

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

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A 1.4 kb positive regulatory element (*ETA<sup>exp</sup>*) that controls staphylococcal exfoliative toxin A (sETA) transcription was cloned from *Staphylococcus aureus*. *ETA<sup>exp</sup>* 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 *petaJ3* containing the 1.7 kb *eta* sequence (*etaJ3*) with a 1.45 kb *ETA<sup>exp</sup>*-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 *ETA<sup>exp</sup>* amplified by PCR (1.7 kb *eta-ETA<sup>exp</sup>*/pUC9), regardless of the orientation of *ETA<sup>exp</sup>* 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-ETA<sup>exp</sup>*/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 *ETA<sup>exp</sup>*-deficient *eta* fragment (*pYT3-etaJ3*) was 2500–4000 times lower than that of *pYT3-etaJ6*.

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## 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

exfoliative toxin (Kondo *et al.*, 1973, 1974, 1975; Kondo & Sakurai, 1976) and to report that they have equal biological activity. The *eta* gene is expressed by a lysogenic bacteriophage integrated within chromosomes (Yoshizawa *et al.*, 2000; Yamaguchi *et al.*, 2000), whereas the *etb* gene is plasmid-linked (O'Toole & Foster, 1986a; Lee *et al.*, 1987). Both the *eta* and *etb* genes have been cloned (O'Toole & Foster, 1986b; Sakurai *et al.*, 1987; Lee *et al.*, 1987) and sequenced (O'Toole & Foster, 1986c; Lee *et al.*, 1987; Sakurai *et al.*, 1988). We have previously shown that cloning the 5.8 kb *eta* sequence from *S. aureus* ZM, the standard ETA-producing strain, into *Escherichia coli* C6008S with pUC9 leads to the production of a large amount of cETA, almost the same amount as produced by the *S. aureus* ZM strain

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 digestion with *AluI* (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 (*ETA<sup>exp</sup>*) 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 *ETA<sup>exp</sup>*-deficient *eta* was 2500–4000 times lower than that of pYT3-*etaJ1*.

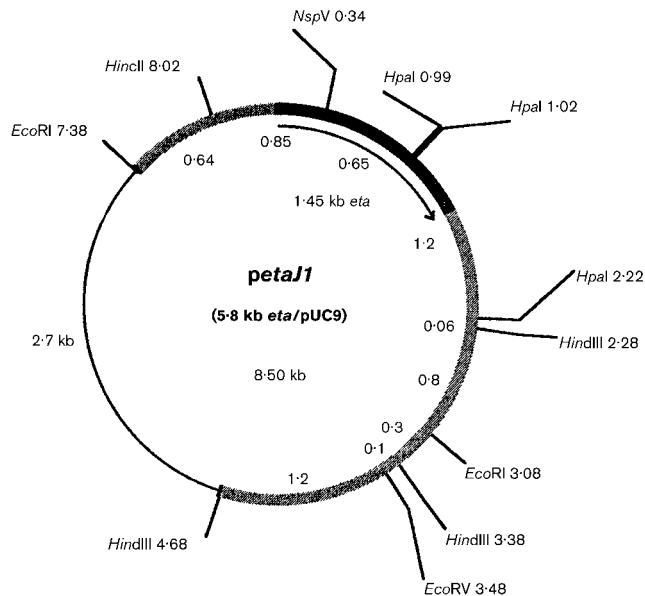
## METHODS

**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<sup>-1</sup>. The *S. aureus* strain FRI-1169 producing toxic shock syndrome toxin-1 (TSST-1) 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-ETA<sup>exp</sup>*/pYT3 (pYT3-*etaJ6*) and 1.7 kb *eta*/pYT3 (pYT3-*etaJ3*) recombinant plasmids was as follows. The 4.2 kb fragment containing the *ETA<sup>exp</sup>* 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 *petaJ1* digested with *EcoRI* or the recombinant plasmid *petaJ3* digested by *EcoRI* were removed using the Kilo-Sequence Deletion Kit (Takara Shuzo), 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-ETA<sup>exp</sup>*) or the 1.7 kb *eta* sequence (*etaJ3*) carrying 1.4 kb *ETA<sup>exp</sup>*-deficient *eta* was inserted into the shuttle plasmid pYT3 (kindly provided by Professor K. Hiramatsu, Department of Bacteriology, Juntendo University, Japan) cleaved at the *SalI* site; the 5' protruding ends



