Physical and Genetic Mapping of the Protein A Gene in the Chromosome of *Staphylococcus aureus* 8325-4

By ARVIND H. PATEL,² TIMOTHY J. FOSTER^{2*} AND
PETER A. PATTEE¹

¹Department of Microbiology, Iowa State University, Ames, Iowa 50011, USA

²Department of Microbiology, Moyne Institute, Trinity College, Dublin 2, Ireland

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The gene coding for protein A (*spa*) has been mapped close to *nov* on the genetic map of the chromosome of *Staphylococcus aureus* 8325-4. A rapid mapping procedure has been developed which first allowed the region of the chromosome carrying the *spa* gene to be identified by blot hybridization of large DNA fragments which had been separated by pulsed-field gel electrophoresis. Restriction endonuclease *Sma*I fragment G was shown to carry the *spa* gene. An insertion mutation in *spa* was constructed by *in vitro* insertion of a fragment of DNA expressing resistance to kanamycin and neomycin. A *spa*::Kan^rNeo^r mutation was isolated in *S. aureus* 8325-4 by allele replacement. This provided a selectable marker which allowed the *spa* gene to be mapped by transformation analysis.

INTRODUCTION

Protein A is a cell-wall-associated protein of the Gram-positive pathogenic bacterium *Staphylococcus aureus* (for reviews see Langone, 1982; Fosgren *et al.*, 1983). Five repeated domains which bind immunoglobulin molecules at a site on the Fc region protrude from the cell surface (Löfdahl *et al.*, 1983; Uhlén *et al.*, 1984). The protein is anchored to the cytoplasmic membrane by a hydrophobic C-terminal domain. Region X, part of which comprises tandemly repeated octapeptide units, is responsible for the molecule spanning the peptidoglycan of the cell wall (Guss *et al.*, 1984).

There is evidence that protein A is a virulence factor of *S. aureus* because it impairs opsonophagocytosis of bacterial cells and because it can induce hypersensitivity, complement activation and histamine release from basophils (Fösgren *et al.*, 1983). There is a correlation between the level of expression of protein A in natural isolates and their resistance to phagocytosis (Peterson *et al.*, 1977). Also, site-specific protein-A-deficient mutants isolated by gene-replacement are more susceptible to phagocytosis *in vitro* (C. G. Gemmell, M. R. Tree, A. H. Patel & T. J. Foster, unpublished).

The genetic map of *S. aureus* strain 8325 has been compiled by recombination analysis using transformation and protoplast fusion (Pattee & Neveln, 1975; Stahl & Pattee, 1983*a, b*; Pattee, 1987). Initially, biosynthetic markers and drug resistance determinants were mapped. Transposon mutagenesis extended the range of markers (Pattee, 1981; Schroeder & Pattee, 1984). However, only one transposon mutation has been reported which affects expression of virulence factors. This strain has a Tn551 insertion which reduces expression of α -toxin (Mallonee *et al.*, 1982) by what is now known to be a mutation in a regulatory locus called *agr* (Recsei *et al.*, 1986).

Abbreviations and genetic symbols: Amp^r, ampicillin resistance; Cml^r, chloramphenicol resistance; Erm^r, erythromycin resistance; Etb^r, ethidium bromide resistance; Kan^r, kanamycin resistance; Mec^r, methicillin resistance; Neo^r, neomycin resistance; Nov^r, novobiocin resistance; PGFE, pulsed-field gel electrophoresis.

Three genes encoding virulence factors have so far been mapped on the *S. aureus* chromosome. The enterotoxin A gene which is associated with a converting bacteriophage (Betley & Mekalanos, 1985) maps in interval 6 (Pattee & Glatz, 1980) but can occupy other chromosomal sites (Mallonee *et al.*, 1982). The α -toxin (*hly*) and lipase (*geh*) genes were inactivated with drug resistance insertion mutations isolated by allele replacement (O'Reilly *et al.*, 1986) and plasmid integration (Lee & Iandolo, 1986), respectively. The integrated drug resistance determinants served as selective markers for the virulence factor loci in mapping experiments (Pattee, 1986, 1987).

