Molecular Cloning and Expression of the Coagulase Gene of *Staphylococcus aureus* 8325-4

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The gene coding for coagulase (coa) was cloned from *Staphylococcus aureus* 8325-4 in a λ replacement vector in *Escherichia coli*. Coagulase (plasma-clotting) activity was measured in λcoa lysates and an immunoreactive protein of 60 kDa was detected by Western immunoblotting with anti-coagulase serum. This protein comigrated with the major immunoreactive protein in supernatants of *S. aureus* 8325-4. The coa gene was subcloned in pUC vectors. One recombinant expressed a 60 kDa immunoreactive protein and plasma-clotting activity. A putative β-galactosidase-coagulase fusion protein and truncated peptides were expressed by variants formed by subcloning. These results are consistent with previously published biochemical data that the prothrombin-binding domain of coagulase is located in the N-terminus of the protein. The cloned coa gene was transferred into *S. aureus* on a shuttle plasmid. Expression of coagulase was higher in a strain with a mutation in the agr locus, which controls the level of several exoproteins in *S. aureus*, suggesting that agr normally regulates coagulase expression negatively.

INTRODUCTION

*Staphylococcus aureus* is a Gram-positive pathogenic bacterium which causes a variety of human tissue infections including furuncles, wound sepsis, osteomyelitis and endocarditis (for reviews see McCartney & Arbuthnott, 1978; Easmon & Adlam, 1983a, b). It is also the major cause of mastitis, an economically important disease of dairy cattle (Anderson, 1983).

The organism produces an array of extracellular and cell-bound proteins which are potentially important in pathogenesis (for reviews see McCartney & Arbuthnott, 1978; Easmon & Adlam, 1983a, b). Coagulase is one such protein (Jeljaszewicz et al., 1986). It has the ability to stimulate the clotting reaction of plasma. This is often used to identify *S. aureus* and to distinguish it from less pathogenic species of staphylococci. Coagulase-negative strains are less virulent than the coagulase-positive *S. aureus* (Kinsman & Arbuthnott, 1980) and it has long been inferred that coagulase is a virulence factor. This is supported by the observations that coagulase-deficient mutants of *S. aureus* are less virulent for mice (Haraldsson & Jonsson, 1984; Hasegawa & San Clemente, 1978; Jonsson et al., 1985; Masuda, 1983) and that the purified protein can cause pathological changes in laboratory animals (Anderson et al., 1982; Smith & Johnstone, 1958).

Coagulase is an extracellular protein which stimulates the clotting reaction in plasma by forming a stoichiometric, non-covalent complex with prothrombin (Hemker et al., 1975). In contrast to the activation of prothrombin by blood-clotting factor Xa to form thrombin, the formation of staphylothrombin does not involve proteolytic cleavage of the blood protein (Hemker et al., 1975). The prothrombin-binding and procoagulant regions of coagulase have been located in the amino terminus of the protein by analysis of chymotrypsin fragments (Kawabata et al., 1986a, b).

The expression of some extracellular proteins by *S. aureus* is dependent on a regulatory locus called agr (Bjorklind & Arvidson, 1980; Recsei et al., 1986). In agr-like regulatory mutants of
strain V8 both protein A and coagulase were derepressed, suggesting that expression is negatively regulated (Bjorklind & Arvidson, 1980).

In order to facilitate molecular genetic analysis of coagulase production we have cloned the coagulase gene from S. aureus 8325-4 in phage λ and plasmid vectors in E. coli. Several truncated and fusion derivatives of coagulase were isolated and their ability to stimulate the clotting reaction was tested. A shuttle plasmid carrying coa was constructed and transferred into S. aureus strains allowing regulation of coagulase expression to be examined.

METHODS

Bacterial strains, plasmids and phages. The bacterial strains are listed in Table 1 and the plasmids in Table 2. The replacement vector AL47.1 (Loenen & Brammar, 1980) was used to clone the coa gene. Transduction in S. aureus was done using phage 80a (obtained from H. Pomeroy, Microbiology Department, Trinity College, Dublin, Ireland).

