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Sequence and mapping of the *aroA* gene of *Staphylococcus aureus* 8325-4

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The *aroA* gene of *Staphylococcus aureus* 8325-4 was cloned. Sequence analysis and the phenotype of directed plasmid insertions 5′ to *aroA* suggest that *aroA* is located in an operon and that it maps 3′ to the *aroC* and *aroB* genes. A revised consensus sequence for the *aroA* gene product EPSP synthase binding site for its substrate (phosphoenolpyruvate) and an inhibitor (glyphosate) is proposed. An *aroA* insertion mutant isolated by allelic replacement was employed in genetic mapping experiments which demonstrated the gene order *thy aroA tyrB* in *Sma*I fragment A of the *S. aureus* 8325-4 chromosome. The *aroA*::Tc^r mutant required aromatic amino acids but remained independent of *p*-aminobenzoic acid (PAB). This could be due to the insertion being located close to the 5′ end of the gene, allowing expression of a truncated protein. The PAB independence may explain the finding that the mutant was not attenuated in mouse infection experiments. It was not possible to isolate a null mutant in *aroA*.

Introduction

In bacteria, aromatic amino acids and *para*-amino benzoic acid (PAB) are synthesized from a common precursor, chorismic acid, which is in turn formed from phosphoenolpyruvate (PEP) and erythrose-4-phosphate by enzymes in the pre-chorismate pathway (reviewed by Pittard, 1989). PAB is a precursor of folic acid, a substance which is not synthesized by chordates. These animals obtain folic acid exogenously or from gut commensals. Mutants which are defective in the enzymes of the prechorismate pathway require aromatic amino acids and PAB for growth. Mutants of pathogenic bacteria defective in any of these genes are capable of only limited growth in mammalian hosts and are thus attenuated (Hoiseth & Stocker, 1981). Furthermore, *aroA* mutants of *Salmonella* spp. are effective oral vaccines (Jones *et al.*, 1991).

In enteric bacteria the *aro* genes are unlinked. The *aroA* gene forms an operon with *serC* (Duncan & Coggins, 1986). In contrast, in *B. subtilis* the *aroA* gene is part of a cluster of genes encoding enzymes involved in the biosynthesis and transport of aromatic compounds (Henner *et al.*, 1986). This 'supraoperon' is comprised of *aroC* (designated *aroF*), *aroB*, *aroH*, the *trp* operon, *hisH*, *tyrA* and *aroA* (designated *aroE*). *aroE* is co-transcribed with *hisH* and *tyrA*. In fungi and yeasts the enzymic activities of individual gene products in bacteria are associated with a single, pentafunctional enzyme (Nakanishi & Yamamoto, 1984; Charles *et al.*, 1986).

The *aroA* gene, which codes for 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19), has been intensively studied. The *aroA* genes of several enteric bacteria have been sequenced (Duncan *et al.*, 1984; Stalker *et al.*, 1985; O'Gaora *et al.*, 1989) in addition to the genes from *Bordetella pertussis* (Maskell *et al.*, 1988), *Mycobacterium tuberculosis* (Garbe *et al.*, 1990), *Bacillus subtilis* (Henner *et al.*, 1986), and *Aeromonas salmonicida* (Vaughan *et al.*, 1993). Sequences are also available from fungi, yeasts and higher plants (Charles *et al.*, 1986; Duncan *et al.*, 1987; Gasser *et al.*, 1988; Gasser & Klee, 1990; Klee *et al.*, 1987). EPSP synthase is of particular interest because it is the site of action of the broad spectrum, post-emergence herbicide glyphosate (*N*-phosphonomethyl glycine, 'Roundup'). Glyphosate is a competitive inhibitor of the enzyme with respect to the substrate, phosphoenolpyruvate (PEP). PEP binds to a conserved stretch of amino acids recognized by comparison of the EPSP synthases from

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Abbreviations: PAB, *p*-amino benzoic acid; PEP, phosphoenolpyruvate; EPSP, 5-enolpyruvylshikimate-3-phosphate; PFGE, pulsed-field gel electrophoresis; S3P, shikimate-3-phosphate.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number L05004.