In this paper we report that the *S. aureus* 8325 chromosome is cut by the restriction enzyme *Sma*I into 13 fragments ranging from 673 kb to 36 kb which have been separated by pulse-field gel electrophoresis. The *Sma*I fragments have been correlated with the *S. aureus* genetic map (P. A. Pattee, unpublished). The physical and genetic mapping of the protein A gene, *spa*, in the *S. aureus* chromosome is reported here.

METHODS

Bacterial strains and plasmids. The *S. aureus* strains are listed in Table 1 and the plasmids in Table 2. Strain 8325-4 is a derivative of strain 8325 which has been cured of three prophages (Novick, 1963). This accounts for the difference in the *Sma*I banding pattern of the two strains. Fragment F (208 kb) in the lysogenic strain 8325 is replaced in strain 8325-4 by a band which comigrates with fragment H (135 kb). Fragment F is known to carry *att* ϕ 11 and may carry *att* ϕ 13. Transduction was performed with phage ϕ 80 α as described by Asheshov (1966).

Genetic mapping. DNA used for transformation was prepared as described by Pattee & Neveln (1975) and transformation was performed as described by Stahl & Pattee (1983*b*), except that 1.0 ml of rabbit serum was added to each 100 ml culture of the recipient strain at an OD₅₄₀ of 0.05, and the cells were routinely harvested at an OD₅₄₀ of 0.1. Phage ϕ 55 was used to promote competence for transformation (Stahl & Pattee, 1983*b*) even with strains that were phage ϕ 11 lysogens. Transformants were selected and scored as described previously (Stahl & Pattee, 1983*b*). The kanamycin (Kan^r) and neomycin (Neo^r) resistance determinant was selected and scored on BHI agar containing both drugs at 10 μ g ml⁻¹. Double selection was necessary to eliminate spontaneous resistant mutants which grew on control plates containing a single aminoglycoside.

Physical mapping. Pulsed-field gel electrophoresis (PFGE) of chromosomal DNA was performed as described by Smith *et al.* (1986). Low-melting-temperature agarose (FMC) plugs (1%) containing cells to be analysed were prepared according to Smith *et al.* (1986). The plugs were incubated at 37 °C for 18 h in EC buffer (Smith *et al.*, 1986) containing lysostaphin (Sigma; 0.3 mg ml⁻¹) instead of lysozyme to lyse the cells *in situ*. They were then transferred to ESP buffer (Smith *et al.*, 1986) and incubated at 50 °C for 48 h. After washing with 10 mM-Tris/HCl, 1 mM-EDTA pH 7.5 (TE) buffer containing phenylmethylsulphonyl fluoride, and then with TE (Smith *et al.*, 1986), the released DNA was cleaved with *Sma*I and analysed by PFGE using the vertical apparatus described by Gardiner *et al.* (1986) at 200 mA and with a 12 s pulse time at 17 °C. Details of the physical map of the *S. aureus* 8325 chromosome will be reported elsewhere (P. A. Pattee, unpublished).

DNA hybridization. This was done by the procedure of Southern (1975) as described in Maniatis *et al.* (1982). Genomic DNA was prepared by the method of O'Reilly *et al.* (1986). Nitrocellulose membranes were used for DNA transfers, while Hybond-N membranes (Amersham) were used for transfers of DNA separated by PFGE. Probes were labelled either by nick-translation (Rigby *et al.*, 1977) using [α -³²P]dATP, hybridization being detected by autoradiography, or with photoactivatable biotin (*N*-[4-azido-2-nitrophenyl]-*N'*-[*N*-d-biotinyl-3-aminopropyl]-*N'*-methyl-1,3-propanediamine; Clontech Laboratories, Palo Alto, California, USA), hybridization being detected using the Blu-Gene system (Bethesda Research Laboratories).