Bacteriological media. E. coli strains were grown in L broth and L agar (Miller, 1972) while S. aureus was routinely cultured in Trypticase Soy Broth and Agar (BBL). λ phages were propagated in λ base and top agar (Miller, 1972). Brain heart infusion broth (BHI) (Oxoid) was used for culturing S. aureus. I phages were propagated in 1 base and top agar.

Chemicals, antibiotics and enzymes. Laboratory chemicals were obtained from Sigma or were the best grade available from BDH. Ampicillin (Ap) was a gift from Beecham. Chloramphenicol (Cm), erythromycin (Em) and tetracycline (Tc) were purchased from Sigma. Restriction enzymes and T4 DNA ligase were obtained from Boehringer and were used according to the manufacturer's instructions. Coagulase protein purified from S. aureus was from Sigma.

Assay for coagulase. Coagulase in S. aureus culture supernatants was measured as follows. Dilutions of supernatants (0.5 ml) were added to 0.5 ml of rabbit plasma diluted 1:3 in phosphate-buffered saline (PBS). The titre was the reciprocal of the highest dilution showing evidence of clotting after 24 h incubation at 37°C (Anderson et al., 1982). In the case of E. coli strains carrying pCOA plasmids, cells from cultures grown for 16 h were concentrated 100-fold and lysed by the procedure used for plasmid DNA preparation (Maniatis et al., 1982). After lysis, benzamidine (2 mM) and phenylmethylsulphonyl fluoride (2 mM) were added. A 30 μl sample was diluted in 0.5 ml PBS. Doubling dilutions were made in PBS and 0.5 ml volumes were added to 0.5 ml of rabbit plasma diluted as above. Coagulase expressed by λcoa was measured in dilutions of lysates prepared as described previously (O'Toole & Foster, 1986a).

Antisera. Anti-coagulase serum raised in rabbits against coagulase purified from strain CN6708 was a gift from Dr C. Adlam (Wellcome, Beckenham, Kent, UK).

Plaque and colony immunoblotting. λ plaques producing coagulase were detected by laying 82 mm nitrocellulose discs (Schleicher and Schuell) onto phage overlay plates. The filters were incubated successively with bovine albumin (Sigma), sheep plasma adsorbed with an extract of E. coli C600, rabbit anti-coagulase serum and peroxidase-conjugated swine anti-rabbit immunoglobulin serum, according to the procedure described by Russell et al. (1985). Sheep plasma was included to block protein-A-producing recombinants. Expression of coagulase by E. coli colonies was detected by the immunoblotting procedure of Helfman et al. (1983).
Cloning coagulase from Staphylococcus aureus

