by S1 nuclease mapping revealed that transcription of the *entD* gene in *Staphylococcus aureus* is initiated 266 bases upstream from the translation start codon.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains are listed in Table 1. For each experiment, freshly prepared overnight cultures were grown in Trypticase soy broth (BBL Microbiology Systems) or Luria broth at 37°C.

Transformations. Transformation of *S. aureus* RN4220 was performed by the method of Chang and Cohen (12). Transformation of *Escherichia coli* LE392 and JM109 was performed by the $CaCl_2$ procedure described by Maniatis et al. (29).

Ethidium bromide curing. Plasmids were eliminated by growth in ethidium bromide as described previously (15). Cells were inoculated into 2.5 ml of brain-heart infusion (BHI) broth containing increasing concentrations of ethidium bromide ranging from 3.2×10^{-6} to 2.5×10^{-5} M. The cultures were agitated for 18 to 24 h in the dark at 37°C. The highest concentration of ethidium bromide in which bacteria grew was used to plate cells onto nonselective BHI agar. Ensuing colonies were replica plated to BHI agar and BHI agar containing penicillin (5 μg/ml); clones were scored for the loss of penicillin resistance.

Hybridization analysis. Plasmid DNA (1.0 μg) prepared from 12 different SED-producing strains was digested with 1.0 U of the restriction endonuclease *Eco*RI at 37°C for 1 h. Electrophoresis of the digested DNA (0.2 μg) was carried out in a 0.6% agarose–TBE (0.089 M Tris, 0.089 M boric acid, 2.5 mM EDTA) gel at 6 V/cm. The DNA fragments were electrophoretically transferred to GeneScreen Plus at 6 V in blotting buffer (10 mM Tris [pH 7.8], 5 mM sodium acetate, 0.5 mM EDTA). The membrane was then incubated in hybridization buffer (1% sodium dodecyl sulfate [SDS], 1 M NaCl, 10% dextran sulfate) containing 600 μg of heat-denatured calf thymus DNA and 2.0×10^6 cpm of heat-denatured ^{32}P-labeled probe DNA (see below) at 65°C overnight. Labeling of DNA was performed by the random primer procedure of Feinberg and Vogelstein (16). Filters were washed two times in 2× SSC (0.3 M NaCl, 30 mM sodium citrate) at room temperature, then two times in 2× SSC–1.0% SDS at 65°C, and finally two times in 0.1× SSC at room temperature. The blot was then exposed to X-ray film for 18 h.

DNA manipulations. Plasmid DNA was isolated from *E. coli* strains by the alkaline lysis method of Birnboim and Doly (6). *S. aureus* plasmid DNA was isolated as described previously (40). Restriction enzymes, T4 DNA ligase, and *Eco*RI linkers (hexamers) were purchased from Bethesda Research Laboratories and used as recommended. Random DNA fragments were obtained by partially digesting pIB485 with *Sau*3A. The 2- to 5-kilobase (kb) fraction was cut from a 0.8% agarose preparatory gel and electrophoretically eluted into a solution containing 5 M NaCl and 20% glycerol. The fragments were ligated into the *Bam*HI site of pBR322 and transformed into *E. coli* LE392, and ampicillin-resistant transformants were selected. Clones containing inserts were

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype[a]	Source or reference
Strains		
S. aureus		
RN4220	Pen^s Cd^s Cm^s SED$^-$	P. A. Pattee
KSI1410	Pen^r Cd^r SED$^+$; contains pIB485	A. D. Johnson-Winegar
KSI1411	KSI1410 cured of pIB485; Pen^s Cd^s SED$^-$	This work
ISP546	*agr* Cm^s	P. A. Pattee
FRI171	SED$^+$	R. Reiser
FRI200	SED$^+$	R. Reiser
FRI205	SED$^+$	R. Reiser
FRI216	SED$^+$	R. Reiser
FRI310	SED$^+$	R. Reiser
FRI358	SED$^+$	R. Reiser
FRI419	SED$^+$	R. Reiser
FRI470	SED$^+$	R. Reiser
FRI472	SED$^+$	R. Reiser
FRI667	SED$^+$	R. Reiser
E. coli		
LE392	Amp^s Tet^s	
JM109	Amp^s	
Plasmids		
pBR322	Contains *bla* and *tet*	9
pUC18	Contains *bla*	45
pLI50	Contains *bla*, *cat*, and *S. aureus* and *E. coli* origins of replication	26
pIT13	Carries a 5.2-kbp *bla* fragment from pI524	This work
pGN12	Carries a 3.5-kbp *cadA* fragment from pI258	S. Silver
pIB485	Contains *bla*, *cad*, and *entD*	This work
pIB486	Carries a 2.6-kbp insert containing *entD* in pBR322	This work
pIB489	Carries a 1.3-kbp insert containing *entD* in pUC18	This work
pIB586	Carries a 1.3-kbp insert containing *entD* in pLI50	This work
pIB476	Carries a 1.23-kbp insert containing *entD* and 244 bp of upstream sequence	This work