Recombinant DNA procedures and construction of pEBM155. *In vitro* manipulation of plasmid DNA was performed by routine procedures (Maniatis *et al.*, 1982). The shuttle plasmid pEBM15, which carries the Δ *spa*::Etb^r mutation linked to pUC18 and pE194, has been described previously (Patel *et al.*, 1987). This plasmid was cleaved partially with *Hind*III and ligated with the 2.6 kb *Hind*III fragment from pBR322-Kan^rNeo^r. Transformants were selected on agar containing ampicillin (50 μ g ml⁻¹) and kanamycin (20 μ g ml⁻¹). Subsequently the presence of the erythromycin resistance (Erm^r) and ethidium bromide resistance (Etb^r) markers was verified. In the recombinant pEBM155 the 2.6 kb *Hind*III Kan^rNeo^r fragment was inserted at a *Hind*III site located within the 2 kb Etb^r insert used in the original inactivation of *spa* (Fig. 1).

Isolation of the *S. aureus* Δ *spa*::Kan^rNeo^r mutation by allele-replacement mutagenesis. The shuttle plasmid carrying the Δ *spa*::Etb^r::Kan^rNeo^r mutation was transformed into the Spa⁻ strain DU5723 which carries the incompatible plasmid pEC1. Double-plasmid-carrying strains were selected on agar containing kanamycin (10 μ g

Table 1. *S. aureus* strains

Strain no.	Parental strain	Genotype	Origin or reference
8325		<i>pig-131</i>	NCTC*
8325-4	8325	<i>pig-131</i>	Novick (1963)
ISP2	8325	<i>pig-131 nov-142</i>	Pattee & Neveln (1975)
ISP86	8325	<i>pig-131 uraA141 hisG15 nov-142 purA102</i>	Pattee (1981)
ISP794†	8325	<i>pig-131</i>	Stahl & Pattee (1983a)
ISP1115	8325	<i>trpE85 uraB232::Tn551 ermB327 Tn4291 nov-142 tmn-3106 rib-127 Ω[Tn551]1051</i>	Luchansky & Pattee (1984)
DU5819	8325-4	<i>pig-131 Δspa-515::Etb^r::Kan^rNeo^r</i>	This study
ISP2073	8325	<i>pig-131 Δspa-515::Etb^r::Kan^rNeo^r</i>	ISP2 × DU5819 DNA; this study
ISP2162	8325	<i>pig-131 Ω[Tn551]1051 Tn4291</i>	ISP794 × ISP1115 DNA; this study
DU5723	8325-4	<i>pig-131 spa-515::Etb^r</i> (carries pEC1)	Patel <i>et al.</i> (1987)

* NCTC, National Collection of Type Cultures, Colindale, London, UK.

† ISP794 was derived from a transformation cross between two auxotrophic mutants [ISP1 8325 *thy-101 pig-131* DNA × ISP2 8325 *nov-142 pig-131*] as described by Stahl & Pattee (1983a). However, genotypically it is identical to NCTC8325 (*pig-131*).

Table 2. *Plasmids*

Plasmid	Markers	Properties	Reference
pEBM15	Amp ^r Etb ^r Erm ^r	pUC18-pE194 <i>spa</i> ::Etb ^r shuttle plasmid	Patel <i>et al.</i> (1987)
pEC1	Cml ^r	pE194 replication origin linked to a Cml ^r fragment	Patel <i>et al.</i> (1987)
pEBM155	Amp ^r Etb ^r Erm ^r Kan ^r Neo ^r	pEBM15 with a 2.6 kb <i>Hind</i> III fragment cloned from pBR322-Kan ^r Neo ^r ; shuttle plasmid	This study
pBR322-Kan ^r Neo ^r	Amp ^r Kan ^r Neo ^r	pBR322 carrying a 2.6 kb <i>Hind</i> III fragment carrying the Kan ^r Neo ^r marker derived from pTC128	Coleman <i>et al.</i> (1985); M. Storrs, pers. comm.
pSPA721	Amp ^r Erm ^r Spa ⁺	pUC18 with a 4.35 kb fragment carrying <i>spa</i> from 8325-4	Patel <i>et al.</i> (1987)
pUC18-EB2	Amp ^r Etb ^r	2 kb <i>Bgl</i> II fragment from pJE1 cloned at <i>Bam</i> HI site in pUC18	Evans & Dyke (1988); K. Sweeney, pers. comm.

ml⁻¹) and chloramphenicol (Cml, 10 µg ml⁻¹). Cultures were grown to stationary phase in Trypticase Soy Broth containing Cml to select for pEC1, diluted 100-fold in fresh Cml broth and grown to stationary phase again. This procedure was repeated until about 100 generations of growth had occurred, and the shuttle plasmid had been eliminated from the population. Double recombinants carrying the *Δspa*::Etb^r::Kan^rNeo^r mutation were identified as Kan^rNeo^rEtb^rErm^s colonies.

