Cloning and expression of the Staphylococcus aureus protein A gene in Escherichia coli

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ABSTRACT

A gene bank of Staphylococcus aureus strain Cowan 1 was established using an E. coli HB101/pBR327 host-vector system. Recombinants expressing staphylococcal protein A (SPA) were detected using an IgG-binding assay. A 3.2 Kb DNA fragment directing the synthesis of SPA in E. coli was identified. SPA produced by E. coli was characterised in minicells and by Western blotting and double diffusion experiments.

INTRODUCTION

Staphylococcal protein A (SPA) is a monomeric protein covalently bound to the cell wall peptidoglycan of most strains of Staphylococcus aureus. Mutant strains containing lesions in the cell wall, which excrete the protein into the growth medium, have been described. Estimates of the molecular weight of SPA vary between 41000 (obtained by physical methods such as equilibrium sedimentation or gel chromatography under denaturing conditions) and much higher values of 55000-36000 (obtained by SDS-polyacrylamide gel electrophoresis). The generally accepted value is 42000. The anomalous behaviour of SPA in SDS-polyacrylamide gels is believed to result from the abnormally extended shape of the molecule, or possibly to post-translational modifications (for reviews of SPA see references 1 and 2).

SPA is of interest because it can interact, with varying specificity, with the Fc region of a wide range of mammalian immunoglobulins. This property, which is quite distinct from the interaction between an antigen and the Fab region of immunoglobulin G, has enabled SPA to be used in a variety of radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) procedures (3). In addition, the extracorporeal perfusion of plasma over immobilised SPA may help cause the regression of certain tumours in humans and other mammals (4; see also references in 1).

SPA is currently produced from large-scale cultures of wild type S. aureus strains by lysis of cell suspensions or from culture supernatants of mutant (excretor) strains. In both cases, purification is usually by affinity chromatography on
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immobilised IgG. Approximately 7% (by weight) of the cell wall of *S. aureus* strain Cowan I, a suitable production strain, is SPA and this is equivalent to approximately 1.7% of total protein (5). The handling of large volumes of *S. aureus* cultures is, however, undesirable because of its pathogenicity. The aim of this project was to introduce the SPA structural gene into *Escherichia coli* and gain its expression in that host. This would provide a safer means of SPA production and also material for studying the properties of the gene.

In this paper we describe the construction of a gene bank of *S. aureus* chromosomal DNA in an *E. coli* host/vector system. A 3.2 Kb fragment of DNA which directs the synthesis of SPA in *E. coli* was identified. The SPA produced was analysed both in minicells and by protein-transfer immunological methods.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids**

Two wild-type strains of *S. aureus* were used: Cowan I (SPA-positive; ATCC 12598, NCTC 8530) and Wood 46 (SPA-negative; ATCC 10832, NCTC 7121). The gene bank was constructed in *E. coli* HB101 (6) using pBR327 and pBR328 as plasmid vectors (7). The minicell-producing *E. coli* strain DS<sub>410</sub> (8) was also used. Cultures of all strains were routinely maintained on L-agar and grown in L-broth supplemented, where appropriate, with the relevant antibiotic.

**Recombinant DNA Procedure**

All recombinant DNA work was done under Category I containment. Chromosomal DNA was prepared from *S. aureus* strain Cowan I by the method of Lindberg *et al.* (9). Throughout the procedure excessive agitation was avoided to ensure minimal shearing of DNA. Restriction enzymes and DNA ligase were from Bethesda Research Laboratories (Science Park, Cambridge) or Cambridge Biotechnology Laboratories (London) and were used according to the manufacturers' recommendations. Calf intestinal alkaline phosphatase (Boehringer Corporation (London) Ltd., Lewes, East Sussex) was used to dephosphorylate endonuclease-cleaved cloning vectors prior to ligation (10). Transformation of the *E. coli* strains HB101 and DS<sub>410</sub> was by the method of Morrison (11).