[a] Phenotypes: Pen, penicillin; Cd, cadmium; Cm, chloramphenicol; Amp, ampicillin; Tet, tetracycline.

obtained by screening the transformants for tetracycline sensitivity. Minilysate preparations (20) of these recombinant plasmids revealed that the smallest (pIB486) contained a 2.6-kilobase-pair (kbp) insert. From this, a 1.3-kbp *entD* fragment was generated by digestion with the restriction enzymes *Eco*RI and *Hin*dIII and subcloned into pUC18. This construct was designated pIB489.

The *S. aureus* promoter region was deleted by first digesting pIB489 with the restriction endonuclease *Fnu*4HI, which cleaved the upstream sequence between nucleotides (nts) −244 and −245 (see Fig. 3). The resulting 5′ overhangs were filled in with the Klenow fragment of *E. coli* DNA polymerase (29), followed by digestion of the DNA with *Hin*dIII, which liberated a 1.23-kbp fragment containing the *entD* structural gene. The fragment was gel purified (see above) and then ligated to pUC18 (previously digested with *Hin*dIII and *Sma*I) to form plasmid pIB476.

Colony blot analysis. Tetracycline-sensitive clones were grown on L-agar plates containing ampicillin and blotted to nitrocellulose membranes. The membranes were placed on Whatman 3MM paper that had been soaked in 20 mM Tris hydrochloride (pH 8.0)–10 mM EDTA–10 mg of lysozyme per ml and incubated at room temperature for 30 min. The nitrocellulose membranes were then immersed in a 0.1% SDS–20% methanol solution for 2 min and washed in blotting buffer (1.0 mM Tris [pH 7.0], 5.0 mM NaCl, 0.2 mM EDTA, and 0.01% NaI) at room temperature for 5 min. They were then incubated for 2 h at room temperature in blotting buffer containing 1.0% hemoglobin to block nonspecific binding sites. Clones producing SED were detected by addition of 200 μl of anti-SED antiserum that had been absorbed with an *E. coli* LE392 cell lysate. Incubation was continued for 6 h at room temperature. The membranes were then washed five times at room temperature for 5 min each time in wash buffer (0.1 M Tris [pH 7.5], 1.0% NaCl, 0.5% Nonidet P-40). This was followed by overnight incubation in blotting buffer containing 1.0% hemoglobin and 10^7 cpm of ^{125}I-labeled protein A. Finally, the membranes were washed an additional five times in wash buffer. Labeled protein A was detected by autoradiography.

Western immunoblot analysis. The presence of SED was detected by SDS-polyacrylamide gel electrophoresis and immunoblotting. Electrophoresis was conducted in a 15% SDS–polyacrylamide gel (24) at 30 mA for 3 h. Proteins were electrophoretically transferred to nitrocellulose membranes in blotting buffer (25 mM Tris [pH 8.3], 0.2 M glycine, 20% methanol) at 0.5 A for 3 h. SED bound to the nitrocellulose membranes was detected as described above.

DNA sequence analysis. A 2.0-kbp *Eco*RI fragment containing the *entD* structural gene was ligated into the *Eco*RI site of the double-stranded replicative intermediate DNA of bacteriophage M13mp19. Following transfection, clones containing both orientations of the fragment were obtained. From these, single-stranded phage DNA was isolated from the two clones, and overlapping deletions were generated as described by Dale et al. (14). Sequencing of both strands was performed by the dideoxy nucleotide chain termination method of Sanger et al. (39) with ^{35}S-labeled dATP.