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Host</th>
<th>Marker(s)*</th>
<th>Relevant properties</th>
<th>Source and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322</td>
<td>E. coli</td>
<td>Ap'Tc'</td>
<td>Cloning vector</td>
<td>Bolivar et al. (1977)</td>
</tr>
<tr>
<td>pUC18</td>
<td>E. coli</td>
<td>Ap'</td>
<td>Cloning vector</td>
<td>Yanish-Perron et al. (1985)</td>
</tr>
<tr>
<td>pUC19</td>
<td>E. coli</td>
<td>Ap'</td>
<td>Cloning vector</td>
<td>Yanish-Perron et al. (1985)</td>
</tr>
<tr>
<td>pSK265</td>
<td>S. aureus</td>
<td>Cm'</td>
<td>Cloning vector</td>
<td>Jones &amp; Khan (1986)</td>
</tr>
<tr>
<td>pCA1</td>
<td>Shuttle</td>
<td>Ap'Cm'</td>
<td>pBR322 linked to pSK265 at EcoRI sites</td>
<td>This study</td>
</tr>
<tr>
<td>pCOA1</td>
<td>E. coli</td>
<td>Ap'</td>
<td>4.2 kb HindIII fragment from (\lambda)coa cloned in pUC18</td>
<td></td>
</tr>
<tr>
<td>pCOA2</td>
<td>E. coli</td>
<td>Ap'</td>
<td>4.2 kb EcoRI fragment from (\lambda)coa cloned in pUC19</td>
<td></td>
</tr>
<tr>
<td>pCOA4</td>
<td>Shuttle</td>
<td>Ap'Cm'</td>
<td>5.2 kb KpnI fragment from (\lambda)coa cloned in pCA1</td>
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</tr>
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<td>pCOA5</td>
<td>E. coli</td>
<td>Ap'</td>
<td>5.2 kb KpnI fragment from pCOA4 in pUC19</td>
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<tr>
<td>pCOA6</td>
<td>E. coli</td>
<td>Ap'</td>
<td>2.7 kb HindIII fragment from pCOA5 in pUC19</td>
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<tr>
<td>pCOA7</td>
<td>E. coli</td>
<td>Ap'</td>
<td>Deletion of 0.85 kb and 1.9 kb (\lambda)coa cloned in pCOA5</td>
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<tr>
<td>pCOA9</td>
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<td>Ap'</td>
<td>Deletion of 0.75 kb XbaI fragment from pCOA5</td>
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<td>pCOA10</td>
<td>E. coli</td>
<td>Ap'</td>
<td>pCOA2 with EcoRI insert in opposite orientation</td>
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</tr>
<tr>
<td>pCOA11</td>
<td>E. coli</td>
<td>Ap'</td>
<td>4.2 kb HindIII fragment from (\lambda)coa cloned in pBR322</td>
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</tr>
<tr>
<td>pCOA12</td>
<td>Shuttle</td>
<td>Ap'Cm'</td>
<td>2 kb Sau3AI fragment from pCC1 cloned in BamHI site of pCOA2</td>
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</tr>
<tr>
<td>pCOA13</td>
<td>E. coli</td>
<td>Ap'</td>
<td>0.95 kb EcoRI fragment deleted from pCOA1</td>
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</tr>
<tr>
<td>pCOA14</td>
<td>E. coli</td>
<td>Ap'</td>
<td>5.2 kb KpnI fragment from pCOA4 in pUC19. Opposite orientation to pCOA5</td>
<td></td>
</tr>
</tbody>
</table>

* Ap, ampicillin; Tc, tetracycline; Cm, chloramphenicol.

**Immunochromatographic analysis of coagulase.** Proteins produced in \(\lambda\)coa lysates were prepared as described previously (O'Toole & Foster, 1986a). Lysates of pCOA-plasmid-carrying strains of E. coli were prepared as described above. The supernatant of a 16 h BH1 broth culture of S. aureus was concentrated by precipitation in 50% saturation ammonium sulphate and dialysed against 1% (w/v) glycine. Proteins were fractionated by SDS-PAGE (Laemmli, 1970) and subjected to Western immunoblotting (Burnette, 1981). Filters were incubated with anti-coagulase serum followed by peroxidase-conjugated protein A.

**Transformation.** Plasmids were transformed into cells of E. coli C600 and TBl made competent by CaCl₂ treatment (Maniatis et al., 1982), and into protoplasts of S. aureus prepared according to the method of O’Reilly et al. (1986).

**Molecular cloning.** A library of S. aureus 8325-4 DNA was constructed in λL47.1 as described previously (O'Toole & Foster, 1986a), except that the genomic DNA was cleaved partially with Sau3AI and the λ replacement vector was cut with BamHI. Subcloning and restriction mapping experiments were done using standard procedures (Maniatis et al., 1982). Recombinant phages from the gene library were plated on E. coli C600 and plaques were screened by filter immunoblotting. Positively reacting plaques were picked, replated and tested for their reaction with pre-immune rabbit serum in order to ensure that the recombinant was not expressing protein A. One phage (\(\lambda\)coa) was studied further: proteins present in a phage lysate were concentrated and tested for plasma-clotting activity and by Western immunoblotting.