**Fig. 1.** Construction and partial restriction endonuclease map of the 5.8 kb *eta* sequence produced by ligating *AluI*-digested fragments of the chromosomal DNA of *S. aureus* ZM, the standard ETA producer, and *SmaI*-cleaved pUC9 with T4 DNA ligase.

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 OD<sub>595</sub> 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 Easyject 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<sup>-1</sup>. The plates were incubated for 48 h at 30 °C (Yoshizawa, 1985; Rhoads & Iandolo, 1990; Schenk & Laddaga, 1992).

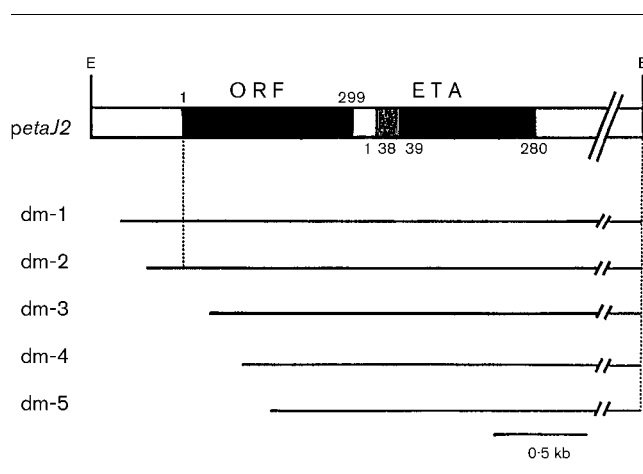
***petaJ3* and *petaJ1* recombinant plasmids.** In all experiments, we used a recombinant pUC9 plasmid containing a 5.8 kb *eta* *AluI* insert (*petaJ1*) or a 1.7 kb *eta* *AluI* insert (*petaJ3*) prepared according to a previously described procedure (Sakurai *et al.*, 1987; Sakurai *et al.*, 1988). The *petaJ1* recombinant plasmid was constructed by

ligating the 5.8 kb *eta* *Alu*I fragment of the chromosomal DNA from *S. aureus* ZM, an ETA-producing strain, and a *Sma*I-cleaved pUC9 with T4 DNA ligase. The *petaJ3* 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*I and *Sma*I-cleaved pUC9. The production of cETA by ampicillin-resistant *E. coli* C6008S transformants (Amp<sup>r</sup>) was assessed with a sandwich ELISA of culture supernatants with rabbit anti-ETA serum, as previously described (Sakurai *et al.*, 1987, 1998).

**Construction of *petaJ2* recombinant plasmid.** The 5.8 kb *eta* sequence was digested with *Eco*RI to remove the 3' terminal sequence (Fig. 1). The resulting 4.2 kb segment (*etaJ2*) was ligated with pUC118 digested with *Eco*RI, and a recombinant plasmid (*petaJ2*) was constructed containing the intact *eta* region, except for the 3' terminal region downstream of the *Eco*RI site. Agglutination tests confirmed that *E. coli* C6008S transformed with *petaJ2* produced cETA in quantities similar to those produced by *E. coli* C6008S transformed with *petaJ1* (data not shown).

**Preparation of deletion mutants.** Deletion mutants for the *etaJ2* insertion were made with the Kilo-Sequence Deletion Kit (Takara Shuzo). Five *ETA*<sup>exp</sup> deletion mutants were obtained with a sandwich ELISA of culture supernatant 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 *ETA*<sup>exp</sup> 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 *Eco*RI 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.