Small amounts of recombinant plasmid DNA for screening purposes were prepared by the method of Birnboim and Doly (12). Larger amounts were prepared from 150 ml spectinomycin-amplified cultures by lysis with lysozyme and Brij-deoxycholate (13) followed by a clearing spin (15,000 rpm at 4°C for 45 min in a Sorval SS24 rotor). The DNA was then banded in a CsCl gradient containing ethidium bromide (200 μg/ml) (13). Plasmid DNA for cloning was prepared from 500ml L-broth cultures without amplification.
Screening Recombinants for Expression of the SPA Gene

The method used to screen recombinants for the expression of SPA was a modification of that described by Broome & Gilbert (14). Colonies were grown overnight on nitrocellulose filters and lysed by sequential exposure to virulent bacteriophage lambda (λ vir) and chloroform. The lysed colonies were then transferred to a polystyrene surface (Nunc bioassay dish; Gibco-Biocult, Paisley, Scotland) which had been coated with IgG using a modification of the method of Neurath & Strick (15). Bioassay dishes were first reacted with 50 ml of methanesulphonic acid at room temperature overnight in a fume cupboard to methylate reactive groups on the polystyrene. This was followed by nitration at room temperature for 15 min with 50 ml of a 2:1 mixture of conc. sulphuric acid : conc. nitric acid. The nitropolystyrene was then reduced to the amino derivative by incubation with 50 ml of acidic stannous chloride solution (100 g of SnCl₂·6H₂O in 100 ml conc. HCl) at room temperature for 2 hr. The surface of each dish was washed extensively with distilled water for 1 hr (at least four changes of 50 ml each) between each step. Finally, the dishes were incubated with 1% glutaraldehyde in 50 mM potassium phosphate buffer, pH 8.5, at room temperature for 2 hr and then at 4°C overnight. After extensive washing in the same buffer, the surface of the dish was covered with a solution of human IgG (Sigma) at 100 µg/ml in phosphate-buffered saline, pH 7.0 (PBS) and incubated at 4°C overnight. The dishes were ready for colony transfer after a final wash with PBS containing 5% bovine serum albumin (Sigma, RIA grade) and 1% Triton X-100 (PBS-BSAT) at room temperature for 1 hr.

Proteins from lysed colonies were transferred to the IgG-coated polystyrene by gently placing the nitrocellulose membrane onto the surface of a bioassay dish. After incubation at 4°C overnight, the filter was removed and colony debris washed off with several changes of PBS-BSAT at room temperature for 1 hr. The surface of each dish was then flooded with PBS-BSAT containing human IgG (2 µg/ml) labelled with 125I to approximately 10⁶ cpm per µg by the chloramine T method (16). The dishes were incubated at 4°C overnight, thoroughly washed with PBS-BSAT, dried and then autographed at -70°C with an intensification screen (17).

Analysis of Recombinants by "Western" Blotting

Colonies scored as positive in the IgG-binding assay were grown overnight in 10 ml of L-broth. The cells were harvested by centrifugation and resuspended in 1 ml of 25% sucrose, 50 mM Tris-HCl, pH 8.0. Lysozyme (final concentration 2.5 mg/ml) was added and the cells incubated at 0°C for 20 min. An addition of 1.5 ml of lysis solution (1% Brij 58 and 0.4% sodium deoxycholate in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was made and the suspension thoroughly mixed. After incubation at 0°C for 30 min, the lysates were centrifuged at 15000 rpm at 4°C for 45 min (Sorvall SM24
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rotor) to give crude cell extracts. Lysates of *S. aureus* strains were prepared in a similar way except that incubation with lysostaphin (final concentration 25 µg/ml) at 37°C replaced the lysozyme treatment.

Proteins in these lysates were separated by electrophoresis on SDS-polyacrylamide gels and then transferred electrophoretically to nitrocellulose filters ("Western" blotting) by the method of Towbin et al. (18). After transfer, filters were soaked at 4°C for 16 hr in PBS-BSAT, incubated with radioiodinated rabbit anti-SPA serum (Sigma), washed, dried and autoradiographed as before.