S1 mapping. Mapping of the *entD* transcription start site was performed by the S1 nuclease mapping procedure described by Berk and Sharp (3). Agarose gel-purified DNA fragments (350 bp) spanning the upstream region of the *entD* structural gene were end labeled with γ-^{32}P-labeled ATP by polynucleotide kinase (30) and hybridized at 30°C to 100 μg of RNA isolated from various *entD* strains. The RNA was then digested with 300 U of S1 nuclease at 30°C for 1 h. Fragments protected from S1 nuclease digestion were ethanol precipitated and subjected to electrophoresis in an 8.0% gel containing 8.0 M urea. Sequencing reactions of the upstream fragments were also included in this gel in order to determine the size of the protected fragments. Bands were visualized by autoradiography.

RESULTS

Localization of *entD*. Analysis of cleared lysate DNA from *S. aureus* KSI1410 (SED$^+$) revealed that it contained a 27.6-kbp plasmid, designated pIB485 (Fig. 1). When cells were cured of pIB485 by treatment with ethidium bromide, they lost resistance to both penicillin and $CdSO_4$. In addition, loss of the ability to produce SED was also observed (data not shown). These results indicated that the genes conferring penicillin resistance and $CdSO_4$ resistance and *entD* or a gene required for the expression of *entD* were contained on pIB485. To confirm these results, pIB485 was transformed into *S. aureus* RN4220. Transformants were selected for $CdSO_4$ resistance and subsequently screened for

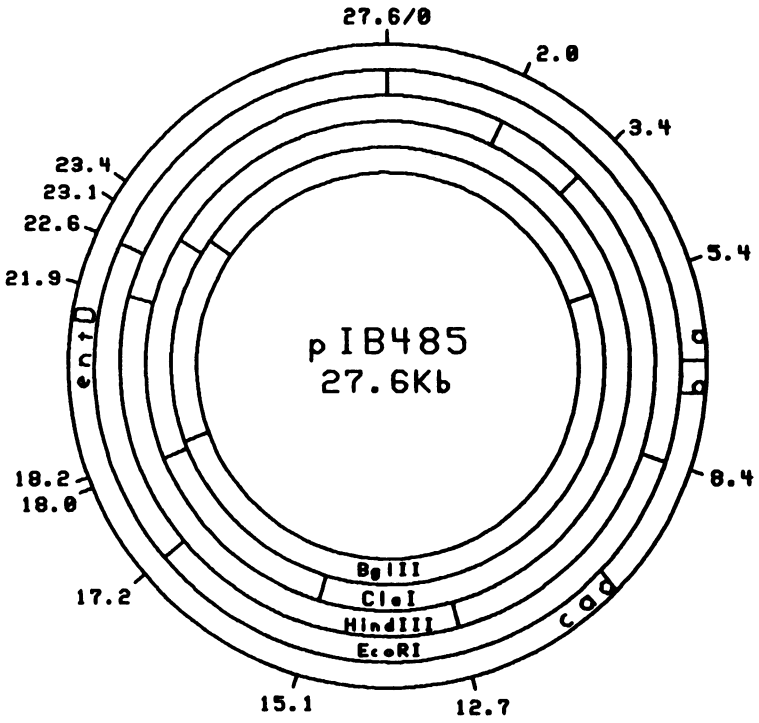

FIG. 1. Restriction endonuclease and genetic map of pIB485. The genetic markers include the genes encoding SED (*entD*), resistance to penicillin (*bla*), and resistance to cadmium (*cad*).

penicillin resistance and SED production. All of the $CdSO_4$-resistant transformants tested also acquired penicillin resistance and produced SED (data not shown).

Cloning of *entD* into *E. coli*. A *Sau*3A restriction endonuclease partial digest of pIB485 was prepared, and the 2- to 5-kbp DNA fragments were isolated by gel electrophoresis. The fragments were then ligated into the *Bam*HI site of pBR322 and transformed into *E. coli* LE392 cells. Ampicillin-resistant transformants were replica-plated onto L-agar containing 10 μg of tetracycline per ml to determine which contained inserts. Of the 55 Tc^s colonies obtained, 5 were shown by colony blots to bind antiserum to SED (data not shown). A protein extract from one of the Tc^s clones containing the recombinant plasmid pIB486 was prepared and analyzed by electrophoresis on a 15% SDS-polyacrylamide gel. Western blot analysis of this gel (data not shown) revealed that SED was produced, confirming that the *entD* gene was contained on the cloned DNA fragment and expressed in *E. coli*.