Table 1. *Bacterial strains*

Strain	Relevant genotype	Properties	Source or reference
Escherichia coli			
JM109	*recA* Δ*lac-proAB*[F′*proAB* *lacI*^{q}Z ΔM15]	Host for pUC plasmids	Yanisch-Perron *et al.* (1985)
AB1321	*proA2 aroA2 his-4 thi-1 lacY1*	Host for *aroA* complementation experiments	Taylor & Thoman (1964)
AB2847	*tsx-354, supE42* λ^- *aroB351 malT354* (λ^r)	Host for *aroB* complementation experiments	Pittard & Wallace (1966)
XacSu$^-$	Δ[pro-lac]$_{XIII}$ *ara arg rpoB gyrA thi*	Host for Tn*5* mutagenesis	Coleman & Foster (1981)
Staphylococcus aureus			
8325-4		NCTC 8325 cured of prophages	Novick (1963)
RN4220		Mutant of 8325-4 capable of accepting chimeric plasmids	Kreiswirth *et al.* (1983)
DU5859	8325-4 *pig-131 aroA-600*::Tc^r	Allele replacement mutant of 8325-4	This study
ISP2504			
ISP2169	8325 *pig-131 tyrB282*::Tn*551* Ω1201[Tn*4001*]	Mapping strain	
ISP794	8325 *pig-131*		
ISP2581	8325 *pig-131 aroA-600*::Tc^r	Transformation of ISP794 with DNA from DU5859	This study
ISP2582	8325 *pig-131 tyrB282*::Tn*551* Ω1201[Tn*4001*] *aroA-600*::Tc^r	Transformation of ISP2169 with DNA from ISP2581	This study
ISP193	8325 *thy-101 thrB106 ilv-129 pig-131* Ω11[Tn*551*] Φ12	Mapping strain	

these diverse organisms (Padgette *et al.*, 1991). Alteration of amino acids in this region by mutagenesis results in resistance to glyphosate.

This paper reports the cloning, genetic mapping and sequence of the *aroA* gene of *Staphylococcus aureus* 8325-4. The *aroA* gene was shown to be part of an operon with *aroB* and *aroC*. An *aroA*::Tc^r mutation was constructed by allele replacement. This mutant was employed in genetic mapping experiments and its virulence for mice was investigated.

Methods

Bacterial strains, plasmids and phage. The bacterial strains, plasmids and bacteriophages used in this study are shown in Tables 1 and 2.

Media and growth conditions. Escherichia coli strains were routinely grown in L-broth and L-agar (Miller, 1972). Antibiotics were from Sigma. These were incorporated into the media where appropriate at the following concentrations: ampicillin (Ap), 100 μg ml^{-1}; chloramphenicol (Cm), 5 μg ml^{-1}; and tetracycline (Tc), 2 μg ml^{-1}. Screening for complementation of the aromatic deficiencies of *E. coli* strains AB1321 or AB2847 was performed on M9 minimal medium agar supplemented with amino acids and vitamins as appropriate. *S. aureus* strains were grown in trypticase soy broth (TSB) or agar, at 37 °C or at 30 °C if carrying a temperature sensitive (*ts*) plasmid. Antibiotics were incorporated as follows: erythromycin (Em), 10 μg ml^{-1}; gentamicin (Gm), 10 μg ml^{-1}; Cm, 5 μg ml^{-1}; kanamycin (Ka), 20 μg ml^{-1}; and Tc, 2 μg ml^{-1}. A defined synthetic medium (DSM) suitable for *S. aureus* has been described by Pattee (1981). Aromatic amino acid auxotrophy (AAA) was detected by the failure of bacteria to grow on DSM lacking tryptophan (Trp) and tyrosine (Tyr). Phenylalanine is essential for the growth of strain 8325-4 and was therefore included. In order to test for PAB auxotrophy, methionine and nucleotides were also removed from the medium. In addition, in order to prevent contamination with PAB, the medium was made with ARISTAR grade chemicals, with HPLC grade amino acids (Sigma) and with grade I filtered water (Barnstead, Nanopure II). The absence of PAB was shown by the failure of *E. coli* AB1321 *aroA* to grow.

DNA manipulations. In vitro manipulation of plasmid DNA was performed by standard procedures (Sambrook *et al.*, 1989). Isolation of plasmid DNA was by the method of Birnboim & Doly (1979). Chromosomal DNA was isolated using the method of Pattee & Neveln (1975). Restriction endonucleases and DNA modifying enzymes were used in accordance with the manufacturers' recommendations (Boehringer or Promega).

Transformation and transduction. S. aureus was transformed with plasmid DNA by electroporation of exponential phase cells as described by Oskouian & Stewart (1990). Phage were propagated and used in transduction experiments as described by Asheshov (1966).

Tn5 mutagenesis. Tn*5* mutagenesis of pCOC2 was done as described by de Bruijn & Lupski (1984), using a high-titre stock of λ467::Tn*5* to deliver the transposon to *E. coli* strain XacSu$^-$ containing the plasmid.