These lysates were also assayed for reactivity against rabbit anti-SPA serum by double diffusion in agarose gels (19).

**Analysis of Plasmid-directed Protein Synthesis in Minicells**

Appropriate plasmids were transformed into *E. coli* DS410. Minicells were prepared and stored by the method of Reeve (20), except that purification was by sedimentation, in 30 ml Corex glass tubes, through three successive sucrose gradients rather than one as described. After storage at -70°C, minicells were thawed and preincubated at 37°C for 60 min in M9 medium (21) containing 0.2% glucose, 0.1% casamino acids and 50 µg/ml D-cycloserine. Polypeptides were labelled by replacing the casamino acids with 35S-methionine (25 µCi; 1100 Ci/mm; Amersham International) and continuing incubation at 37°C for 30 min. This was followed by a 5 min chase with unlabelled methionine (0.4 mg/ml final concentration). Labelled minicells were recovered by centrifugation and resuspended either in disruption mix (2% SDS, 3% sucrose, 500 mM 2-mercaptoethanol, 50 mM Tris-HCl pH 7.0) for electrophoresis, or in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) for binding to IgG-Sepharose. Lysates of *S. aureus* Cowan I were pulse-labelled with 35S-methionine under similar conditions without D-cycloserine.

Cell suspensions for IgG binding were lysed as described for the Western blotting procedure and mixed gently at room temperature for 4 hr with IgG-Sepharose CL, the gift of Dr. M. Scawen (0.1 g wet weight/500 µl lysate). The IgG-Sepharose CL was then recovered, washed four times with 1% Triton X-100 in TE buffer and boiled for 4 min in disruption mix (0.1 g wet weight/200 µl) for electrophoresis as described (18). Labelled polypeptides were detected by fluorography using PPO (17).

**RESULTS**

**Establishment of a Gene Bank and Identification of SPA-producing Colonies**

Chromosomal DNA of *S. aureus* Cowan I was partially digested with Sau3A using a ratio of DNA (µg) to enzyme (BRL units) of 40:1. To ensure a representative distribution of cleavage fragments, one-third aliquots of the digest were removed after 5, 10 and 20 minutes of incubation at 37°C and mixed in a tube containing 50 µl
Figure I. Detection of SPA production by recombinant colonies using the IgG-binding assay. Transformants carrying recombinant plasmids were grown on nitrocellulose filters and lysed in situ. Proteins from lysed colonies were transferred to IgG-coated polystyrene dishes, which were then probed with radiiodinated IgG and autoradiographed. Positive recombinant colonies are indicated by arrows. Colonies around the outside of the filter were positive controls (S. aureus Cowan I).

of buffer-saturated phenol. Digested DNA was fractionated on a neutral sucrose gradient and the fractions were analysed by agarose gel electrophoresis (22). Fractions containing DNA fragments in the range 8-10 Kb were pooled, extracted with phenol and precipitated with ethanol. This size range was selected as optimum for cloning as it gave a probability greater than 90% that the whole S. aureus genome would be represented in a gene bank of 2000 colonies, yet was small enough to ensure a reasonable transformation frequency. Recovered DNA fragments were subsequently ligated to BamHI-cleaved dephosphorylated pBR327 DNA and transformed into E. coli HB101. Colonies containing recombinant DNA molecules were selected and grown in L-broth in the individual wells of microtitre plates. After the addition of glycerol (final concentration 15%) plates were stored at -70°C. Approximately 1800 colonies were picked and screened by the IgG-binding assay (Figure I). Three colonies, designated 7-D4, 8-G4 and 10-C9, were identified as positive and were also shown to give a positive reaction against rabbit anti-SPA serum by Ouchterlony double diffusion assay (Figure 2). This indicated the presence of a protein immunologically identical to SPA of S. aureus origin in extracts of these E. coli strains.

