INTRODUCTION

The exfoliative toxins A and B (ETA and ETB) produced by *Staphylococcus aureus* cause staphylococcal scalded-skin syndrome (Johnson et al., 1975; Melish et al., 1976). We were the first to report the ETA and ETB serotypes of *S. aureus* from an 8-kb *eta* gene (etaJ1) obtained from the chromosomal DNA of *S. aureus* ZM, the standard ETA-producing strain. The cETA prepared from an *Escherichia coli* transformant into which the recombinant plasmid petaJ1 (5-8 kb *eta*pUC9) had been introduced was expressed at high levels in the culture supernatant and the ammonium-sulfate-precipitated culture supernatant fraction as shown by immunoblotting and the single radial immunodiffusion test. However, cETA produced by the recombinant plasmid petaJS containing the 1·7 kb *eta* sequence (etaJ3) with a 1·45 kb ETAEXP-deficient *eta* fragment (1·7 kb *eta*pUC9) obtained from the 5·8 kb *eta* sequence by subcloning was not detected in either the culture supernatant or the ammonium-sulfate-precipitated culture supernatant fraction (167-fold concentrate of the culture supernatant) by immunoblotting or the single radial immunodiffusion test. A large amount of cETA was produced by the 1·7 kb *eta* sequence when it was linked to ETAEXP amplified by PCR (1·7 kb *eta*-ETAEXP/pUC9), regardless of the orientation of ETAEXP insertion. Northern blot hybridization showed lower levels of the transcripts of the 1·7 kb *eta* sequence than of the 5·8 kb *eta* sequence. The rsETA prepared from an *S. aureus* transformant into which the recombinant plasmid 3·4 kb *eta*-ETAEXP/pYT3 (pYT3-etaJ6) had been introduced was expressed at high levels in the culture supernatant fraction as shown by the latex agglutination test. However, the agglutination titre in the culture supernatant fraction of rsETA produced by the recombinant plasmid (1·7 kb *eta*pYT3) containing the 1·7 kb *eta* sequence carrying the 1·4 kb ETAEXP-deficient *eta* fragment (pYT3-etaJ3) was 2500–4000 times lower than that of pYT3-etaJ6.

A 1·4 kb positive regulatory element (ETAEXP) that controls staphylococcal exfoliative toxin A (sETA) transcription was cloned from *Staphylococcus aureus*. ETAEXP is located upstream of the cloned 5·8 kb *eta* gene (etaJ1) obtained from the chromosomal DNA of *S. aureus* ZM, the standard ETA-producing strain. The cETA prepared from an *Escherichia coli* transformant into which the recombinant plasmid petaJ1 (5-8 kb *eta*pUC9) had been introduced was expressed at high levels in the culture supernatant and the ammonium-sulfate-precipitated culture supernatant fraction as shown by immunoblotting and the single radial immunodiffusion test. However, cETA produced by the recombinant plasmid petaJS containing the 1·7 kb *eta* sequence (etaJ3) with a 1·45 kb ETAEXP-deficient *eta* fragment (1·7 kb *eta*pUC9) obtained from the 5·8 kb *eta* sequence by subcloning was not detected in either the culture supernatant or the ammonium-sulfate-precipitated culture supernatant fraction (167-fold concentrate of the culture supernatant) by immunoblotting or the single radial immunodiffusion test. A large amount of cETA was produced by the 1·7 kb *eta* sequence when it was linked to ETAEXP amplified by PCR (1·7 kb *eta*-ETAEXP/pUC9), regardless of the orientation of ETAEXP insertion. Northern blot hybridization showed lower levels of the transcripts of the 1·7 kb *eta* sequence than of the 5·8 kb *eta* sequence. The rsETA prepared from an *S. aureus* transformant into which the recombinant plasmid 3·4 kb *eta*-ETAEXP/pYT3 (pYT3-etaJ6) had been introduced was expressed at high levels in the culture supernatant fraction as shown by the latex agglutination test. However, the agglutination titre in the culture supernatant fraction of rsETA produced by the recombinant plasmid (1·7 kb *eta*pYT3) containing the 1·7 kb *eta* sequence carrying the 1·4 kb ETAEXP-deficient *eta* fragment (pYT3-etaJ3) was 2500–4000 times lower than that of pYT3-etaJ6.

Abbreviations: ETA, exfoliative toxin A; ETB, exfoliative toxin B; cETA, recombinant ETA produced by *E. coli*; sETA, staphylococcal exfoliative toxin A produced by *S. aureus* ZM; rsETA, recombinant ETA prepared from an *S. aureus* transformant or recombinant plasmid; TSST-1, toxic shock syndrome toxin 1.

The GenBank/DDBJ accession number for the sequence reported in this paper is AB070631.