RESULTS

Isolation and characterization of the Δspa::Etb^r::Kan^rNeo^r mutation

The Etb^r marker originally used to inactivate the *spa* gene is expressed very poorly in the single-copy state and is unsuitable as a selective marker in genetic crosses (Patel *et al.*, 1987). Thus a *spa* mutation was constructed by the insertion of a DNA fragment containing a Kan^rNeo^r marker. A 2.6 kb *Hind*III fragment carrying the Kan^rNeo^r determinant of pBR322-Kan^rNeo^r was inserted into one of the *Hind*III sites located within the Etb^r fragment of

Fig. 1. Structure of pEBM155 and isolation of *S. aureus* *spa*::*Kan*^r*Neo*^r by allele replacement. The parallel lines represent staphylococcal DNA from the *spa* region in the shuttle plasmid pEBM155 and in the chromosome. The thin line shows the 2 kb fragment carrying the *Etb*^r marker which substituted for a 1.2 kb *Bcl*I fragment in *spa* forming Δ *spa*::*Etb*^r (Patel *et al.*, 1987). The cross-hatched areas indicate the parts of the *spa* gene remaining in the substitution mutation. The crosses indicate the regions in which recombination must have occurred in generating the Δ *spa*::*Kan*^r*Neo*^r mutation in the chromosome. The large triangles indicate the positions of the inserted *Kan*^r*Neo*^r fragment and plasmid pE194. The *Hind*III site in the 2 kb *Etb*^r region which was involved in insertion of the *Kan*^r*Neo*^r fragment was not determined. Restriction sites are abbreviated as follows: P, *Pst*I; H, *Hind*III; E, *Eco*RI; B, hybrid *Bcl*I/*Bgl*III site.

Fig. 2. Southern hybridization. Genomic DNA was cut with *Eco*RI and probed with ³²P-labelled plasmid DNA. The *spa* probe was from pSPA721 (tracks A, B, C and D), the *Etb*^r probe from pUC18-EB2 (tracks E, F, G and H) and the *Kan*^r*Neo*^r probe from pBR322-*Kan*^r*Neo*^r (tracks I, J, K and L). Genomic DNA was from strains 8325-4 (tracks A, E and I), DU5819 (tracks B, F and J), ISP2073 (tracks C, G and K) and ISP2 (tracks D, H and L). Standard size markers (kb) are indicated.

pEBM15 to form pEBM155 (Fig. 1). This shuttle plasmid was transferred into *S. aureus* Δ *spa*::*Etb*^r (DU5723) and a recombinant (DU5819) carrying the *Kan*^r*Neo*^r marker at its chromosomal *spa* locus was isolated. The resulting Δ *spa*::*Etb*^r::*Kan*^r*Neo*^r mutation was then transformed from the 8325-4 background into the 8325 derivative ISP2 to form ISP2073. The structure of the altered *spa* locus in strain DU5819 and ISP2073 was verified by Southern hybridization as indicated below.

Genomic DNA was cleaved with *Eco*RI, fractionated by agarose gel electrophoresis and hybridized with the following probes: a 4.3 kb *Eco*RI fragment (*spa* probe) carrying the *spa* gene

Fig. 3. PFGE in agarose gel and hybridization analysis of *spa-515::Kan^rNeo^r*. A, PFGE gel after staining with ethidium bromide; B, hybridization analysis of the gel in A using biotinylated pBR322-Kan^rNeo^r DNA as a probe. Track 1, DU5819; track 2, ISP2073; track 3, ISP794. The letters at the left identify the *Sma*I fragment of the *S. aureus* 8325 chromosome: A, 673 kb; B, 361 kb; C, 324 kb; D, 262 kb; E, 257 kb; F, 208 kb; G, 175 kb; H, 135 kb; I, 117 kb; J, 80 kb; K, 76 kb; L, 44 kb; M, 36 kb.