**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 (*ETA*<sup>exp</sup>), codons from ATG (+1) to the TAA stop codon of the ORF (*ETA*<sup>exp</sup>); positions 1 and 38 on ETA, signal sequence of ETA; positions 39 and 280 on ETA, mature ETA; E, *Eco*RI.

**Construction of 1.7 kb *eta-ETA*<sup>exp</sup>/pUC9 and *ETA*<sup>exp</sup>/pUC9 recombinant plasmids.** The inserted sequence on the recombinant pUC9 plasmid containing a 1.7 kb *eta* at the *Sma*I insertion site (*petaJ3*) was digested with *Hind*III or *Eco*RI, and a flush end at the *Hind*III or *Eco*RI site was created with the Kilo-Sequence Deletion Kit (Takara Shuzo). The primers used to amplify the *ETA*<sup>exp</sup> fragment were 5'-CATTGGATGTC AATGC-3' (primer 1), corresponding to nucleotides -432 to -416 of the *ETA*<sup>exp</sup> sequence, and 5'-AACGTTTCTATTTCAAAC-3' (primer 2), corresponding to nucleotides 946 to 964 of the *ETA*<sup>exp</sup> sequence, as counted from the putative initiation codon, residue +1 (ATG). The *ETA*<sup>exp</sup> 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 MgCl<sub>2</sub>, 0.01% (w/v) gelatin, 200 µM of each of the dNTPs, 0.2 µM of each of the *ETA*<sup>exp</sup>-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 *ETA*<sup>exp</sup> 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, *petaJ3* digested with *Hind*III or *Eco*RI, or pUC9 digested with *Sma*I, and the recombinant plasmids (*petaJ4*, *petaJ5* or *ETA*<sup>exp</sup>/pUC9) were constructed. The cETA production rates of *petaJ4* and *petaJ5* were determined by the single radial immunodiffusion test. *ETA*<sup>exp</sup>/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 *Sma*I site was digested with *Eco*RI and *Hind*III, after which the 1.7 kb *eta Eco*RI-*Hind*III fragment was isolated electrophoretically with Whatman DE81 paper. pHY300PLK was digested with *Eco*RI and *Hind*III; the recombinant plasmid (*petaJ3*-300) was constructed by incubating pHY300PLK with calf intestine alkaline phosphatase followed by ligation with the 1.7 kb *eta Eco*RI-*Hind*III 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<sup>-1</sup>, 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 14 000 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 14 000 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 TY medium, as reported previously (Kondo *et al.*, 1973), and then centrifuged at 8000 g for 20 min at 4 °C. Five

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 antiserum 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 *petaJ3*.** 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 RNeasy 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* 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 rETA 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.

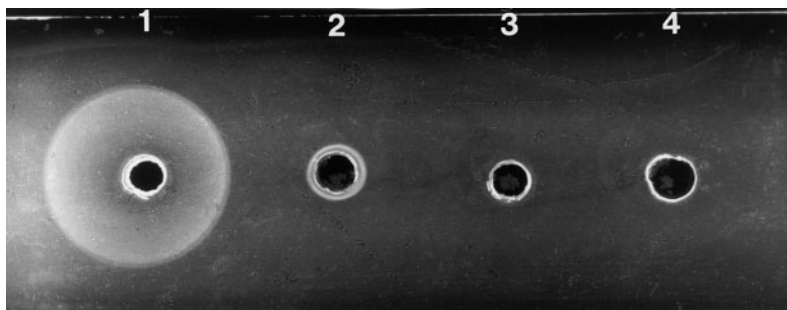
## RESULTS

### 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 *ETA<sup>exp</sup>* and deletion mutants

The 1.4 kb *ETA<sup>exp</sup>* 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 GGCAA (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. 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 *ETA<sup>exp</sup>*. 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 *ETA<sup>exp</sup>*.