A novel positive regulatory element for exfoliative toxin A gene expression in *Staphylococcus aureus*

Susumu Sakurai,1 Hitoshi Suzuki,2 Toshiaki Hata,3 Yukio Yoshizawa,4 Ritsuko Nakayama,3 Katsuhiko Machida,5 Shogo Masuda6 and Takashi Tsukiyama1

1Department of Molecular Genetics, Kohn Clinical Medicine Research Institute, Shinagawa-ku, Tokyo 140-0001, Japan
2Division of Bioscience, Graduate School of Environmental Earth Science, Hokkaido University, Kita-ku, Sapporo 060, Japan
3,4,5Department of Molecular Genetics, Radioisotope Research Center, Department of Laboratory Medicine and Department of Microbiology (II), The Jikei University School of Medicine, Minato-ku, Tokyo 105-8461, Japan

Received 2 December 2002
Revised 9 December 2003
Accepted 16 December 2003

The exfoliative toxins A and B (ETA and ETB) produced by *Staphylococcus aureus* cause staphylococcal scalded-skin syndrome (Johnson et al., 1975; Melish et al., 1976). We were the first to report the ETA and ETB serotypes of *S. aureus* from an 8-kb *eta* gene (etaJ1) obtained from the chromosomal DNA of *S. aureus* ZM, the standard ETA-producing strain. The cETA prepared from an *Escherichia coli* transformant into which the recombinant plasmid petaJ1 (5-8 kb *eta*pUC9) had been introduced was expressed at high levels in the culture supernatant and the ammonium-sulfate-precipitated culture supernatant fraction as shown by immunoblotting and the single radial immunodiffusion test. However, cETA produced by the recombinant plasmid petaJS containing the 1·7 kb *eta* sequence (etaJ3) with a 1·45 kb ETAEXP-deficient *eta* fragment (1·7 kb *eta*pUC9) obtained from the 5-8 kb *eta* sequence by subcloning was not detected in either the culture supernatant or the ammonium-sulfate-precipitated culture supernatant fraction (167-fold concentrate of the culture supernatant) by immunoblotting or the single radial immunodiffusion test. A large amount of cETA was produced by the 1·7 kb *eta* sequence when it was linked to ETAEXP amplified by PCR (1·7 kb *eta*-ETAEXP/pUC9), regardless of the orientation of ETAEXP insertion. Northern blot hybridization showed lower levels of the transcripts of the 1·7 kb *eta* sequence than of the 5·8 kb *eta* sequence. The rsETA prepared from an *S. aureus* transformant into which the recombinant plasmid 3·4 kb *eta*-ETAEXP/pYT3 (pYT3-etaJ6) had been introduced was expressed at high levels in the culture supernatant fraction as shown by the latex agglutination test. However, the agglutination titre in the culture supernatant fraction of rsETA produced by the recombinant plasmid (1·7 kb *eta*pYT3) containing the 1·7 kb *eta* sequence carrying the 1·4 kb ETAEXP-deficient *eta* fragment (pYT3-etaJ3) was 2500–4000 times lower than that of pYT3-etaJ6.

A 1·4 kb positive regulatory element (ETAEXP) that controls staphylococcal exfoliative toxin A (sETA) transcription was cloned from *Staphylococcus aureus*. ETAEXP is located upstream of the cloned 5·8 kb *eta* gene (etaJ1) obtained from the chromosomal DNA of *S. aureus* ZM, the standard ETA-producing strain. The cETA prepared from an *Escherichia coli* transformant into which the recombinant plasmid petaJ1 (5-8 kb *eta*pUC9) had been introduced was expressed at high levels in the culture supernatant and the ammonium-sulfate-precipitated culture supernatant fraction as shown by immunoblotting and the single radial immunodiffusion test. However, cETA produced by the recombinant plasmid petaJS containing the 1·7 kb *eta* sequence (etaJ3) with a 1·45 kb ETAEXP-deficient *eta* fragment (1·7 kb *eta*pUC9) obtained from the 5-8 kb *eta* sequence by subcloning was not detected in either the culture supernatant or the ammonium-sulfate-precipitated culture supernatant fraction (167-fold concentrate of the culture supernatant) by immunoblotting or the single radial immunodiffusion test. A large amount of cETA was produced by the 1·7 kb *eta* sequence when it was linked to ETAEXP amplified by PCR (1·7 kb *eta*-ETAEXP/pUC9), regardless of the orientation of ETAEXP insertion. Northern blot hybridization showed lower levels of the transcripts of the 1·7 kb *eta* sequence than of the 5·8 kb *eta* sequence. The rsETA prepared from an *S. aureus* transformant into which the recombinant plasmid 3·4 kb *eta*-ETAEXP/pYT3 (pYT3-etaJ6) had been introduced was expressed at high levels in the culture supernatant fraction as shown by the latex agglutination test. However, the agglutination titre in the culture supernatant fraction of rsETA produced by the recombinant plasmid (1·7 kb *eta*pYT3) containing the 1·7 kb *eta* sequence carrying the 1·4 kb ETAEXP-deficient *eta* fragment (pYT3-etaJ3) was 2500–4000 times lower than that of pYT3-etaJ6.