(purified from pSPA721), a 2 kb *Bgl*/II *Etb^r* fragment (*Etb^r* probe) used in the construction of $\Delta spa::Etb^r$ (purified from pUC18-EB2) and a plasmid carrying the 2.6 kb *Hind*III Kan^rNeo^r fragment (Kan^rNeo^r probe) used in constructing $\Delta spa::Etb^r::Kan^rNeo^r$ (purified from pBR322-Kan^rNeo^r).

A fragment of 4.3 kb hybridized with the *spa* probe in both 8325-4 and in ISP2 (Fig. 2, tracks A and D) whereas a 7.7 kb fragment hybridized in strains DU5819 and ISP2073 (Fig. 2, tracks B

Table 3. Mapping *spa*, *purA102* and *nov-142* markers by transformation of DU5819 (*Neo^r Kan^r Nov^s Pur⁺*) with DNA from ISP86 (*Neo^s Kan^s Pur⁻ Nov^r*), selecting for novobiocin resistance

The transformation frequency was 280 Nov^r transformants per 10⁹ c.f.u. and the reversion frequency was <2.9 Nov^r colonies per 10⁹ c.f.u. Excess DNA and 1.7 × 10⁹ c.f.u. were used in 1 ml of the transformation suspension.

Recombinant class	Phenotype*	Percentage of total†
A	Neo ^r Kan ^r Pur ⁺ Nov ^r	35.1
B	Neo ^r Kan ^r Pur ⁻ Nov ^r	38.7
C	Neo ^s Kan ^s Pur ⁻ Nov ^r	26.1
D	Neo ^s Kan ^s Pur ⁺ Nov ^r	0.2

* See Table 1 for genotypes of the parental strains. The Neo^rKan^r phenotype reflects the presence of the insertional inactivated *spa* allele; Pur⁻ and Nov^r reflect the presence of *purA102* and *nov-142* alleles, respectively.

† A total of 476 Nov^r recombinants were analysed.

Table 4. Mapping Ω 1051, *spa* and Tn4291 markers by transformation of DU5819 (*Neo^r Kan^r Erm^s Mec^s*) with DNA from ISP2162 (*Neo^s Kan^s Erm^r Mec^r*) with selection for erythromycin resistance

The transformation frequency was 200 Erm^r transformants per 10⁹ c.f.u. and the reversion frequency was 0.5 Erm^r colonies per 10⁹ c.f.u. Excess DNA and 2.0 × 10⁹ c.f.u. were used per ml of the transformation suspension.

Recombinant class	Phenotype*	Percentage of total†
A	Neo ^r Kan ^r Erm ^r Mec ^s	24.3
B	Neo ^s Kan ^s Erm ^r Mec ^s	15.3
C	Neo ^r Kan ^r Erm ^r Mec ^r	1.8
D	Neo ^s Kan ^s Erm ^r Mec ^r	58.6

* See Table 1 for genotypes of the parental strains. The Neo^rKan^r phenotype reflects the presence of the insertional inactivated *spa* allele; Erm^r indicates the presence of Ω [Tn551]1051, and Mec^r reflects the presence of Tn4291.

† A total of 399 Erm^r recombinants were analysed.

and C). A 7.7 kb fragment also hybridized to the *Etb^r* probe and the Kan^r Neo^r probe in DU5819 and ISP2073 (Fig. 2, tracks F, G, J, K), while the *Spa⁺* strains 8325-4 and ISP2 did not hybridize to these probes (Fig. 2, tracks E, H, I, L). This confirmed that the Δ *spa*::*Etb^r*::Kan^rNeo^r mutation was present as predicted in the chromosome of strain 8325-4.

Physical mapping

Chromosomal DNA from the *Spa⁻* strains DU5819 and ISP2073 along with the *Spa⁺* control strain were analysed by PFGE and Southern hybridization. The filters were probed with biotinylated pBR322-Kan^rNeo^r plasmid DNA. The 175 kb *Sma*I fragment G of strain ISP2073 and the corresponding fragment of the 8325-4 derivative DU5819 hybridized with the Kan^rNeo^r-specific probe (Fig. 3, tracks A1, B1, A2, B2). In the multiple lysogen 8325, *Sma*I-G spans the chromosome from Ω [Tn551]1023 to Ω [Tn551]1051 and thus includes Tn4291 (methicillin resistance [*Mec^r*]; Trees & Iandolo, 1988), *purA102* and *nov-142* (Fig. 4; Pattee, 1987; P. A. Pattee, unpublished).