**RESULTS**

**Analysis of phage-specified polypeptides**

\(\lambda\)coa lysates expressed a coagulase titre of 64. In addition, \(\lambda\)coa produced a polypeptide which migrated at the same rate as the coagulase expressed in S. aureus 8325-4 culture supernatants...
Table 3. Coagulase titres in E. coli lysates

Each titre is the reciprocal of the highest dilution showing evidence of clotting after 24 h incubation at 37 °C (see Methods).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Coagulase titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C600</td>
<td>&lt;2</td>
</tr>
<tr>
<td>λcoa</td>
<td>64</td>
</tr>
<tr>
<td>C600(pCOA1)</td>
<td>10240</td>
</tr>
<tr>
<td>C600(pCOA2)</td>
<td>&lt;2</td>
</tr>
<tr>
<td>C600(pCOA4)</td>
<td>1024</td>
</tr>
<tr>
<td>C600(pCOA5)</td>
<td>1024</td>
</tr>
<tr>
<td>C600(pCOA6)</td>
<td>512</td>
</tr>
<tr>
<td>C600(pCOA9)</td>
<td>4096</td>
</tr>
<tr>
<td>C600(pCOA11)</td>
<td>1024</td>
</tr>
<tr>
<td>C600(pCOA14)</td>
<td>128</td>
</tr>
</tbody>
</table>

* Nos are the means of three independent experiments.

(Fig. 1, tracks 6 and 7). It should be noted that although the coagulase proteins produced by strains 8325-4 and CN6708 cross-react immunologically, they migrate at slightly different positions in SDS-polyacrylamide gels (Fig. 1, tracks 1, 2, 6 and 7).

Subcloning the coagulase gene in plasmid vectors and analysis of plasmid-specified polypeptides

λcoa DNA was cleaved with KpnI and ligated with KpnI-cut pUC19 vector DNA and transformed into E. coli strain TB1. Lac− colonies were picked and screened by colony immunoblotting using anti-coagulase serum as the primary antibody. Positively reacting colonies were shown to carry a 5.2 kb KpnI fragment from λcoa. Recombinants having the insert in both orientations were found and one of each (pCOA5 and pCOA14) was kept for further analysis. Extracts of cells carrying these plasmids expressed plasma-clotting activity, although the titres differed (Table 3). Many attempts to detect an immunoreactive polypeptide in lysates of pCOA5-carrying cells failed, possibly due to poor expression or degradation, or both. However, an immunoreactive polypeptide which comigrated with the major protein in S. aureus 8325-4 culture supernatants was detected in extracts of E. coli carrying pCOA9, which was derived from pCOA5 by deleting a 0.7 kb XbaI fragment (Fig. 1, tracks 5 and 7). This shows that the intact coa gene had been subcloned from the phage.

A 4.2 kb HindIII fragment from λcoa and a 2.7 kb HincII fragment from pCOA5 were cloned into HindIII-cut and HincII-cut pUC19, forming pCOA1 and pCOA6, respectively. The HindIII fragment was also cloned into the HindIII site of pBR322 to form pCOA11. pCOA1-, pCOA11- and pCOA6-carrying cells reacted positively in colony immunoblotting tests with anti-coagulase serum and extracts clotted rabbit plasma with titres of 10240, 1024 and 512, respectively. However, none of the lysates contained the 60 kDa polypeptide seen in λcoa and pCOA9 extracts. pCOA1 expressed immunoreactive proteins of 45 kDa and 39 kDa but repeated attempts to identify immunoreactive proteins in extracts of pCOA6 failed. pCOA1 probably produces truncated coagulase peptides which retain immunoreactivity and functional activity. If this is the case, the HindIII site lies in the promoter-distal 3′ region of the coding sequence. pCOA6 presumably produces similar, but shorter, truncated polypeptides. Alignment of the cloned fragments with the restriction map of pCOA5 and λcoa (Fig. 2) suggests that the coagulase-coding sequence spans the 0.9 kb EcoRI–HindIII fragment. If this is the case, transcription of the coa gene proceeds in the direction indicated in Fig. 2.