Abbreviations: ETA, exfoliative toxin A; ETB, exfoliative toxin B; cETA, recombinant ETA produced by *E. coli*; sETA, staphylococcal exfoliative toxin A produced by *S. aureus* ZM; rsETA, recombinant ETA prepared from an *S. aureus* transformant or recombinant plasmid; TSST-1, toxic shock syndrome toxin 1.

The GenBank/DDBJ accession number for the sequence reported in this paper is AB070631.
(Sakurai et al., 1987). The cETA produced by the 5.8 kb eta sequence ligated with pUC9 (petaJ1) is detectable with both the micro-Ouchterlony gel diffusion test and biological activity tests (Sakurai et al., 1987). However, we have found that although the cETA produced by the 1.7 kb fragment carrying the 1.45 kb eta sequence obtained from the 5.8 kb eta sequence by digesting Alul (1.7 kb eta) can be detected by ELISA, the amount of cETA produced by the 1.7 kb eta sequence is much smaller than that produced by the 5.8 kb eta sequence (Hata, 1994). Because the 1.7 kb eta is cleaved from the genomic insertion of the 5.8 kb eta, it is believed to be flanked by excess sequences.

In this paper, we report that the upstream region (ETAexp) of the 5.8 kb eta is required for genetic expression of eta and that the rsETA prepared from an S. aureus transformant into which the recombinant plasmid pYT3-etaJ6 had been introduced was expressed at high levels in the culture supernatant fraction in quantities similar to those produced by E. coli C6008S transformed with petaJ1 as shown by the latex agglutination test. However, the latex agglutination titre of rsETA in the culture supernatant of S. aureus transformed with the recombinant plasmid pYT3-etaJ3 containing the 1.7 kb eta sequence carrying 1.4 kb ETAexp-deficient eta was 2500–4000 times lower than that of pYT3-etaJ1.

**METHODS**

**Bacterial strains and media.** The ETA-producing strain S. aureus ZM (phage type II) was used to produce ETA (referred to below as sETA). E. coli C6008S was cultured in a medium consisting of 1.6% (w/v) bactotryptone (Difco), 1% (w/v) yeast extract (Difco) and 0.5% (w/v) NaCl, pH 7.2 (2 × TY). Transformants were cultured in 2 × TY medium containing 50 μg ampicillin ml⁻¹. The S. aureus strain FRI-1169 producing toxic shock syndrome toxin-1 (TSS; Sakurai et al., 1987) and staphylokinase (SAK) was obtained from Tokyo Metropolitan Research Laboratory of Public Health and used as a recipient of cloned DNA in plasmid shuttle vector.

To determine exoprotein production S. aureus was cultivated in LB medium (Sigma-Aldrich; Sambrook et al., 1989) at 30 °C for 48 h.

**Molecular biology techniques.** Standard techniques were used for DNA isolation, molecular cloning, transformation in E. coli, electrophoresis, and DNA sequencing (Sambrook et al., 1989).

**Construction of recombinant plasmids pYT3-etaJ6 and pYT3-etaJ3.** Construction of the 3.4 kb eta-ETAexp/pYT3 (pYT3-etaJ6) and 1.7 kb eta/pYT3 (pYT3-etaJ3) recombinant plasmids was as follows. The 4.2 kb fragment containing the ETAexp gene and the eta gene was obtained by EcoRI digestion of petaJ1. The recombinant plasmid petaJ3 (1.7 kb eta/pUC9) was digested by EcoRI. The 5’ protruding ends of the 4.2 kb fragment obtained from petaJ3 digested with EcoRI or the recombinant plasmid petaJ3 digested by EcoRI were removed using the Kilo-Sequence Deletion Kit (Takara Shuzou), and then the 4.2 kb fragment or petaJ3 was digested by HindIII (Fig. 1). The resulting 3.4 kb fragment (3.4 kb eta-ETAexp) or the 1.7 kb eta sequence (etaJ3) carrying 1.4 kb ETAexp-deficient eta was inserted into the shuttle plasmid pYT3 (kindly provided by Professor K. Hiramoto, Department of Bacteriology, Juntendo University, Japan) cleaved at the SalI site; the 5’ protruding ends were converted to blunt ends, and then the fragment was digested with HindIII. Agglutination tests confirmed that E. coli C6008S transformed with pYT3-etaJ6 produced cETA in quantities similar to those produced by E. coli C6008S transformed with petaJ1 (data not shown). However, cETA produced by pYT3-etaJ3 was not detected in the culture supernatant fraction. pYT3-etaJ3 or the pYT3-etaJ6 recombinant plasmid was transformed into S. aureus strain FRI-1169.