Restriction and genetic mapping of pIB485. Mapping of pIB485 was achieved by digestion with the restriction endonucleases *Eco*RI, *Hin*dIII, *Pst*I, *Cla*I, and *Bgl*II (Fig. 1). The DNA fragments were separated in a 0.6% agarose gel, with the *Eco*RI and *Hin*dIII fragments of bacteriophage lambda used as size markers. In order to determine the position of the genes located on pIB485, single digestions of pIB485 were carried out with the above-listed restriction enzymes. These fragments were separated in a 0.6% agarose gel and then subjected to Southern blot analysis. *E. coli* plasmid constructs containing *bla*, *cad*, and *entD* sequences (pIT13, pGN12, and pIB489, respectively) were used as probes to map the location of each gene. As indicated in Fig. 1 and 2B (lane 2), *entD* was located on the 5.4-kbp *Eco*RI fragment of pIB485.

The location of *entD* in 10 independent isolates of SED-producing strains (kindly supplied by Raoul Reiser, Food Research Institute, Madison, Wis.) was also analyzed. Plasmid DNA was prepared from each strain and digested with *Eco*RI. These digests were then separated by agarose gel electrophoresis, and the patterns were compared with the *Eco*RI-generated restriction pattern of pIB485. All strains contained a 27.6-kbp plasmid with the same restriction

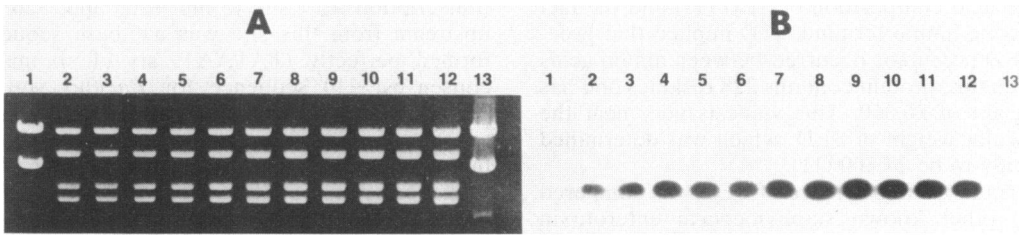

FIG. 2. (A) Agarose gel electrophoresis of *Eco*RI-digested plasmid DNA isolated from SED^+ strains. Lanes: 2, KSI1410; 3, FRI171; 4, FRI200; 5, FRI205; 6, FRI216; 7, FRI310; 8, FRI358; 9, FRI419; 10, FRI470; 11, FRI472; and 12, FRI667. Lanes 1 and 13 contain bacteriophage lambda DNA digested with *Hin*dIII. (B) Southern blot analysis of the DNA fragments shown in panel A. The DNA was blotted to GeneScreen Plus and probed with ^{32}P-labeled pIB486.