Table 2. *Plasmids*

Plasmid	Host	Markers	Relevant properties	Source or reference
pCW59	*S. aureus*	Cm^r Tc^r	Cloning vector	Wilson *et al.* (1981)
pTV1*ts*	*S. aureus*	Cm^r Em^r	*ts* pE194 replicon	Youngman (1987)
pRN8013	*S. aureus*	Tc^r	*ts* derivative of pT181	Novick *et al.* (1986)
pBR322	*E. coli*	Ap^r Tc^r	Cloning vector	Bolivar *et al.* (1977)
pUC18	*E. coli*	Ap^r	Cloning vector	Yanisch-Perron *et al.* (1985)
pBluescript SK^+ & KS^+	*E. coli*	Ap^r	Cloning vectors	Short *et al.* (1988)
pTS1	*ts* shuttle	Ap^r Cm^r	*ts* shuttle plasmid derived from pTV1*ts*. 4·2 kb *Bam*HI–*Pst*I fragment in pUC18	This study
pBRKan Neo	*E. coli*	Ap^r Ka^r Ne^r	2·6 kb *Hin*dIII fragment expressing Ka^r in pBR322	Storrs *et al.* (1988)
pCOC101	*E. coli*	Ap^r	3 kb *Hin*dIII *aroA* fragment in pBR322	This study
pCOC102	*E. coli*	Ap^r	3 kb *Hin*dIII *aroA* fragment in pUC18	This study
pCOC104	*E. coli*	Ap^r Ka^r Ne^r	Δ*aroA*::Ka^r formed by ligating Ka^r fragment into *Acc*I-cut pCOC102	This study
pCOC106	*E. coli*	Ap^r	pBluescript KS^+ with 440 bp *Pst*I–*Eco*RV *aroA* fragment	
pCOC107	*ts* shuttle	Ap^r Cm^r	4·2 kb *Pst*I–*Bam*HI fragment of pTV1*ts* in pCOC106	This study
pCOC109	*S. aureus*	Tc^r Ka^r Ne^r	Δ*aroA*::Ka^r from pCOC104 in pRN8013	This study
pCOC201	*E. coli*	Ap^r	6·2 kb *Eco*RI *aroA* fragment in pBluescript SK^+	This study
pCOC203	*E. coli*	Ap^r Tc^r	*aroA*::Tc^r in pBluescript KS^+. Tc^r fragment from pCW59 in *Hin*dIII site	This study
pCOC204	*ts* shuttle	Ap^r Cm^r Tc^r	*aroA*::Tc^r from pCOC203 in pTS1	This study
pCOC206	*E. coli*	Ap^r	6·2 kb *Eco*RI *aroA* fragment in pUC18 variant lacking *Hin*dIII and *Bam*HI sites	This study
pCOC207	*E. coli*	Ap^r	Deletion between *Acc*I sites internal to *aroA* fragment in pCOC206	This study
pCOC208	*E. coli*	Ap^r Tc^r	2·6 kb Tc^r fragment of pCW59 in *Bam*HI site of pCOC207	This study
pCOC209	*ts* shuttle	Ap^r Cm^r Tc^r	Tc^r Δ*aroA* from pCOC208 linked to pTS1 at *Eco*R1 sites	This study

In this host the phage is unable to replicate and acts as a suicide delivery system for the transposon. Ka^r transformants were pooled and plasmid DNA isolated. This was transformed into *E. coli* AB1321, selecting on L-agar plates containing Ka. Colonies were replica-plated on to M9 agar plates to screen for derivatives which failed to complement the *aroA* lesion. Plasmid DNA was isolated from putative mutants. The point of insertion of the transposon was identified by restriction mapping (de Bruijn & Lupski, 1984).

Physical mapping. Pulsed-field gel electrophoresis (PFGE) of chromosomal DNA was done as described by Smith *et al.* (1986) and Patel *et al.* (1989).

DNA hybridization. This was done as described in Sambrook *et al.* (1989). 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-^{32}P]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) (hybridization being detected using the Blu-gene system; BRL).

DNA sequencing. DNA sequencing was carried out using the chain termination method of Sanger *et al.* (1977) with the modification of Biggin *et al.* (1983) for the incorporation of [α-^{35}S]dATP. Double-stranded sequencing was done initially on subcloned DNA fragments in pBluescript KS^+, using universal primer (Boehringer). The sequence obtained from these clones was used to synthesize primers on an Applied Biosystems PCRmate oligonucleotide synthesizer to complete

the sequence of the *aroA* gene on both strands. DNA sequence was also obtained from plasmid sub-clones of DNA lying 5′ to *aroA* on one strand only. Sequencing reactions used modified T_7 polymerase (Tabor & Richardson, 1987) from Promega. [α-^{35}S]dATP was from Amersham. Plasmid DNA was denatured by the addition of 5 μl 1 M-NaOH, 1 mM-EDTA to 20 μl DNA (15–20 μg) and incubation at room temperature for 5 mins. This was then neutralized by centrifuging through a Sepharose-CL 6B spin column at 200 *g* as described by Murphy & Kavanagh (1988). DNA was used immediately in sequencing reactions or stored at −20 °C until required.