**Electroporation procedure.** S. aureus was grown in 2 × LB medium (Sigma-Aldrich) at 37 °C until mid-exponential phase, after which 10 ml of the preculture was inoculated into 100 ml 2 × LB medium prewarmed to 37 °C and shaken. When the shaking culture had reached an OD₅₉₅ of 0.6, the cells were chilled on ice and centrifuged for 5 min at 6000 r.p.m. The pellets were washed three times at 4 °C with 25 ml deionized water, followed by two washes with 5 ml 10% (v/v) glycerol. Finally, the cells were resuspended in 200 μl 10% (v/v) glycerol, and made electrocompetent. Fifty-microlitre aliquots of the electrocompetent cells were frozen at −80 °C. A tube of cells was mixed with 0.5 μg DNA and transferred to a 1 mm cuvette chilled on ice. The cuvette was pulsed at 1.8 kV with an Easyjet T Prima (EquiBio). Immediately after pulsing, 0.95 ml Brain Heart Infusion (Becton Dickinson) containing 0.5 M sucrose was added to the cuvette. The suspension was transferred to a polypropylene tube and incubated for 2 h at 30 °C. The cells were plated onto an LB agar plate containing 1 μg tetracycline ml⁻¹. The plates were incubated for 48 h at 30 °C (Yoshizawa, 1985; Rhoads & Landolo, 1990; Schenk & Laddaga, 1992).

**petaJ3 and petaJ1 recombinant plasmids.** In all experiments, we used a recombinant pUC9 plasmid containing a 5.8 kb eta Alul insert (petaJ1) or a 1.7 kb eta Alul insert (petaJ3) prepared according to a previously described procedure (Sakurai et al., 1987; Sakurai et al., 1988). The petaJ1 recombinant plasmid was constructed by
ligating the 5·8 kb *eta* *Alu* fragment of the chromosomal DNA from *S. aureus* ZM, an ETA-producing strain, and a SmaI-cleaved pUC9 with T4 DNA ligase. The *petαJ* recombinant plasmid was constructed by ligating the 1·7 kb fragment carrying the 1·45 kb *eta* (1·7 kb *eta*) obtained from the 5·8 kb *eta* fragment after partial digestion by *Alu* and SmaI-cleaved pUC9. The production of cETA by ampicillin-resistant *E. coli* C6008S transformants (Amp') was assessed with a sandwich ELISA of culture supernatants with rabbit anti-ETA serum, as previously described (Sakurai et al., 1987, 1998).

Construction of *petαJ* recombinant plasmid. The 5·8 kb *eta* sequence was digested with EcoRI to remove the 3' terminal sequence (Fig. 1). The resulting 4·2 kb segment (*etaJ2*) was ligated with pUC118 digested with EcoRI, and a recombinant plasmid (*petαJ*) was constructed containing the intact *eta* region, except for the 3' terminal region downstream of the EcoRI site. Agglutination tests confirmed that *E. coli* C6008S transformed with *petαJ* produced cETA in quantities similar to those produced by *E. coli* C6008S transformed with *petαI* (data not shown).

Preparation of deletion mutants. Deletion mutants for the *etaJ2* insertion were made with the Kilo-Sequence Deletion Kit (Takara Shuzo). Five *ETAexp* deletion mutants were obtained with a sandwich ELISA of culture supernatants with rabbit anti-ETA serum, and their cETA production rates were assessed with the single radial immunodiffusion test or the latex agglutination test. Fig. 2 shows a schematic representation of the *etaJ2* insertion and the deletion mutants (dm-1, -2, -3, -4 and -5).

Determination of nucleotide sequences of the *etaJ2* insertion and *ETAexp* deletion mutants. The nucleotide sequences of the *etaJ2* insertion and the five deletion mutants were determined with the dideoxy chain-termination method (Sambrook et al., 1989) and the Kilo-Sequence Deletion Kit (Takara Shuzo) following the manufacturer's instructions. The entire nucleotide sequence upstream of the EcoRI site in the *etaJ2* insertion to the end of the leader sequence (containing part of the −35 promoter sequence for the 1·45 kb *eta* sequence) was determined.