DNA Restriction Analysis and Subcloning of the SPA Gene

The DNA inserts in recombinant plasmids 7-D4, 8-G4 and 10-C9 were
Figure 2. Identification of SPA produced in *E. coli* by double diffusion assay. Samples were located in gels of 1% agarose in barbitone buffer (19) and incubated in humid conditions at room temperature for 16-40 hr. Gels were washed in saline and dried before staining with Coomassie blue R250 for photography. Centre well, rabbit anti-SPA serum (Sigma, 1:8 dilution in saline). Outer wells, anticlockwise from top; SPA (Pharmacia, 50 μg/ml in PBS); *S. aureus* Cowan I cell extract; *E. coli* HB101/10-C9 cell extract; SPA (Pharmacia, 50 μg/ml in PBS); *E. coli* HB101/pBR327 cell extract; *S. aureus* Wood 46 cell extract.

approximately 8.9, 5.9 and 6.6 Kb respectively. Each plasmid gave a different fragmentation pattern when digested with the same restriction enzyme, indicating that three overlapping chromosomal DNA fragments had been cloned. Digestion with *PstI* gave a fragment of approximately 3.2 Kb that was common to all three plasmids. It was assumed that this fragment contained the SPA gene and it was therefore subcloned into the *PstI* site of pBR328 to give a recombinant plasmid designated SPA-3. Colonies of *E. coli* HB101 containing SPA-3 were identified as positive in the IgG-binding assay. The cleavage sites of different restriction enzymes were subsequently mapped within the insert of SPA-3 (Figure 3).

**Analysis of Recombinants by Western Blotting**

Cell extracts of *S. aureus* Cowan I and Wood 46 and of *E. coli* HB101 carrying

![Restriction map of *S. aureus* DNA insert in SPA-3. Cleavage sites for a number of restriction enzymes within the 3.2Kb *PstI* insert of SPA-3 were mapped by analysis of the fragments generated by single and double digests with these enzymes.](https://academic.oup.com/nar/article-abstract/11/10/3065/2379148/Cloning-and-expression-of-the-staphylococcal)}
Figure 4. Analysis of recombinants by Western blotting. Proteins in samples were separated by electrophoresis in 12.5% SDS-polyacrylamide gels, transferred to nitrocellulose filters and probed with radioiodinated rabbit anti-SPA serum.

(a) Lanes contained (1) HB101/pBR327, (2) HB101/7-D4, (3) HB101/8-G4, (4) HB101/10-C9, (5) S. aureus Cowan I, (6) HB101/pBR328, (7) HB101/SPA-3.

(b) Lanes contained (1) HB101/pBR327, (2) HB101/10-C9, (3) E. coli lysis mixture containing no cells, (4) lysozyme standard, 10 µg, (5) HB101/pBR327 (sonicate), (6) HB101/10-C9 (sonicate), (7) S. aureus Wood 46, (8) S. aureus Cowan I, (9) SPA (Pharmacia), 1 µg.

(c) Lanes contained (1) HB101/10-C9 (overnight exposure), (2) HB101/10-C9 (10-day exposure).

All cell extracts of E. coli were prepared by the lysozyme/detergent method unless otherwise stated.
Figure 5. Analysis of S-methionine labelled polypeptides from minicells. Lysates of S-methionine pulse-labelled E. coli DS410 containing the following plasmids were analysed by electrophoresis on a 12.5% SDS-polyacrylamide gel. Lanes contained (1) and (9) 14C-labelled molecular weight standards (New England Nuclear), (2) DS410, (3) DS410/pBR327, (4) DS410/7-D4, (5) DS410/8-G4, (6) DS410/10-C9, (7) DS410/SPA-3, (8) DS410/pBR328, (10) polypeptides isolated from S-methionine pulse-labelled lysate of S. aureus Cowan I by IgG-Sepharose binding.