Nucleotide and deduced amino acid sequences were analysed using the PC/GENE software package (Intelligenetics) which includes the multiple alignment programme CLUSTAL (Higgins & Sharp, 1988).

Plasmid constructions. Chromosomal DNA isolated from *S. aureus* 8325-4 was cut with *Hin*dIII and ligated with pBR322 which had been cleaved with the same enzyme. The ligated DNA was transformed into *E. coli* AB1321 *aroA*, and Ap^r transformants were selected. These were replica-plated on to minimal medium to identify transformants carrying plasmids which complemented the aromatic deficiency of the strain. Aro$^+$ transformants were selected and plasmid DNA was isolated. A pBR322 clone carrying a 3 kb insert was designated pCOC101. Genomic DNA was cut with *Eco*RI and ligated with *Eco*RI-cut pBluescript SK$^+$. An *aroA*-carrying recombinant (pCOC201) was recognized by colony hybridization using the *Hin*dIII fragment from pCOC101 as a probe.

Plasmids capable of integrating into the *S. aureus* chromosome were constructed (Fig. 1). A 440 bp *Pst*I–*Eco*RV fragment internal to the *aroA* gene was cloned into pBluescript KS$^+$ cut with the same enzymes. The resultant plasmid was cleaved with *Pst*I and *Bam*HI and ligated with a 4·2 kb *Pst*I–*Bam*HI fragment of pTVI*ts* which encodes a Cm resistance determinant and a temperature-sensitive Rep protein. The ligated DNA was transformed into *E. coli* JM109, and Ap^r transformants were screened for resistance to Cm. Plasmid DNA was checked by restriction mapping and one derivative, pCOC107, was kept for further use (Fig. 1).

Plasmid pCOC102 (carrying the 3 kb *aroA Hin*dIII fragment) was cleaved with *Eco*RV to create a deletion in *aroA* and ligated with the 2·6 kb *Hin*dIII fragment of pBR Kan Neo (containing a gene from *S. aureus* which expresses resistance to Ka) that had been blunted following treatment with DNA polymerase I Klenow fragment. The ligated DNA was transformed into *E. coli* JM109, selecting on L-agar containing Ap. The transformants were then screened for resistance to Ka. After checking the structure, one plasmid designated pCOC104 was retained. The *aroA* deletion is flanked by 1·0 and 1·1 kb chromosomal DNA. Plasmid pRN8013 is a temperature-sensitive derivative of pT181, a plasmid which harbours a Tc resistance determinant. The 4·8 kb *Hin*dIII fragment from pCOC104 containing Δ*aroA*::Ka^r was ligated with pRN8013 which had been partially digested with *Hin*dIII. The ligated DNA was electroporated directly into *S. aureus* RN4220 and Tc^r transformants selected at 30 °C. Three Tc^r Ka^r recombinants were isolated. Plasmid DNA was prepared and a plasmid (pCOC109) with the appropriate structure was kept (Fig. 1). Plasmid integrants were selected by plating a stationary phase culture of 8325-4 (pCOC109) on TSA containing Tc and incubating at 42 °C.

The 6·2 kb *Eco*RI fragment carrying *aroA* in pCOC201 was cloned into the *Eco*RI site of a pUC18 derivative which lacked the *Hin*dIII–*Bam*HI portion of the multiple cloning site to generate pCOC206. This plasmid was cleaved with *Acc*I, treated with DNA polymerase I Klenow fragment and allowed to ligate back on itself in order to delete the 1·8 kb *Acc*I fragment which carries much of the *aroA* gene. Flanking sequences of 3 kb and 1·4 kb remained. This plasmid was designated pCOC207. A unique *Bam*HI site is located approximately 100 bp from the *Acc*I deletion. Plasmid pCOC207 was cleaved with *Bam*HI, treated with DNA polymerase I Klenow fragment, and blunt-end-ligated with a 2·6 kb *Hin*dIII fragment of pCW59 containing a Tc^r marker that had also been treated with DNA polymerase I Klenow fragment. The ligated DNA was transformed into *E. coli* JM109. One Ap^r Tc^r recombinant with the appropriate structure (pCOC208) was retained for further use. This plasmid was cleaved with *Eco*RI to release the Tc^r Δ*aroA*-containing fragment which was inserted into the unique *Eco*RI site of pTSI, a *ts* shuttle vector containing the *Pst*I–*Bam*HI fragment of pTVI*ts* joined to pUC18. The ligated DNA was electroporated into *S. aureus* RN4220 and transformants were selected on TSA containing both Tc and Cm at 30 °C. One recombinant with the expected structure (pCOC209) was kept (Fig. 1). This plasmid was transferred to strain 8325-4 by transduction. Putative integrants were isolated at a frequency of 10^{-2} by plating on Tc agar and incubating at 42 °C.