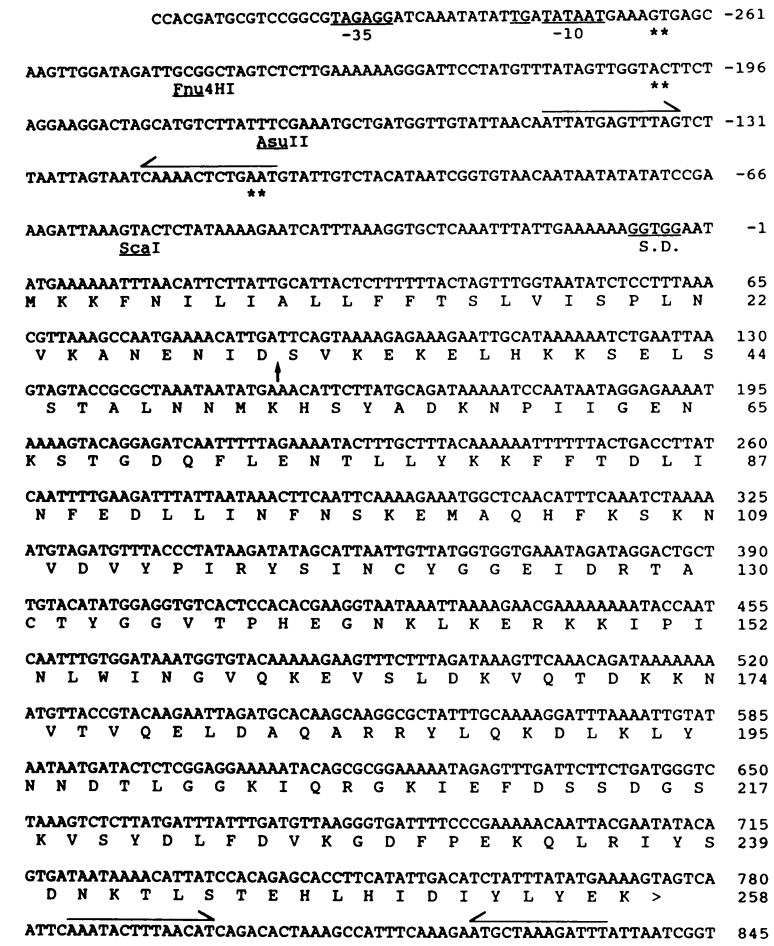

FIG. 3. Sequence analysis of the *entD* gene. Shown is the nucleotide sequence of the DNA fragment containing the *entD* open reading frame and the derived amino acid sequence. Horizontal arrows indicate regions of dyad symmetry. Those near the 3′ end of the *entD* gene indicate a possible transcription termination signal. Transcription start sites are denoted by asterisks. The −10 and −35 sequences recognized in *S. aureus* and the Shine-Dalgarno ribosome-binding site (S.D.) are underlined. A vertical arrow indicates the leader peptide cleavage site.

endonuclease pattern as pIB485 (Fig. 2A). This gel was also subjected to Southern blot analysis with the 2.0-kbp *entD* fragment from pIB488 used as a probe for the *entD* gene. In each case, a 5.4-kbp band hybridized to the probe, indicating the presence of the *entD* gene (Fig. 2B).

DNA sequencing of *entD*. The nucleotide sequence of a 2.0-kbp fragment containing *entD* was determined by analysis of overlapping deletion clones. An open reading frame was found (start and stop codons at nucleotides 1 and 775, respectively) that contained the presumptive nucleotide sequence of *entD* (Fig. 3). This open reading frame was 774 nucleotides long and encoded a 258-amino-acid protein. The published amino acid composition of SED (11) and the fact that serine is at the amino terminus (11) implied that processing of the SED precursor occurred between amino acids 30 and 31. The mature protein contains 228 residues and has a molecular weight of 26,360. This value is very near the published molecular weight of SED, which was determined electrophoretically to be 27,300 (11).

The deduced amino acid sequence of SED was compared with those the other known staphylococcal enterotoxin sequences and streptococcal pyrogenic exotoxin A (SPEA) (Fig. 4). The alignment was set up to minimize the number of gaps required to align all six sequences. Individual comparisons of these sequences by the algorithm of Wilbur and Lipman (44) showed that SED shared between 53.1 and 55.0% sequence identity with SEA and SEE and only between 39.5 and 41.2% identity with SEB, SEC_1, and SPEA.

Localization of the *entD* transcription start site. The transcription start site of the *entD* gene was localized by S1 nuclease mapping. The results revealed the presence of one major protected fragment (the faint, faster-migrating bands were not reproducible) when RNA isolated from *S. aureus* KSI1410 was used (Fig. 5, lane 2). The size of this fragment, determined by comparison with the simultaneously run sequencing ladder of the *entD* upstream region, mapped the transcription start site to nts −266 and −265 (Fig. 3). Just upstream from this site was a 6-base sequence that conformed perfectly (TATAAT, six of six matches) to the consensus −10 sequence for *Bacillus* spp. and *E. coli* promoters (19, 38). Although a sequence similar to the consensus −35 sequence was also present, it contained mismatches (three of six) and was separated from the −10 sequence by only 14 nts rather than the consensus separation of 17 to 18 nts (19). When RNA isolated from the *E. coli* clone JM109(pIB488) was used, three protected fragments were observed (Fig. 5, lane 3). These fragments identify transcription start sites at nt −266, as observed with *S. aureus* RNA, and also at nts −109 and −200 (Fig. 3).