the recombinant and vector plasmids were analysed by Western blotting (Figure 4). Extracts of the Cowan I strain of S. aureus gave a single major band of molecular weight approximately 54000 (Figure 4a, lane 5), whereas those of E. coli strains containing 7-D4, 8-G4, 10-C9 or SPA-3 gave complex patterns (Figure 4a, lanes 2, 3, 4, 7). The major protein species present had molecular weights of 43000 and 41000; however, prolonged autoradiographic exposure revealed a minor band comigrating with SPA from S. aureus (Figure 4c). These bands were not present in extracts of either E. coli control strains (Figure 4a, lanes 1 and 6) or an SPA-negative strain of S. aureus (Figure 4b, lane 7). The band of molecular weight approximately 15000, which was present in all E. coli extracts, corresponded to the lysozyme used during the extraction process (Figure 4b, lanes 3 and 4). In addition, the pattern of bands in a sonicated extract of HB101/10-C9 (Figure 4b, lane 6) was identical, apart from the 15000 band, to a lysate of the same strain prepared by the lysozyme/detergent method (Figure 4b, lane 2). Similar results (not shown) were obtained using either pooled human IgG (Sigma) or the purified Fc fragment of human IgG (Dynatech Ltd.)
Table I. Sizes of Polypeptides produced in Minicells

<table>
<thead>
<tr>
<th>Lysate</th>
<th>Molecular sizes of $^{35}$S-labelled polypeptides (kilodaltons)</th>
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<tbody>
<tr>
<td>S. aureus Cowan I</td>
<td></td>
</tr>
<tr>
<td>DS410/pBR327</td>
<td>$45^a$</td>
</tr>
<tr>
<td>DS410/7-D4</td>
<td>$32^b$ 29.5 26.5</td>
</tr>
<tr>
<td>DS410/8-G4</td>
<td>59 56.5 47 45 32$^b$ 29.5 26.5</td>
</tr>
<tr>
<td>DS410/10-C9</td>
<td></td>
</tr>
<tr>
<td>DS410/SPA-3</td>
<td>56 53 47 45 29.5 26$^c$</td>
</tr>
<tr>
<td>DS410/pBR328</td>
<td>32$^b$ 29.5 26$^c$</td>
</tr>
</tbody>
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Sizes of polypeptides were calculated from Figure 5.

a This is the main polypeptide bound by Ig-Sepharose from a pulse-labelled lysate of S. aureus Cowan I.

b This is the $\beta$-lactamase encoded by pBR327 and pBR328.

c This is the chloramphenicol acetyltransferase encoded by pBR328 which masks the polypeptide of molecular weight 26500 associated with tetracycline resistance.

as probe, in place of rabbit anti-SPA serum.

In a separate experiment, whole and papain-digested preparations of human IgG were fractionated on a polyacrylamide gel and, after blotting to nitrocellulose, probed with radioiodinated cell extracts of HB101/SPA-3. Preferential binding of label to the Fc fragment of the papain-digested preparation was observed.

Analysis of Recombinant DNA using Minicells

Polypeptides encoded by recombinant plasmids containing the SPA gene were analysed by SDS-polyacrylamide gel electrophoresis of $^{35}$S-methionine pulse-labelled lysates of minicells (Figure 5). Minicells containing plasmids pBR327 and pBR328 expressed only the polypeptides associated with antibiotic resistance (Lanes 3 and 8), while those containing 7-D4, 8-G4, 10-C9 or SPA-3 expressed additional polypeptides (lanes 4, 5, 6 and 7), the sizes of which are summarised in Table I. The polypeptide corresponding to $\beta$-lactamase (molecular weight 32000) was present in all plasmid-containing strains of E. coli except DS410/SPA-3, where the S. aureus DNA fragment
was inserted at the PstI site in the β-lactamase gene. The main polypeptide which
bound to IgG-Sepharose from a pulse-labelled lysate of S. aureus Cowan I (lane 10)
comigrated with a polypeptide (f) of molecular weight 45000 present in all lysates of
minicells containing the recombinant plasmids. This polypeptide, while clearly
present in the lysate of DS410/SPA-3, became apparent in the lysates of DS410
containing 7-D4, 8-G4 or 10-C9 only after prolonged autoradiographic exposure. All
six polypeptides (a-f) were removed from lysates of minicells containing recombinant
plasmids by binding to IgG-Sepharose, and could be recovered and visualised on SDS-
polyacrylamide gels by dissociation of the complex with IgG-Sepharose by boiling in
disruption mix (not shown).

