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 λ coa 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, 1983*a, 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, 1983*b*). Coagulase is one such protein (Jeljaszewicz *et al.*, 1983). 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.*, 1986*a, 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

Table 1. *Bacterial strains*

Strain	Genotype	Relevant properties	Source and reference
<i>S. aureus</i> 8325-4	<i>coa</i> ⁺ <i>agr</i> ⁺	NCTC 8325 cured of prophages. Plasmid-free	Novick (1963)
RN4220	<i>coa</i> ⁺	Mutant of 8325-4 capable of accepting shuttle plasmids from <i>E. coli</i>	Kreiswirth <i>et al.</i> (1983)
ISP546	<i>coa</i> ⁺ <i>agr</i> ::Tn551	Mutant of 8325-4	Brown & Pattee (1980) Recsei <i>et al.</i> (1986)
<i>E. coli</i> C600	<i>lac thr leu thi</i> <i>tonA hspR hspM</i>		Appleyard (1954)
TB1	<i>ara thi rpsL Δlac-pro_{XIII}</i> (ϕ 80 <i>dlacI ΔM15</i>) <i>hspR</i>	Host for detecting chimaeric pUC plasmids	Yanish-Perron <i>et al.</i> (1985)

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 λ L47.1 (Loenen & Brammar, 1980) was used to clone the *coa* gene. Transduction in *S. aureus* was done using phage 80 α (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* to measure coagulase expression in culture supernatants.

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* CN6708 (Anderson *et al.*, 1982) was a gift from Dr C. Adlam (Wellcome, Beckenham, Kent, UK).

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 μ l 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 *lcoa* 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. Before use in tests with recombinant phages or plasmids in *E. coli* the serum was adsorbed with a concentrated extract of *E. coli* C600. Peroxidase-conjugated swine anti-rabbit globulin was purchased from Dakopatts (Glostrup, Denmark). Peroxidase-conjugated protein A was obtained from Sigma.

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).

Table 2. *Plasmids*

Plasmid	Host	Marker(s)*	Relevant properties	Source and reference
pBR322	<i>E. coli</i>	Ap ^r Tc ^r	Cloning vector	Bolivar <i>et al.</i> (1977)
pUC18	<i>E. coli</i>	Ap ^r	Cloning vector	Yanish-Perron <i>et al.</i> (1985)
pUC19	<i>E. coli</i>	Ap ^r	Cloning vector	Yanish-Perron <i>et al.</i> (1985)
pSK265	<i>S. aureus</i>	Cm ^r	Cloning vector	Jones & Khan (1986)
pCA1	Shuttle	Ap ^r Cm ^r	pBR322 linked to pSK265 at <i>Eco</i> RI sites	This study
pCOA1	<i>E. coli</i>	Ap ^r	4.2 kb <i>Hind</i> III fragment from <i>λcoa</i> cloned in pUC18	
pCOA2	<i>E. coli</i>	Ap ^r	4.2 kb <i>Eco</i> RI fragment from <i>λcoa</i> cloned in pUC19	
pCOA4	Shuttle	Ap ^r Cm ^r	5.2 kb <i>Kpn</i> I fragment from <i>λcoa</i> cloned in pCA1	
pCOA5	<i>E. coli</i>	Ap ^r	5.2 kb <i>Kpn</i> I fragment from pCOA4 in pUC19	
pCOA6	<i>E. coli</i>	Ap ^r	2.7 kb <i>Hinc</i> II fragment from pCOA5 in pUC19	
pCOA7	<i>E. coli</i>	Ap ^r	Deletion of 0.85 kb and 1.9 kb <i>Acc</i> I fragment from pCOA5	
pCOA9	<i>E. coli</i>	Ap ^r	Deletion of 0.75 kb <i>Xba</i> I fragment from pCOA5	
pCOA10	<i>E. coli</i>	Ap ^r	pCOA2 with <i>Eco</i> RI insert in opposite orientation	
pCOA11	<i>E. coli</i>	Ap ^r	4.2 kb <i>Hind</i> III fragment from <i>λcoa</i> cloned in pBR322	
pCOA12	Shuttle	Ap ^r Cm ^r	2 kb <i>Sau</i> 3AI fragment of pC221 cloned in <i>Bam</i> HI site of pCOA2	
pCOA13	<i>E. coli</i>	Ap ^r	0.95 kb <i>Eco</i> RI fragment deleted from pCOA1	
pCOA14	<i>E. coli</i>	Ap ^r	5.2 kb <i>Kpn</i> I fragment from pCOA4 in pUC19. Opposite orientation to pCOA5	