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          1         10        20        30        40        50
SED       SVKEKELHKKSELSSTALN-NMKHSYADKNPIIGENKSTGDQFLENTLLYKKFFTDLINF
SEA  SEKSEEIN  D R      QG   G- L QI YYNEKAKT   ESH     QH I F G     HSWY
SEB    SQPDP PD    S KF-- G ME   VL D NHVSAINV  I-     YFD I SIKD K G Y
SEC1   SQPDPTPD    A KF-- G ME   VL D HYVSATKV  V- K  AHDI   NISDKK K Y
SEE     SEEIN  D R      QRN  S- LRNI YYNEKA T   ESD          F G   GHPWY
SPEA    QQDPDPSQ  R- S V-KN Q- IYFL EGDPVTHENV  V-  L SHD I NVS--G-P Y

     60        70        80        90                   100       110
SED  EDLLINFNSKEMAQHFKSKNVDVYPIRYSINCYGGEID-----------RTACTYGGVTPHEGNK
SEA  N   VD D  DIVDKY G K  L GAY GYQ A  T-PN----------K    M     L DN R
SEB  DNVRVE KN DL DKY D Y   FGAN YYQ  FSKKTNDINSHQTDKRK - M     E N  Q
SEC1 DKVKTELLNEGL KKY DEV    GSN YV   FSSK NVGKV--TGG-K - M   I K    H
SEE  N   VDLG  DATNKY G K  L GAY GYQ A  T-PN----------K    M     L DN R
SPEA DK KTELKNQ   TLF D    I GVE YHL  LC --NAE-------- S  I     N    H

          120           130       140       150       160       170
SED  LKERKKIPINLWI----NGVQKEVSLDKVQTDKKNVTVQELDAQARRYLQKDLKLYNNDTLGGKI
SEA   T E  V     L----D K NT P ET K N          L        EKYN   S VFD  V
SEB   DKYRS TV----RVFED K-NLL F -    N  K  A    YLT H  V NK   EFNNSPYET
SEC1 FDNGNLQNV- - RVYE -KRNTI FE-    N  S  A    IK  NF INKKN  EFNSSPYET
SEE   T E  V       ----D K TT PI   K S  E       L   H  HGKFG   S SF   V
SPEA  EIP   VVKVS ----D I S-L F -IE N  M  A    YKV K  TDNKQ  T GPSKYET

          180       190       200       210       220
SED  QRGKIEFDSSDGSKVSYDLFDVKGDFPEKQLRIYSDNKTL-STEHLHIDIYLYEK*
SEA     L V HT TEPS N    GAQ QYSNTL    R    IN - NM        TS*
SEB  GYI FIE-NENSFWYDMMPAPGDKFDQS Y MM N   MVD -KDVK EV  TT K*
SEC1 GYI FIENNGNTFWYDMMPAPGDKFDQS Y MM N    VD -KSVK EVH TT NG*
SEE     L V H  E  T      AQ QY DTL    R    IN - N    L   TT*
SPEA GYI FIPKNKESFWFDFFPEPE--FTQS Y M  K  E  D -NTSQ EV  TT *
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FIG. 4. Comparison of the amino acid sequence of SED with those of SEA (5), SEB (20), SEC_1 (7), SEE (12), and SPEA (37). The algorithm of Wilbur and Lipman (38) was used with a *K*-tuple of 1 and a window size of 20. The comparisons were adjusted to give the maximum amount of similarity between each of the sequences. Regions of identity are indicated by blank spaces, and the dashed lines indicate a gap of one amino acid. Asterisks are placed at the C-terminus of each protein. The numbers correspond to the amino acid residues of the mature form of SED.

Sequences upstream from both of these transcription start sites that were similar to the consensus −10 and −35 sequences were also present.

In order to determine whether the transcription start sites localized at nts −109 and −200 were involved in *entD* expression in *S. aureus*, a promoter deletion contruct was made by digesting the *entD* plasmid pIB489 with the restriction enzymes *Fnu*4HI (cleavage site shown in Fig. 3) and *Hin*dIII (cleaves 3′ to the *entD* gene). This digestion isolated the *entD* structural gene and the promoters at nts −109 and −200 on a single DNA fragment while omitting the promoter at nt −266. This fragment was then ligated into the shuttle vector pLI50 to form pIB476.

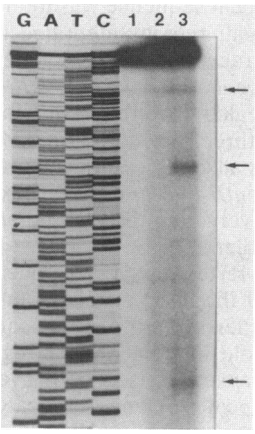

FIG. 5. Analysis of the transcription start site of the *entD* gene. Total RNA was isolated from *S. aureus* KSI1410 (lane 2) and *E. coli* KSI1454 (lane 3) and used to hybridize to ^{32}P-labeled fragments containing the upstream sequences of *entD*. A hybridization was also carried out with no protecting RNA (lane 1). The samples were digested with S1 nuclease and separated on a sequencing gel. Protected fragments were detected by autoradiography.