DISCUSSION

Evidence is presented here for the cloning and expression in E. coli of the SPA
structural gene from S. aureus. The gene was localised to a 3.2 Kb PstI DNA
fragment which was then cloned into the PstI site of pBR328. E. coli strains carrying
this recombinant plasmid synthesised proteins which reacted with rabbit anti-SPA
serum, with pooled human IgG and with purified Fc fragment of human IgG in a
manner identical to SPA from S. aureus. The level of expression of the cloned SPA
gene in E. coli, estimated by titration in a double diffusion assay (not shown), was 10-
fold lower than in S. aureus Cowan I and equivalent to approximately 0.2% of the
total protein. This was similar in strains carrying all three original recombinant
plasmids (7-D4, 8-G4 and 10-C9) and SPA-3, indicating that expression might be from
a S. aureus promoter.

The molecular weights of the main E. coli polypeptides that bound anti-SPA
serum were lower (43000 and 41000) than that of SPA from S. aureus (54000) when
analysed by Western blotting. However, a full-size SPA-like protein was present in
E. coli at a very low level. The molecular weight estimate of 54000 obtained here for
SPA by SDS-polyacrylamide gel electrophoresis agrees with published figures
obtained by the same method. The main polypeptide bound by immobilised human IgG
from pulse-labelled lysates of S. aureus Cowan I, however, migrated with an apparent
molecular weight of 45000. This comigrated with a polypeptide (f) present in lysates
of E. coli minicells containing any one of the four recombinant plasmids tested. Other polypeptides which reacted with IgG, ranging in size from 47000 to 59000, were
also present.

The reasons for the heterogeneity of SPA-like polypeptides produced from
recombinant DNA in minicells and as analysed by Western blotting are not clear.
Neither is it clear why different values for the molecular weight of SPA from
S. aureus should be obtained by the two different methods. The pulse-labelled
polypeptides detected in minicells may represent primary translation products of cloned DNA sequences, whereas those from cell lysates used for Western blotting may have undergone subsequent processing or degradation. The interpretation of results obtained in minicells, however, requires caution as artefacts may arise in several ways. For example, fusion polypeptides may be produced where a cloned gene is transcribed from a vector promoter, or small polypeptides may be produced due to premature termination or processing (23). Minicells are non-growing cells, in which normal regulatory mechanisms may not apply, and artefacts could also arise as a result of this (24).

The possibility remains that only part of the SPA coding sequence has been cloned. This would account for the smaller polypeptides identified by Western blotting. It is unlikely, however, since the original cloning experiment gave three recombinant plasmids which contained DNA inserts with different restriction enzyme fragmentation patterns, but which produced identical polypeptide patterns both in minicells and by Western blotting.

Experiments designed to investigate these possibilities further are in progress.

ACKNOWLEDGEMENTS

The idea for this work originally came from Dr. Jon Oram. We would like to thank Dr. Andrew Shuttleworth for demonstrating the Western blotting technique, and Dr. Peter Greenaway and Professor Tony Atkinson for encouragement throughout the course of the work. We are grateful to Dr. Peter Greenaway for constructive criticism in the preparation of the manuscript.

REFERENCES


POSTSCRIPT

After submission of this manuscript there appeared a report of the cloning in E. coli of the SPA gene from S. aureus strain 8325-4 (Lofdahl et al. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 697-701). Restriction enzyme mapping indicates some differences between this gene and that from strain Cowan I reported here. Different molecular weights for SPA produced by E. coli and S. aureus were also observed by Lofdahl et al.