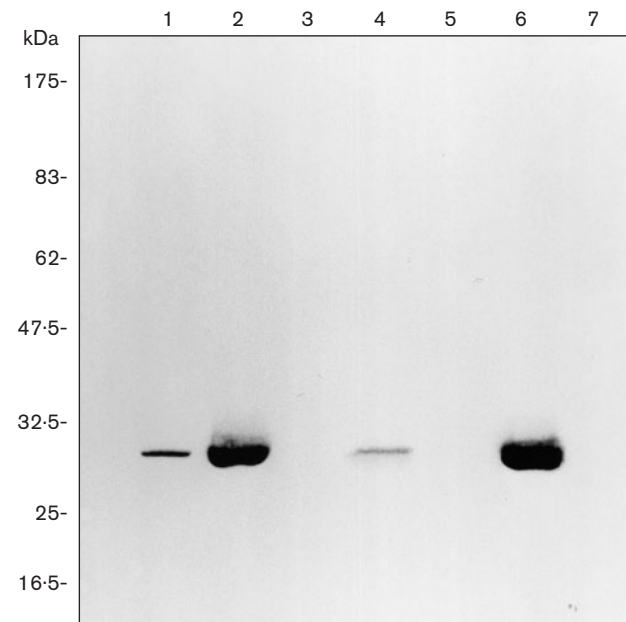
**Fig. 3.** Single radial immunodiffusion test of the ammonium-sulfate-precipitated cell extract fractions from *petaJ1* and three deletion mutants. Well 1, pETAJ1; well 2, dm-1; well 3, dm-2; well 4, dm-3.

### 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



**Fig. 4.** Immunoblot analysis of the culture supernatant, the ammonium-sulfate-precipitated (ASP) culture supernatant fraction and the ASP cell extract fraction prepared from *E. coli* containing *petaJ1* or *petaJ3*. Lane 1, purified sETA of *S. aureus* ZM as a molecular mass standard; lane 2, ASP culture supernatant fraction from *E. coli* containing *petaJ1*; lane 3, ASP culture supernatant fraction from *E. coli* containing *petaJ3*; lane 4, culture supernatant of *E. coli* containing *petaJ1*; lane 5, culture supernatant of *E. coli* containing *petaJ3*; lane 6, ASP cell extract fraction from *E. coli* containing *petaJ1*; lane 7, ASP cell extract fraction from *E. coli* containing *petaJ3*.

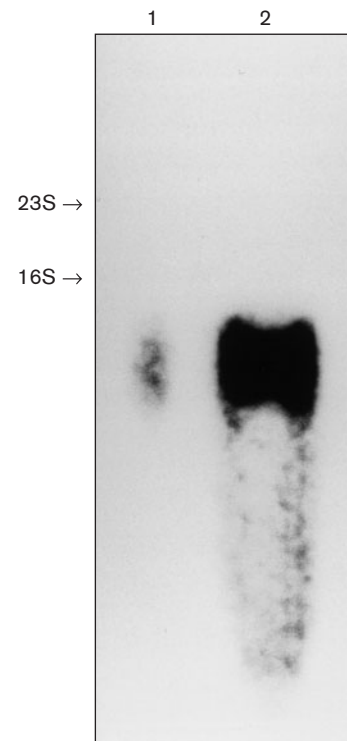
*E. coli* C6008S transformed with *petaJ1* than in *E. coli* C6008S transformed with *petaJ3* (Fig. 5). The *eta* transcript in *E. coli* C6008S transformed with *petaJ1* was approximately 1 kb in size and was apparently monocistronic, because the molecular mass of the mature secreted form of cETA is 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*-*ETA*<sup>exp</sup>/pUC9 recombinant plasmid