Construction of 1·7 kb *eta-ETAexp/pUC9* and *ETAexp/pUC9* recombinant plasmids. The inserted sequence on the recombinant pUC9 plasmid containing a 1·7 kb *eta* at the SmaI insertion site (*petαJ3*) was digested with HindIII or EcoRI, and a flush end at the HindIII or EcoRI site was created with the Kilo-Sequence Deletion Kit (Takara Shuzo). The primers used to amplify the *ETAexp* fragment were 5’-CATTGGAATGCAATAGC-3’ (primer 1), corresponding to nucleotides −432 to −416 of the *ETAexp* sequence, and 5’-AAGTCTTATTTCAACAG-3’ (primer 2), corresponding to nucleotides 946 to 964 of the *ETAexp* sequence, as counted from the putative initiation codon, residue +1 (ATG). The *ETAexp* fragment was amplified with 1 μg nucleic acid as the template in a 50 μl reaction mixture of the following composition: 50 mM KCl, 10 mM Tris/HCl (pH 8·3), 1·5 mM MgCl2, 0·01% (w/v) gelatin, 200 μM of each of the dNTPs, 0·2 μM of each of the *ETAexp*-specific oligonucleotide primers, and 1·25 units of *Taq* polymerase (Toyobo). The sample was denatured for 0·5 min at 94°C, and PCR was performed for 30 cycles at 94°C for 1·0 min, at 57°C for 1·0 min and 72°C for 1·0 min with autoextension in a MiniCycler (MJ Research). A single adenine 3'-end overhang on the 1·4 kb *ETAexp* sequence amplified with PCR was removed with a Sure Clone Ligation Kit (Amersham Pharmacia Biotech), and the amplified DNA fragments were isolated by 1% (w/v) agarose gel electrophoresis using DEAE ion-exchange chromatography (DE81, Whatman). The purified PCR products were ligated with recombinant pUC9 plasmid, *petαJ* digested with HindIII or EcoRI, or pUC9 digested with SmaI, and the recombinant plasmids (*petαJ4*, *petαJ5* or *ETAexp*/pUC9) were constructed. The cETA production rates of *petαJ4* and *petαJ5* were determined by the single radial immunodiffusion test. *ETAexp*/pUC9 was used for simultaneous transformation with 1·7 kb *eta/pHY300PLK.

Construction of the 1·7 kb *eta/pHY300PLK* recombinant plasmid. The insertional sequence of the recombinant pUC9 plasmid containing the 1·7 kb *eta* sequence at the SmaI site was digested with EcoRI and HindIII, after which the 1·7 kb *eta EcoRI–HindIII* fragment was isolated electrophoretically with Whatman DE81 paper. *pHY300PLK* was digested with EcoRI and HindIII; the recombinant plasmid (*petαJ3*-300) was constructed by incubating *pHY300PLK* with calf intestine alkaline phosphatase followed by ligation with the 1·7 kb *eta* EcoRI–HindIII fragment.

Preparation of crude toxin. The recombinant plasmid was transformed into *E. coli* C6008S; the transformant was grown for 18 h at 37°C in 500 ml 2×TY broth containing 50 μg ampicillin ml−1, then centrifuged at 8000 g for 20 min at 4°C. The culture supernatant and the precipitated cell fractions were used to prepare toxin solution, as reported previously (Sakurai et al., 1998). Five hundred millilitres of culture supernatant was saturated by adding 342 g ammonium sulfate with stirring on ice, followed by centrifugation at 8000 g for 20 min at 4°C (ammonium-sulfate-precipitated culture supernatant fraction). The precipitated cell fractions were suspended in 10 ml distilled water, sonicated with an ultrasonic disruptor (UD 201, Tomy Seiko) on ice, and then centrifuged at 14000 g for 20 min at 4°C. The resulting supernatant was saturated by the addition of 8·5 g ammonium sulfate with stirring on ice followed by centrifugation at 14000 g for 20 min at 4°C (ammonium-sulfate-precipitated cell extract fraction). The ammonium-sulfate-precipitated fractions were dissolved in 20 ml distilled water and dialysed against 2 l distilled water at 4°C for 16 h with a VT 801 dialysis tube (molecular sieving of 8000) while being stirred with a magnetic stirrer at 4°C overnight. The dialysate was lyophilized, and the dried material was dissolved in 3 ml distilled water and subjected to the single radial immunodiffusion test and immunoblot analysis. The ETA-producing strain *S. aureus* ZM was grown at 37°C for 48 h in 500 ml 1½ TY medium, as reported previously (Kondo et al., 1973), and then centrifuged at 8000 g for 20 min at 4°C. 

**Fig. 2.** Schematic representation of the *etaJ2* insertion and its deletion mutants (dm-1, -2, -3, -4 and -5). Positions 1–299 on the ORF (*ETAexp*), codons from ATG (+1) to the TAA stop codon of the ORF (*ETAexp*); positions 1 and 38 on ETA, signal sequence of ETA; positions 99 and 280 on ETA, mature ETA; E, EcoRI.
hundred millilitres of the culture supernatant was saturated by the addition of 342 g ammonium sulfate with stirring on ice, followed by centrifugation at 8000 g for 20 min at 4 °C. The ammonium-sulfate-precipitated culture supernatant fraction from S. aureus ZM (sETA) was dissolved in 20 ml distilled water and dialysed against 2 l distilled water, after which the dialysate was lyophilized as described above. The dried material was dissolved in 3 ml distilled water and then used for immunoblot analysis. The methods of isolation and purification of sETA and the immunization procedure were as described previously (Kondo et al., 1973).