After introduction of pIB476 into *S. aureus* RN4220 and *E. coli* JM109, the strains were assayed for ability to make SED. As shown in Fig. 6A (lane 3), SED was produced by the *S. aureus* RN4220 strain containing the *entD* plasmid pIB586 (contains all promoter sequences determined in Fig. 5). However, when the promoter at nt −266 was absent, as in *S. aureus* RN4220(pIB476), the ability to express *entD* was reduced by 94% (Fig. 6A, lane 4). When the wild-type and deleted promoter constructs were placed in the *E. coli* JM109 background, both expressed the *entD* gene, as predicted by the S1 nuclease protection analysis (Fig. 6B, lanes 2 and 3). The expression of *entD* observed with pIB476 in *E. coli* was probably the result of transcription initiation from the promoters at nts −109 and −200. Therefore, considering the data in Fig. 5 and 6, transcription of the *entD* gene in *S. aureus* appears to be initiated exclusively at nts −266 and −265.

Regulation of *entD* by *agr*. The *S. aureus* regulatory gene *agr* (37), also described as *exp* (7), has been shown to control the expression of a number of extracellular protein genes (10, 17, 32, 33, 37). The effect of *agr* on *entD* expression was

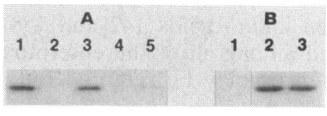

FIG. 6. Western immunoblot analysis of the expression of *entD* in *S. aureus* and *E. coli* strains containing *entD* plasmid constructs. (A) Equal amounts of extracellular proteins from 18-h cultures of *S. aureus* strains. Lanes: 1, partially purified SED (100 μg); 2, RN4220 (pLI50); 3, RN4220(pIB586); 4, RN4220(pIB476); 5, ISP546(pIB586). (B) Total proteins from 4-h cultures of *E. coli* strains. Lanes: 1, JM109(pLI50); 2, JM109(pIB586); 3, JM109(pIB476). SED was detected by using anti-SED antiserum and ^{125}I-labeled protein A.

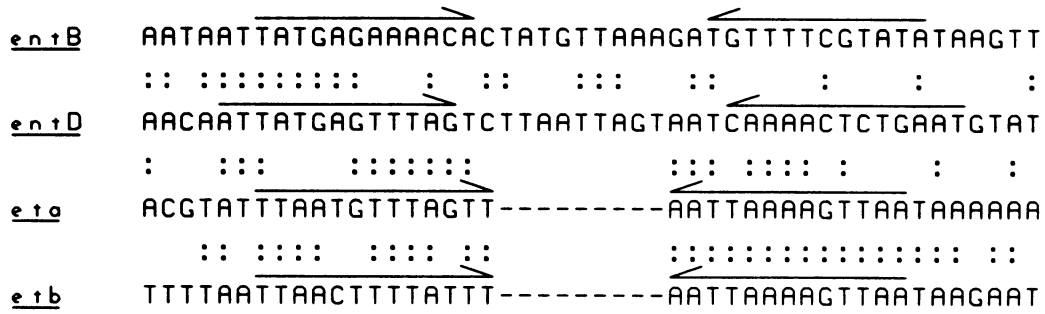

FIG. 7. Comparison of the nucleotide sequences that span the inverted repeat regions found upstream from the *entB*, *entD*, *eta*, and *etb* open reading frames. The inverted repeat sequences are indicated by arrows.

tested by transforming pIB586 into the *agr S. aureus* mutant ISP546 (28). Extracellular proteins were isolated from this strain and subjected to Western blot analysis. Densitometric scanning of this blot indicated that expression of *entD* was reduced 82% in ISP546 (Fig. 6, lane 5) compared with expression of *entD* in RN4220 (Fig. 6, lane 3). Since RN4220 has been shown to exhibit a leaky *agr* phenotype (17), further reduction in the expression of *entD* compared with that in the *agr* wild-type strain 8325-4 was expected.