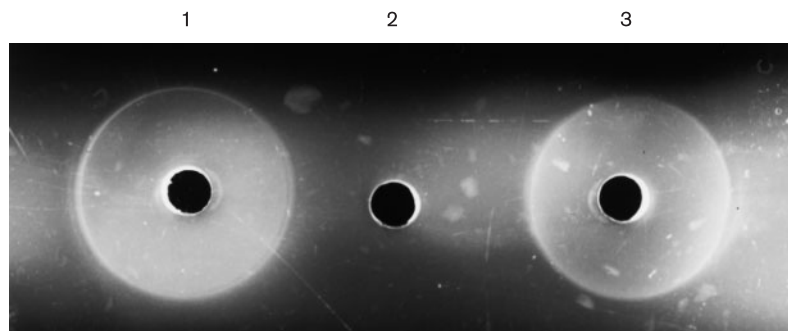
An extremely large amount of cETA was produced when *ETA*<sup>exp</sup> was linked to an upstream (*petaJ4*; Fig. 6, well 1) or downstream (*petaJ5*; Fig. 6, well 3) 1.7 kb *eta* sequence, regardless of the orientation of insertion of the *ETA*<sup>exp</sup> amplified with PCR.

### 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



**Fig. 5.** Northern blot analysis of whole-cell RNA from *E. coli* containing *petaJ1* (lane 2) or *petaJ3* (lane 1). DIG-labelled 1.7 kb *eta* was used as probe.



**Fig. 6.** Single radial immunodiffusion test of cETA. An extremely large amount of cETA was produced when *ETA<sup>exp</sup>* was linked upstream (*petaJ4*; well 1) or downstream (*petaJ5*; well 3) of 1.7 kb *eta*. Centre well, 1.7 kb *eta*.

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 (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 *ETA<sup>exp</sup>*-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 *ETA<sup>exp</sup>* gene (Fig. 5). These results suggest that the *ETA<sup>exp</sup>* gene product increases *eta* transcription by encoding a positive regulatory protein that interacts with the *eta* promoter region. Alternatively, the *ETA<sup>exp</sup>* 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 *ETA<sup>exp</sup>* gene product binds to the *eta* promoter region and activates transcription, we developed a model of the secondary and tertiary structure of the *ETA<sup>exp</sup>* gene product predicted by computer analysis of the *ETA<sup>exp</sup>* nucleotide sequence. The most striking feature of the *ETA<sup>exp</sup>* 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 *ETA<sup>exp</sup>* 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 *ETA<sup>exp</sup>* insert (Fig. 6). These findings suggest that *ETA<sup>exp</sup>* activates the promoter of *etaJ3* as a transcriptional activator. However, when *ETA<sup>exp</sup>*/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 *ETA<sup>exp</sup>* 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* C6008S 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 *ETA<sup>exp</sup>*-deficient *eta*) was 2500–4000 times lower than that of pYT3-*etaJ6*. pYT3-*etaJ6* introduced into *S. aureus* clinical isolates that produce  $\alpha$ -toxin, TSST-1, staphylococcal enterotoxin B (SEB) and SAK had no effect on the rate of production of  $\alpha$ -toxin, SEB, TSST-1, SAK, protein A or coagulase (data not shown). These results indicate that a functional *ETA<sup>exp</sup>* 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  $\beta$ -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  $\alpha$ -haemolysin, serine protease, TSST-1,  $\delta$ -haemolysin, nuclease,  $\beta$ -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; Janson *et al.*, 1989). Our analysis has shown that the nucleotide sequence of *ETA<sup>exp</sup>* 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 *ETA<sup>exp</sup>* in the 5.8 kb *eta* sequence and

the 1.7 kb *eta* sequence was detected only with ELISA; however, the cETA produced by the deletion mutants and the 1.7 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 *ETA<sup>exp</sup>* in the upstream region of *eta* is required for high expression of the *eta* gene. Our Southern blot analysis using *eta* and *ETA<sup>exp</sup>* probes showed that *eta* and *ETA<sup>exp</sup>* are located only on the 4 kb *Hind*III 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  $\phi$ -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  $\phi$ -ZM-1 carries both the *eta* gene and *ETA<sup>exp</sup>* is unknown. *ETA<sup>exp</sup>* 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 *petaJ1* recombinant plasmid binds to the 451 bp promoter fragment obtained from the 1.7 kb *eta* after digestion with *Rsa*I. We are now isolating and characterizing the *ETA<sup>exp</sup>* protein.

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