**Single radial immunodiffusion test of cETA.** A single radial immunodiffusion test was performed on a glass slide with 3 ml 1% (w/v) agarose gel in 10 mM Tris/HCl–0·5 M NaCl, pH 7·5, containing rabbit anti-sETA serum. The concentration of antisera in the agarose gel was adjusted to 1:75, and the plate was examined for the formation of turbid haloes around the wells with the ammonium-sulfate-precipitated cell extract fraction in a moisture chamber at room temperature.

**Immunoblot analysis of cETA.** A 10 µl sample of the culture supernatant, the ammonium-sulfate-precipitated culture supernatant fraction, and the ammonium-sulfate-precipitated cell extract fraction of petaJ1 and petaJ3 were used to assess production of cETA. The ammonium-sulfate-precipitated culture supernatant fraction from S. aureus ZM (sETA) was used as a control. Samples were separated with SDS-PAGE according to the method of Laemmli (1970), electrophoretically transferred to PVDF membranes using the Semi-Dry Blot Apparatus (ATTO) according to the manufacturer’s instructions, and then incubated with rabbit anti-sETA IgG followed by a goat anti-rabbit IgG–horseradish peroxidase conjugate. sETA was purified with a procedure previously reported (Kondo et al., 1973) and used as the molecular mass standard.

**Northern blot analysis of RNA from petaJ1 and petaJ2.** RNA from petaJ3 or petaJ1 transformants that had grown to the early stationary phase in 2×TY medium at 37 °C was extracted with the RNasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. A 30 µg sample of RNA was electrophoresed on a gel containing 1-0% (w/v) agarose, 20 mM MOPS, pH 7-0, and 2-2 M formaldehyde (Sambrook et al., 1989). The RNA was transferred to a nylon membrane (Amersham Pharmacia Biotech) with a model 785 vacuum blotter (Bio-Rad). The filters were prehybridized and then hybridized with digoxigenin (DIG; Roche Diagnostics)-labelled 1·7 kb eta probe prepared according to the manufacturer’s instructions. Signals were visualized with a DIG Luminescent Detection Kit (Roche Diagnostics) according to the manufacturer’s instructions.

**Assay of rsETA by latex agglutination test.** S. aureus was cultured in LB medium at 30 °C for 48 h, and the culture supernatant was used for the latex agglutination test, performed with a latex agglutination kit (Denka-Seiken) according to the manufacturer’s instructions.

**RESULTS**

**Single radial immunodiffusion test of cETA from petaJ1 and deletion mutants**

An extremely large amount of cETA was produced by the 5·8 kb eta sequence (Fig. 3). The first deletion mutant, dm-1, produced only a slight halo, whereas the cETA produced by two mutants, dm-2 and dm-3, did not form precipitation haloes around the wells containing anti-sETA serum.

**Nucleotide sequences of ETAexp and deletion mutants**

The 1·4 kb ETAexp fragment was sequenced after the 5·8 kb etaJ1 sequence was digested with EcoRI to remove the 3’-terminal sequence (Fig. 1). The resulting 4·2 kb segment was ligated with pUC118 digested with EcoRI, and the nucleotides in the etaJ2 insertion from the EcoRI site to the end of the leader sequence of eta, which was common to that of the etaJ3 insertion, were sequenced. One large ORF extending from the putative initiation codon, residues +1 (ATG) to 894, just before the stop codon TAA, with the potential to encode a 33 kDa polypeptide, was identified. The probable ATG methionine initiation codon (Stormo et al., 1982) is preceded 7 bp upstream by the sequence GCCAA (position −8 to −13), which is a potential ribosome-binding site (Shine & Dalgarno, 1974). A potential stem–loop structure can be formed from nucleotides 907 to 932, as counted from the putative initiation codon. This stem–loop structure was 12 bp distal to the stop codon TAA. Translation of the ORF identified from the DNA sequence yielded a 298 amino acid polypeptide with a molecular mass of 33 kDa. Deletion mutants were created to determine whether any of the proposed regulatory sequences were functional, either in vivo or in vitro. The first mutation (dm-1) at position −312 had a deletion in the sequence upstream of the 1·4 kb ETAexp. The second mutation (dm-2) lacked the sequence upstream of position −134, and the other three mutants (dm-3, −4 and −5) lacked the initial sequence to the positions downstream of the putative +1 initiation codon, ATG. The fact that deletion mutant dm-1 decreased cETA production (Fig. 3) suggests that a sequence upstream from the dm-1 site at position −311 might be the most important structural element for the activation of ETA production by ETAexp.