Construction of aroA::Tc^r. Plasmid pCOC201 (pBluescript with the 6·2 kb *aroA Eco*RI fragment) was partially digested with *Hin*dIII in order to linearize the plasmid. The DNA was ligated with a 2·6 kb *Hin*dIII fragment of pCW59 which contains a Tc^r determinant and transformed into *E. coli* JM109. One plasmid (pCOC203) of thirty tested had the fragment inserted in the *Hin*dIII site in *aroA*. The *ts* shuttle plasmid pTS1 was cleaved with *Eco*RI and ligated with the *Eco*RI fragment of pCOC203 carrying *aroA*::Tc^r, and the DNA was used to transform *E. coli* JM109, selecting for Ap^r. One derivative (pCOC204) with the appropriate structure was retained for further use (Fig. 1). Plasmid pCOC204 was electroporated into *S. aureus* RN4220. Selection on TSA with Tc at 42 °C allowed allelic replacement recombinants to be isolated.

Genetic mapping. S. aureus was transformed with chromosomal DNA as described by Stahl & Pattee (1983) and modified by Patel *et al.* (1989). Transformants were selected and scored as described previously (Stahl & Pattee, 1983) and linkage was calculated as described by Patel *et al.* (1989).

Animal infection models. Strain 8325-4 *aroA*::Tc^r was tested for virulence in intraperitoneal and subcutaneous mouse infection models (Patel *et al.*, 1987). Intraperitoneal diffusion chambers were constructed and implanted in the mouse peritoneal cavity (de Azavedo & Russell, 1988).

Results

Cloning the aroA gene

The *aroA* gene of *S. aureus* 8325-4 was cloned on a 3 kb *Hin*dIII fragment in pBR322 by complementation of the Aro$^-$ phenotype of *E. coli* AB1321. To identify the position of the *aroA* gene, the cloned fragment was cloned into pUC18 (forming pCOC102) and mutagenized using Tn*5*. Thirteen individual insertions in the *Hin*dIII fragment spanning a region of 1·3 kb were mapped (Fig. 2). This is sufficient to encode a protein of approximately 46 kDa, which is the size of the EPSP synthase of *E. coli* (Duncan *et al.*, 1984). Problems experienced when subcloning or while attempting to construct a nested set of deletions for sequencing suggested that the cloned *aroA* gene might be unstable. A discrepancy of a single *Cla*I site between the restriction map of the 3 kb *Hin*dIII fragment and that of the chromosomal *aroA* locus was observed in Southern blotting experiments (data not shown). Thus the *aroA*