The location of the coa gene in pCOA1 was confirmed by deleting a 0.9 kb EcoRI fragment (i.e. including the EcoRI–HindIII referred to above) to yield pCOA13 (Fig. 2). This plasmid did not express either clotting activity or an immunoreactive protein, showing that the coa gene is located in this region.

In order to localize coa more precisely a 4.2 kb EcoRI fragment was subcloned from λcoa into pUC19. Several immunoreactive colonies were purified for further analysis. All had the 4.2 kb
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Fig. 1. Western immunoblots: lane 1, coagulase purified from strain CN6708 (12 μg); lanes 2 and 6, λ<sub>coa</sub> lysate (80 μg protein); lane 3, lysate of *E. coli*<sub>pCOA2</sub> (14 μg protein); lane 4, lysate of *E. coli*<sub>pCOA1</sub> (45 μg protein); lane 5, lysate of *E. coli*<sub>pCOA9</sub> (47 μg protein); lane 7, *S. aureus* 8325-4 culture supernatant (13-5 μg protein).

EcoRI fragment in the same orientation. A representative recombinant, pCOA2, expressed a polypeptide of 60 kDa which reacted strongly with anti-coagulase serum but extracts lacked plasma-clotting activity.

The orientation of the inserted EcoRI fragment in pCOA2 was reversed. This plasmid (pCOA10) did not express the immunoreactive 60 kDa protein. In addition, insertion of a 2 kb Sau3AI fragment from pC221 into the BamHI site located between the lac promoter of pUC19 and the EcoRI cloning site (forming pCOA12) eliminated expression of the 60 kDa immunoreactive polypeptide. These results clearly demonstrated that expression of this protein is dependent on the vector's promoter, unlike coagulase produced by pCOA1 and pCOA5. It seems likely that the 60 kDa protein is a hybrid formed between the N-terminal residues of β-galactosidase and coagulase.

Two other plasmids were constructed which helped to confirm the location of the coa gene. pCOA7 was formed by deleting two AccI fragments from pCOA5, which eliminated both coagulase activity and immunoreactivity, whereas deleting the 0-7 kb XbaI fragment of pCOA5 (to give pCOA9) did not affect either property.

Expression of the cloned coa gene in *S. aureus*

In order to investigate the regulation of expression of coagulase in *S. aureus* the 5-2 kb KpnI fragment which carries the intact coagulase gene was cloned into the shuttle plasmid pCA1. This derivative (pCOA4) was initially selected in *E. coli* and then transformed into protoplasts of *S. aureus* RN4220. It was then transduced into *S. aureus* 8325-4 <sup>agr</sup> and into ISP546 (<sup>agr</sup> ::Tn551).

The level of expression of coagulase in broth culture supernatants was measured by titrating plasma-clotting activity (Table 4). The plasmid-free <sup>agr</sup> strain 8325-4 expressed a low level of
Fig. 2. Maps of \( \lambda \)coa and pCOA plasmids. Cloned \( S. \) aureus sequences are represented by the narrow parallel lines and \( \lambda \) sequences by the broader parallel lines. Plasmid vector sequences are shown by the single thin line. The proposed direction of transcription of the coa gene and its position are indicated by the filled arrow. The open arrows indicate the position and direction of the lac promoter in the pUC vector. The expression of plasma-clotting activity is indicated by COA, while the presence of immunoreactive products is indicated by CRM. Restriction endonuclease cleavage sites are abbreviated as follows: E, EcoRI; H, HindIII; B, BamHI; S, Sau3A1; K, KpnI; Hc, HincII; Ac, AccI; Pv, PvuII; X, XbaI; Ps, PstI.
Cloning coagulase from Staphylococcus aureus

Table 4. Regulation of coagulase expression in S. aureus

Each titre is the reciprocal of the highest dilution showing evidence of clotting after 24 h incubation at 37 °C (see Methods).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Without plasmid</th>
<th>With pCOA4</th>
</tr>
</thead>
<tbody>
<tr>
<td>8325-4</td>
<td>51 200</td>
<td>307 200</td>
</tr>
<tr>
<td>ISP546</td>
<td>307 200</td>
<td>1 024 000</td>
</tr>
</tbody>
</table>

* Nos are the means of three independent experiments.

coagulase compared to ISP546. When pCOA4 was introduced into 8325-4 and ISP546 the coagulase titres obtained were higher than those obtained with the plasmid-free strains (Table 4). Both these results are consistent with the hypothesis that the coagulase gene is negatively regulated by the agr system.