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

Immunochemical analysis of coagulase. Proteins produced in *λcoa* 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 BHI 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 TB1 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 *λ*L47.1 as described previously (O'Toole & Foster, 1986a), except that the genomic DNA was cleaved partially with *Sau*3AI and the *λ* replacement vector was cut with *Bam*HI. 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 (*λcoa*) 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

λcoa lysates expressed a coagulase titre of 64. In addition, *λcoa* 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).

Strain	Coagulase titre*
C600	<2
<i>λcoa</i>	64
C600(pCOA1)	10240
C600(pCOA2)	<2
C600(pCOA4)	1024
C600(pCOA5)	1024
C600(pCOA6)	512
C600(pCOA9)	4096
C600(pCOA11)	1024
C600(pCOA14)	128

* 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 *Kpn*I and ligated with *Kpn*I-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 *Kpn*I 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 *Xba*I fragment (Fig. 1, tracks 5 and 7). This shows that the intact *coa* gene had been subcloned from the phage.

A 4.2 kb *Hind*III fragment from *λcoa* and a 2.7 kb *Hinc*II fragment from pCOA5 were cloned into *Hind*III-cut and *Hinc*II-cut pUC19, forming pCOA1 and pCOA6, respectively. The *Hind*III fragment was also cloned into the *Hind*III 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 *Hind*III 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 *Eco*RI-*Hind*III 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 *Eco*RI fragment (i.e. including the *Eco*RI-*Hind*III 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 *Eco*RI fragment was subcloned from *λcoa* into pUC19. Several immunoreactive colonies were purified for further analysis. All had the 4.2 kb

Fig. 1. Western immunoblots: lane 1, coagulase purified from strain CN6708 (12 µg); lanes 2 and 6, *coa* lysate (80 µg protein); lane 3, lysate of *E. coli*(pCOA2) (14 µg protein); lane 4, lysate of *E. coli*(pCOA1) (45 µg protein); lane 5, lysate of *E. coli*(pCOA9) (47 µg protein); lane 7, *S. aureus* 8325-4 culture supernatant (13.5 µg protein).

*Eco*RI 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 *Eco*RI fragment in pCOA2 was reversed. This plasmid (pCOA10) did not express the immunoreactive 60 kDa protein. In addition, insertion of a 2 kb *Sau*3AI fragment from pC221 into the *Bam*HI site located between the *lac* promoter of pUC19 and the *Eco*RI 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 *Acc*I fragments from pCOA5, which eliminated both coagulase activity and immunoreactivity, whereas deleting the 0.7 kb *Xba*I 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 *Kpn*I 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 *agr*⁺ and into ISP546 (*agr*::Tn551).

The level of expression of coagulase in broth culture supernatants was measured by titrating plasma-clotting activity (Table 4). The plasmid-free *agr*⁺ strain 8325-4 expressed a low level of

Fig. 2. Maps of λcoa and pCOA plasmids. Cloned *S. aureus* sequences are represented by the narrow parallel lines and λ 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, *Sau3AI*; K, *KpnI*; Hc, *HincII*; Ac, *AccI*; Pv, *PvuII*; X, *XbaI*; Ps, *PstI*.

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).

Strain	Coagulase titre*	
	Without plasmid	With pCOA4
8325-4	51 200	307 200
ISP546	307 200	1 024 000

* 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 λ coa 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 *Hind*III fragment in pCOA1 subcloned from λ coa expressed truncated immunoreactive proteins but retained plasma-clotting activity. This suggests that a *Hind*III site is located in the 3' part of the *coa* gene. A deletion from a presumably more promoter-proximal *Hinc*II 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 *Eco*RI 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 *Kpn*I and *Hind*III fragments subcloned from λ coa 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*::Tn551 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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