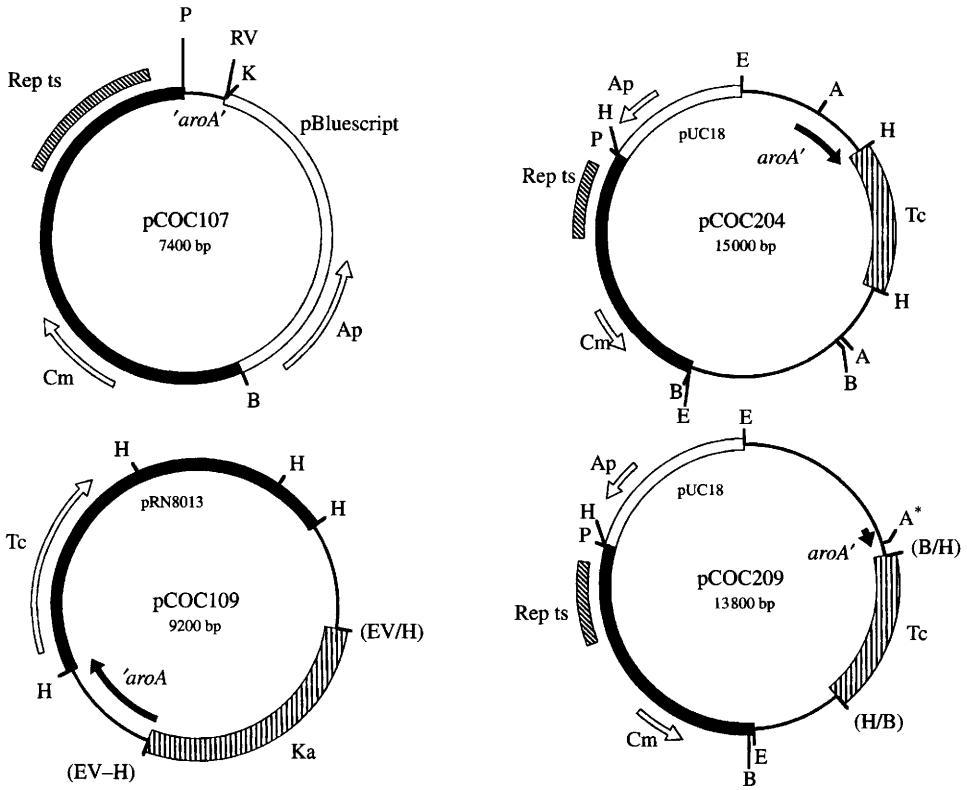

Fig. 1. Maps of integrating plasmids. *E. coli* plasmid vector sequences are indicated by parallel lines, while *S. aureus* vector sequences are filled. *S. aureus* chromosomal DNA is represented by a thin line. Deletion of the *aroA* gene is indicated by the apostrophe (*aroA'*, *'aroA*). Antibiotic resistance markers inserted in *aroA* are represented by a thick box with vertical dashed lines. Restriction sites are abbreviated as follows; H, *Hin*dIII; EV, *Eco*RV; P, *Pst*I, A, *Acc*I, K, *Kpn*I and B, *Bam*HI. A* indicates the *Acc*I fragment deletion in pCOC209. The locations of genes coding for ampicillin resistance (Ap), chloramphenicol resistance (Cm), kanamycin resistance (Ka) and tetracycline resistance (Tc) are indicated. Note that the plasmids are not drawn to the same scale.

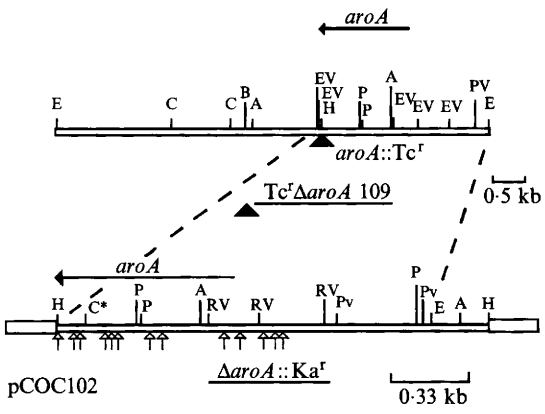

Fig. 2. Restriction map of the 3 kb *Hin*dIII fragment inserted in pCOC102 and the 6·2 kb *Eco*RI fragment inserted in pCOC201 which carry the *aroA* gene. The flanking boxes denote vector sequences. The double line indicates cloned DNA. Vertical arrows indicate the positions of Tn*5* insertions in pCOC102. The insertion points of Tc^r markers in *aroA*::Tc^r and pCOC109 are indicated by filled arrows, and deletions are shown as horizontal lines. Restriction sites are abbreviated as follows; H, *Hin*dIII; C, *Cla*I; P, *Pst*I; B, *Bam*HI; A, *Acc*I; E, *Eco*RI; EV, *Eco*RV; PV, *Pvu*II. C* indicates the *Cla*I site present in the *Hin*dIII fragment which is absent from the *Eco*RI fragment and the chromosomal *aroA* locus.

gene was recloned on a 6·2 kb *Eco*RI fragment pBluescript SK$^+$, forming pCOC201. The restriction map of the 6·2 kb *Eco*RI fragment was found to be almost identical to that of the *Hin*dIII fragment in the region where they overlapped (Fig. 2). However, the single *Cla*I site located in the *Hin*dIII fragment was absent. All sequencing was carried out using the *Eco*RI fragment.