DISCUSSION

The gene encoding SED (*entD*) is located on a 27.6-kbp penicillinase plasmid designated pIB485. This plasmid was found in all SED-producing strains analyzed. This finding is similar to the discovery of the staphylococcal plasmids that contain the structural genes encoding SEB and SEC_1 (1) and the gene encoding exfoliative toxin B (42). Other unique elements containing enterotoxin genes in *S. aureus* appear to be quite common. It has been shown that *entA* (encodes SEA) is encoded by the temperate bacteriophage PS42D (4, 4a). In addition, Johns and Khan (22) have shown that the gene encoding SEB (*entB*) is contained on a large element unique to SEB^+ strains. They demonstrated, by Southern blot analysis of different SEB-producing isolates, that *entB* is flanked by approximately 25 kbp of similar sequences in each strain. Although these data strongly suggest association of *entB* with a bacteriophage or an integrated plasmid, no such element has been isolated. The relationship of pIB485 to the elements that carry *entA* and *entB* is currently being investigated.

Amino acid sequence comparison of the different enterotoxin molecules showed that there is a large amount of sequence similarity among the proteins. Direct comparisons of individual amino acid matches of the enterotoxins reveal that they fall into at least two groups. One group is composed of SEB, SEC_1, and SPEA from *S. pyogenes*, while SEA, SED, and SEE constitute another group. Two stretches of amino acids within SED (from residues 101 to 114 and between amino acids 142 and 158) appear to be highly conserved among all of the enterotoxins, indicating that these sequences may be required for their biological activity.

Not surprisingly, the antigenic cross-reactivity of the enterotoxins can be related in a similar manner. SEB and SEC_1 have been shown to share common epitopes, as do SEA and SEE (2). SED does not cross-react with SEB or SEC_1, but does share some minor epitopes with SEA and SEE (2). The significance of these groupings, if any, is unknown.

Further characterization of *entD* by S1 nuclease protection analysis indicates that in *S. aureus* transcription is initiated 266 nts upstream from the *entD* translation start codon. However, RNA isolated from the *E. coli* clone producing SED revealed three protected fragments. Although one fragment was identical to that protected by the *S. aureus* RNA, the other two indicated transcription initiation at different sites. A number of hypotheses could explain this observation. One is that the additional sites are utilized differentially at various phases of the *S. aureus* growth cycle. However, this seems unlikely because SED production is almost completely eliminated (a 94% reduction) when the promoter at nt −266 is deleted. Another possibility is that the additional promoters could be utilized in response to environmental signals (nutritional factors, serum factors, low iron, etc.) not present in our growth medium. A third possibility is that the A+T content of *S. aureus* DNA is so high (70%) that the RNA polymerase(s) of *E. coli* erroneously recognizes these regions as transcription initiation sites. Since *E. coli* DNA is approximately 50% A+T, *E. coli* RNA polymerases may lack the promoter recognition specificity that is inherent in the *S. aureus* RNA polymerases. At this time we have no evidence to support or reject these last two possibilities.

The region upstream from *entD* has other interesting features besides its transcriptional initiation sites. In particular, an inverted repeat sequence was found which compares favorably with similar regions identified upstream of other staphylococcal extracellular protein genes. Nucleotide sequence analysis of *geh* (26), *nuc* (41), *eta* (27, 34), *etb* (27), and *entB* (23) revealed that they contain inverted repeats which, in the last three, are quite similar at the nucleotide level (Fig. 7). They also have similar arm lengths (12 to 13 bp), although the *entB* and *entD* arms are separated by 12 and 14 bp, respectively, and the *eta* and *etb* repeat arms are adjacent to each other.

The role of the inverted repeat sequence located within the untranslated end of the *entD* mRNA transcript is uncertain at this point. In other systems, such sequences have been shown to play a role in translational regulation (18) or to stabilize mRNA transcripts (36). However, the fact that a similar inverted repeat sequence is located upstream from the transcription start site of *entB* (17) suggests that they could also function to regulate transcription. In fact, transcriptional regulation of many extracellular protein genes in *S. aureus* has recently been characterized. Control is mediated by the product of the *agr* gene (37), also referred to as *exp* by Morfeldt et al. (31). We show that this gene also regulates the expression of *entD* (Fig. 6, lane 5). The

regulatory significance (if any) of the inverted repeat sequences interacting with the *agr* gene product or any other gene product awaits a more detailed molecular analysis of the upstream regions of other extracellular protein genes from *S. aureus*.

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