![Fig. 3. Single radial immunodiffusion test of the ammonium-sulfate-precipitated cell extract fractions from petaJ1 and three deletion mutants. Well 1, pETAJ1; well 2, dm-1; well 3, dm-2; well 4, dm-3.](image-url)
Immunoblot analysis of cETA

On immunoblotting, cETA produced by E. coli C6008S transformed with petaJ1 was identical in size to the purified sETA from S. aureus ZM (Fig. 4, lane 1). cETA was expressed at high levels in the culture supernatant (Fig. 4, lane 4), the ammonium-sulfate-precipitated culture supernatant fraction (Fig. 4, lane 2), and the ammonium-sulfate-precipitated cell extract fraction from E. coli C6008S transformed with petaJ1 (Fig. 4, lane 6). The amount of cETA produced by petaJ1 (Fig. 4, lane 2) was almost the same as that produced by the control S. aureus ZM strain (data not shown). However, the cETA in E. coli C6008S transformed with petaJ3 could not be detected in the culture supernatant (Fig. 4, lane 5), the ammonium-sulfate-precipitated culture supernatant fraction (Fig. 4, lane 3), or the ammonium-sulfate-precipitated cell extract fraction (Fig. 4, lane 7).

Northern blot analysis

Northern blot analysis of mRNA from early-stationary-phase cultures with DIG-labelled 1·7 kb eta as a probe showed that eta transcripts were much more abundant in E. coli C6008S transformed with petaJ1 than in E. coli C6008S transformed with petaJ3 (Fig. 5). The eta transcript in E. coli C6008S transformed with petaJ1 was approximately 1 kb in size and was apparently monocistronic, because the molecular mass of the mature secreted form of cETA is 26·9 kDa. The concentration of eta mRNA produced by E. coli C6008S was much lower when cells were transformed with petaJ3 than when transformed with petaJ1, a finding consistent with the results of the immunoblot analysis showing that an extremely large amount of cETA was produced in E. coli C6008S transformed with petaJ1 (Fig. 4, lanes 2 and 6).

Single radial immunodiffusion test of the 1·7 kb eta-ETAexp/pUC9 recombinant plasmid

An extremely large amount of cETA was produced when ETAexp was linked to an upstream (petaJ4; Fig. 6, well 1) or downstream (petaJ5; Fig. 6, well 3) 1·7 kb eta sequence, regardless of the orientation of insertion of the ETAexp amplified with PCR.

Assay of rsETA produced by S. aureus strain FRI-1169 transformed with pYT3-etaJ3 or pYT3-etaJ6

The rsETA prepared from the S. aureus strain FRI-1169 transformant into which the recombinant plasmid
pYT3-etaJ6 had been introduced was expressed at high levels in the culture supernatant fraction in quantities similar to those produced by _E. coli_ C600085 transformed with petaJ1, as shown by the latex agglutination test (results not presented). The latex agglutination titre of rsETA in the culture supernatant of the _S. aureus_ strain FRI-1169 transformed with pYT3-etaJ6 was 20 000. However, the latex agglutination titre of rsETA in the culture supernatant of _S. aureus_ FRI-1169 transformed with pYT3-etaJ3 (containing the 1·7 kb eta sequence with the 1·4 kb ETAexp-deficient eta) was 2500–4000 times lower than that of pYT3-etaJ6.

**DISCUSSION**

We have presented evidence that the _eta_ gene is positively regulated at the transcriptional level by the ETAexp gene (Fig. 5). These results suggest that the ETAexp gene product increases _eta_ transcription by encoding a positive regulatory protein that interacts with the _eta_ promoter region. Alternatively, the ETAexp gene product may produce a physiological state in the cell which is necessary for high _eta_ promoter activity. Miller & Mekalanos (1984) showed that the positive regulatory gene toxR in _Vibrio cholerae_ regulates toxin expression by controlling transcription of the ctxAB promoter, and Miller et al. (1987) presented evidence that the toxR gene product (ToxR) is a 32 527 Da DNA-binding, integral membrane protein that also binds a tandemly repeated sequence located upstream of the cholera toxin promoter region.

To test the hypothesis that the ETAexp gene product binds to the _eta_ promoter region and activates transcription, we developed a model of the secondary and tertiary structure of the ETAexp gene product predicted by computer analysis of the ETAexp nucleotide sequence. The most striking feature of the ETAexp protein is a helix–turn–helix motif that is present in several other previously characterized DNA-binding proteins (Pabo & Sauer, 1984). Many DNA-binding proteins recognize dyad symmetrical sequences, and an inverted sequence is present between the −10 region corresponding to the consensus sequence of the _E. coli_ promoter and the SD sequence of etaJ3 (Sakurai et al., 1987). These findings suggest that the ETAexp protein binding to the promoter region is a necessary step in the transcriptional activation of the _eta_ promoter.