Sequencing the aroA gene

A total of 1735 bp was sequenced (Fig. 3) including the 1·1 kb *Eco*RV fragment encompassing the region marked by the Tn*5* insertions in pCOC102. Two open reading frames (ORFs) are contained within this region. The translated product of one ORF is 43% identical to the *aroE* gene product of *B. subtilis* and 29% identical to the *aroA* gene product of *E. coli*. An alignment of the products of these genes is shown in Fig. 4. A putative ribosome binding site is located 8 bp 5′ to the ATG start codon. A TAA stop codon marks the end of the gene, which is immediately followed by a second small ORF which could code for a protein of 68 amino acids (pI 4·05). A search of the EMBL database did not

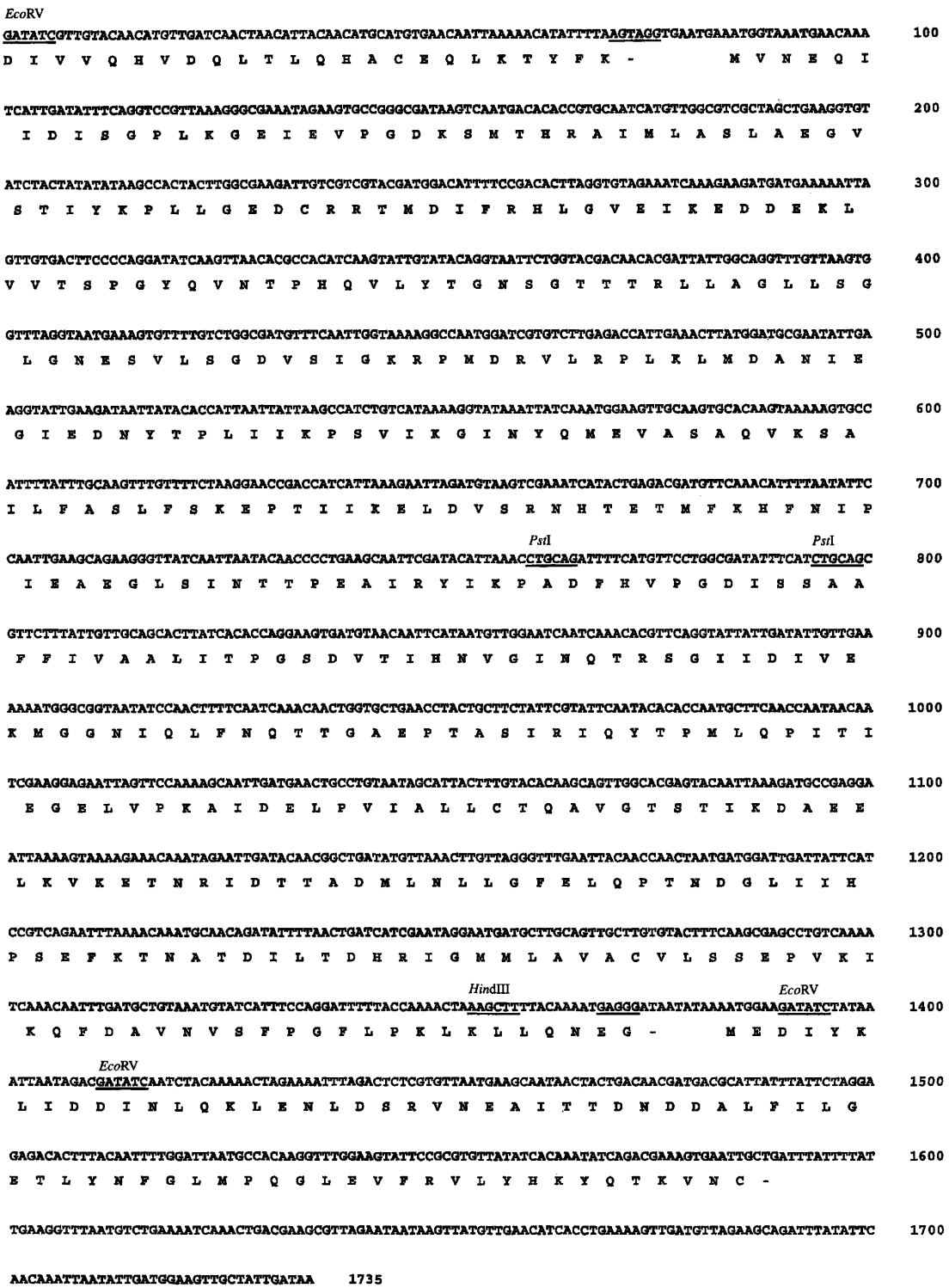

Fig. 3. Sequence of the *aroA* gene of *S. aureus* 8325–4 and its deduced amino acid product. The *Eco*RV site at the start of the sequence corresponds to the central *Eco*RV site of the 3 kb *Hin*dIII insert in pCOC102. ORFs 5′ and 3′ to *aroA* are shown with their predicted amino acid sequences. Potential ribosome binding sites are underlined.

suggest the identity of this protein. A potential ribosome binding site is located 10 nucleotides 5′ to the start codon. There are no detectable repeat sequences of the type characteristic of rho-independent terminators in the DNA adjacent to the end of either the *aroA* gene or the 3′ ORF. Further work is required to determine if this