Genetic mapping

The Δ *spa*::*Etb^r*::Kan^rNeo^r mutation was originally isolated in strain 8325-4 to form DU5819. The *spa* mutation was then transformed into the novobiocin-resistant (Nov^r) strain ISP2. It was observed that selection for the introduction of *spa*::Kan^rNeo^r in ISP2 sometimes caused a Nov^s phenotype, suggesting linkage between *spa* and *nov*. This finding is consistent with the physical mapping experiment described above.

Linkage between *nov* and *spa* was confirmed and quantified by transforming DU5819 (*Spa⁻*) with DNA isolated from ISP86 (*Ura⁻ His⁻ Nov^r Pur⁻*) and selecting for Nov^r (Table 3). While

Fig. 4

Fig. 5

Fig. 4. Abbreviated chromosome map of *S. aureus* 8325.

Fig. 5. Linkage relationship of *spa*-515::Kan^rNeo^r to Ω[Chr::Tn551]1051, Tn4291 (formerly *mec*-4916), *purA*102 and *nov*-142 (Luchansky & Pattee, 1984; Kuhl *et al.*, 1978; Trees & Iandolo, 1988). Map distances are calculated from the data in Tables 3 and 4. Map distances between two markers A and B are expressed as 1 minus the estimated co-transformation frequency (C), where C = frequency of co-transformation of two markers A and B (i.e. C = AB/A or C = AB/B where AB is the recombinant class containing both donor markers A and B).

*uraA*141 and *hisG*15 segregated independently, the results placed *purA*102 0.35 map units to the 'left' of *nov*-142 as previously shown by Stahl & Pattee (1983a). The results also placed *spa* (scored as Kan^rNeo^r) 0.74 map units from *nov*-142 and on the same side as *purA*102. When DU5819 was transformed with DNA isolated from ISP2162 (Ω1051 Erm^r Mec^r), with selection for Erm^r, the results (Tables 3 and 4) placed Ω1051 and *spa* to the 'left' of Tn4291 in the order *spa*-Ω1051-Tn4291. Fig. 5 summarizes the linkage relationships of these markers; Fig. 4 shows the relative location of this region on the chromosome map of *S. aureus*.

DISCUSSION

This paper describes the application of PFGE to mapping chromosomal genes in *S. aureus*. This technique allows any gene which has been cloned and/or inactivated by insertion mutagenesis to be localized to a segment of the chromosome in a single Southern blot experiment. This information can be used to choose markers for subsequent genetic mapping experiments and may obviate the need to use the laborious procedure of multi-factorial analysis by protoplast fusion (Stahl & Pattee, 1983a, b) to locate the region of the chromosome involved.

The gene coding for protein A is only the third exoprotein virulence factor gene of *S. aureus* to be mapped on the chromosome. The availability of an insertion mutation isolated by allele replacement facilitated these mapping experiments. Allele-replacement mutagenesis is a generally applicable technique for isolating insertion mutations in chromosomal genes, but requires the gene in question to be cloned. Despite efforts by several groups, transposon-generated mutations affecting virulence factor or exoprotein genes of *S. aureus* have not been reported, apart from a single Tn551 mutation in *agr* which causes a pleiotropic regulatory mutation (T. J. Foster & P. A. Pattee, unpublished). It seems that staphylococcal transposons may show some degree of insertion site specificity.

There is no evidence that virulence factor genes are clustered in any particular region of the chromosome. Here we show that the protein A gene is not linked to genes coding for lipase (Pattee, 1987) or α-toxin (Pattee, 1986). Furthermore, sequence analysis of chromosomal genes which code for exoproteins suggests that they are monocistronic (Uhlén *et al.*, 1984; Lee & Iandolo, 1986; Kaida *et al.*, 1987). In many cases putative transcription termination signals have been identified adjacent to the coding sequences.

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