When petaJ4 or petaJ5 was cloned into _E. coli_, the transformant produced a large amount of cETA on the single radial immunodiffusion test regardless of the orientation of the ETAexp insert (Fig. 6). These findings suggest that ETAexp activates the promoter of etaJ3 as a transcriptional activator. However, when ETAexp/pUC9 and the petaJ3-300 recombinant plasmid were simultaneously cloned into _E. coli_, the latex agglutination titre of the ammonium-sulfate-precipitated cell extract fraction from the transformant was 5000 to 10 000 times lower than that of petaJ1 (data not shown), suggesting that ETAexp must be located nearby upstream or downstream of the _eta_ gene.

In the above latex agglutination experiment, the rsETA prepared from an _S. aureus_ transformant into which the recombinant plasmid pYT3-etaJ6 had been introduced was expressed at high levels in the culture supernatant fraction in quantities similar to those produced by _E. coli_ C600085 transformed with petaJ1. However, the latex agglutination titre of rsETA in the culture supernatant of _S. aureus_ FRI-1169 transformed with pYT3-etaJ3 (containing the 1·7 kb eta with the 1·4 kb ETAexp-deficient eta) was 2500–4000 times lower than that of pYT3-etaJ6. pYT3-etaJ6 introduced into _S. aureus_ clinical isolates that produce _z_-toxin, TSST-1, staphylococcal enterotoxin B (SEB) and SAK had no effect on the rate of production of _z_-toxin, SEB, TSST-1, SAK, protein A or coagulase (data not shown). These results indicate that a functional ETAexp element is required for expression of the _eta_ gene, although we did not examine the production of other exoproteins, such as serine protease, nuclease and _β_-haemolysin. An accessory gene regulator (agr), a chromosomal locus of _S. aureus_, is required for the high-level post-exponential-phase expression of several exoproteins, such as _z_-haemolysin, serine protease, TSST-1, _δ_-haemolysin, nuclease, _β_-haemolysin and enterotoxin B (Recsei et al., 1986; Morfeldt et al., 1988). The agr gene has also been cloned in _E. coli_, and its nucleotide sequence has been determined (Peng et al., 1988; Janzon et al., 1989). Our analysis has shown that the nucleotide sequence of ETAexp is distinct from the nucleotide sequence of three global regulatory loci of _S. aureus_ (agr, sar and Mgr; data not shown). In this experiment, the ETA produced by five deletion mutants of ETAexp in the 5·8 kb eta sequence and

---

**Fig. 6.** Single radial immunodiffusion test of cETA. An extremely large amount of cETA was produced when ETAexp was linked upstream (petaJ4; well 1) or downstream (petaJ5; well 3) of 1·7 kb eta. Centre well, 1·7 kb _eta_.

---

**S. Sakurai and others**
the 1-kb eta sequence was detected only with ELISA; however, the cETA produced by the deletion mutants and the 1-kb eta did not form precipitation haloes on the single radial immunodiffusion test (Fig. 6, well 2), and their exfoliative activity could not be detected with an in vivo assay (data not shown) performed with a method reported previously (Kondo et al., 1973). These results indicate that the integrity of ETAexp in the upstream region of eta is required for high expression of the eta gene. Our Southern blot analysis using eta and ETAexp probes showed that eta and ETAexp are located only on the 4-kb HindIII fragment of chromosomal DNA from ETA-producing strains of S. aureus (data not shown). The SAK gene is located on a staphylococcal serotype B bacteriophage (Kondo & Fujise, 1977), and the nucleotide sequences of the lytA gene in staphylococcal serotype B phage 80, the eta gene and the SAK gene have homologous downstream regions (Bon et al., 1997). Yoshizawa et al. (2000) have reported that when the restriction-minus, non-ET-producing S. aureus 1039 strain was lysogenized with the temperate phage ϕ-ZM-1 from an ETA-producing ZM strain, clones of 6 of 10 lysogens produced ETA and the eta gene fragment could be amplified with PCR. Why the temperate phage ϕ-ZM-1 carries both the eta gene and ETAexp is unknown. ETAexp may act as an activator of some other gene or gene(s) in the phage genome. In a recent preliminary experiment, we found that the ammonium-sulfate-precipitated cell extract fraction from the E. coli transformed with the petaI1 recombinant plasmid binds to the 451 bp promoter fragment obtained from the 1-kb eta after digestion with Rsal. We are now isolating and characterizing the ETAexp protein.

REFERENCES