DISCUSSION

This paper reports the cloning and expression of the coagulase gene of S. aureus 8325-4. Lysates of E. coli formed by the growth of λcoa produced an immunoreactive polypeptide band in SDS-polyacrylamide gels which comigrated with the major immunoreactive band in supernatants of early stationary phase cultures of S. aureus. The HindIII fragment in pCOA1 subcloned from λcoa expressed truncated immunoreactive proteins but retained plasma-clotting activity. This suggests that a HindIII site is located in the 3' part of the coa gene. A deletion from a presumably more promoter-proximal HincII site also expressed coagulase and immunoreactive activity in colony blots. However, this protein was not detectable by Western immunoblotting.

In contrast, pCOA2 produced an immunoreactive peptide of 60 kDa which lacked plasma-clotting activity. It is possible that this protein is a fusion between β-galactosidase from pUC19 and coagulase. Cloning at the EcoRI site in pUC19 would generate a fusion protein in which the N-terminal 22 amino acids of β-galactosidase are fused to coagulase lacking its N-terminus. Expression would then be dependent on the vector’s lac promoter and translation initiation signals. Elimination of expression of the 60 kDa protein in insertion mutant pCOA12 is consistent with this. Fortuitously, this lacZ'-coa' fusion protein is the same size as the mature staphylococcal coagulase.

The properties of the proteins expressed by pCOA1, pCOA2 and pCOA12 were consistent with biochemical data which showed that prothrombin-binding activity is located in the N-terminus of coagulase (Kawabata et al., 1986a, b). An N-terminal 43 kDa chymotrypsin fragment retained both prothrombin-binding and clotting activity, whereas shorter peptides lacking the N-terminus lost the ability to clot plasma. DNA sequencing currently being done in this laboratory will determine if this is correct.

Coagulase activity was expressed by the KpnI and HindIII fragments subcloned from λcoa irrespective of their orientation in pUC vectors. The coa promoter must be active in E. coli as are other S. aureus exoprotein gene promoters (O'Toole & Foster, 1986a; Lofdahl et al., 1983; Kreiswirth et al., 1983), with the exception of those for epidermolytic toxin B (O'Toole & Foster, 1986b) and enterotoxin B (Ranelli et al., 1985). There is no obvious explanation for the finding that coagulase was expressed at higher levels by pCOA5 where the lac promoter of the vector opposed the direction of coa transcription.

It has been reported that coagulase expression by strain V8 is regulated in a similar fashion to protein A because it is expressed predominantly during the exponential phase of growth and because higher titres are expressed by certain regulatory mutants (Bjorklind & Arvidson, 1980). Here we show that an agr::Tn.551 mutation in strain 8325-4 allows higher expression of coagulase than the agr+ strain, both from the single copy chromosomal gene and when the
strains harbour the coa gene on a multicopy plasmid. This is similar to the behaviour of the protein A (spa) gene (Uhlen et al., 1984; Patel et al., 1987) and is consistent with the suggestion that coa is negatively regulated by the product of the agr locus. However, it is not known if control is exerted at the level of the initiation of transcription of the coa gene. The cloned coa gene will be useful as a probe in Northern hybridization experiments. Also, it will be interesting to compare the promoter regions of the coa and spa genes for sequence similarities.

The cloning and mapping of the coagulase gene described here is facilitating the construction of specific coa mutations in virulent strains of S. aureus by recombinational allele replacement. This will permit the role of coagulase in the pathogenesis of staphylococcal infections to be determined.

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