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E. coli      MESLTLQPIA-RVDGTINLPGSKTVSNRALLLASLAHGKTVLTNLLDSDD    49
S. aureus    MVNEQIIDISGPLKGEIEVPGDKSMTHRAIMLASLAEGVSTIYKPLLGED    50
B. subtilis  MKRDKV----QTLHGEIHIPGDKSISHRSVMFGALAAGTTTVKNFLPGAD    46
             * .  .      ..*.*..**.*....*......** * ... . * ..*

             VRHMLNALTALGVSYTLSADRTRCEIIGNGGPLHAEGALELFLGNAGTAM    99
             CRRTMDIFRHLGV--EIKEDDEKLVVTSPGYQ-VNTPHQVLYTGNSGTTT    97
             CLSTIDCFRKMGV--HIEQSSSDVVIHGKGIDALKEPESLLDVGNSGTTI    94
                 .. .  .**   .... .   . . *     .    *  **.**.

             RPLAAALCLGSNDIVLTGEPRMKERPIGHLVDALRLGGAKITYLEQENYP   149
             RLLAGLLSGLGNESVLSGDVSIGKRPMDRVLRPLKLMDANIEG-IEDNYT   146
             RLMLGILAGRPFYSAVAGDESIAKRPMKRVTEPLKKMGAKIDGRAGGEFT   144
             * . . *       ...*. .. .**. ..  .*.  .*.*.    ....

             PLRLQGNFTGGNVDVDGSVSSQFLTALLMTAPLAPEDTVIRIKGDLVSKP   199
             PLIIKPSVIKG-INYQMEVASAQVKSAILFASLFSKEPTI-IKELDVSRN   194
             PLSVSGASLKG-IDYVSPVASAQIKSAVLLAGLQAEGTTT-VTEPHKSRD   192
             ** .  .   * ..    *.*. ... .. * * ..... ...   *.

             YIDITLNLMKTFGVEIENQHYQQFVVKGGQSYQSPGTYLVEGDASAASYF   249
             HTE---TMFKHFNIPIEAEGLSINTTPEAIRYIKPADFHVPGDISSAAFF   241
             HTE---RMLSAFGVKLSEDQTSVSIAGGQK--LTAADIFVPGDISSAAFF   237
             ...    ... *.. ....     .. .     ....  * ** *.*..*

             LAAAAIK-GGTVKVTGIGRNSMQGDIRFADVLEKMGATI-----------   287
             IVAALITPGSDVTIHNVGINQTRSGI--IDIVEKMGGNIQLF-NQTTGAE   288
             LAAGAMVPNSRIVLKNVGLNPTRTGI--IDVLQNMGAKLEIKPSADSGAE   285
             ..*. .  .. . . ..* *  ...*   *....**...

             CWGDDYISCTRGELNAIDMDMNHIPDAAMTIATAALF---AKGTTRLRNI   334
             PTASIRIQYTPM-LQPITIEGELVPKAIDELPVIALLCTQAVGTSTIKDA   337
             PYGDLIIE-TSS-LKAVEIGGDIIPRLIDEIPIIALLATQAEGTTVIKDA   333
               ..  *  *.  *..... . .*    .... **.   * **. ...

             YNWRVKETDRLFAMATELRKVGAEVEEGHDYIRITPPEKLNFA-EIATYN   383
             EELKVKETNRIDTTADMLNLLGFELQPTNDGLIIHPSEFKTNATDILT--   385
             AELKVKETNRIDTVVSELRKLGAEIEPTADGMKVYGKQTLKGGAAVSSHG   383
              . .****.*. . .. *. .* *.. . * . .   .  . . .. . .

             DHRMAMCFSLVA-LSDTPVTILDPKCTAKTFPDYFEQLARISQAA   427
             DHRIGMMLAVACVLSSEPVKIKQFDAVNVSFPGFLPKLKLLQNEG   430
             DHRIGMMLGIASCITEEPIEIEHTDAIHVSYPTFFEHLNKLSKKS   428
             ***..* ....  ....*..* . . .  ..*... .*  . . .
```

Fig. 4. Clustal alignment of the EPSP synthases from *S. aureus*, *E. coli* and *B. subtilis*. Amino acids which are identical in all three sequences are indicated by asterisks and conservative amino acid substitutions by dots. Gaps introduced to maximise homology are shown as hyphens. The regions involved in binding the substrates shikimate-3-phosphate (centered around K22 in the *E. coli* sequence) and phosphoenolpyruvate (centered around G96) are underlined.

ORF is expressed. No sequences similar to *S. aureus* promoters (Novick, 1990) were identified.

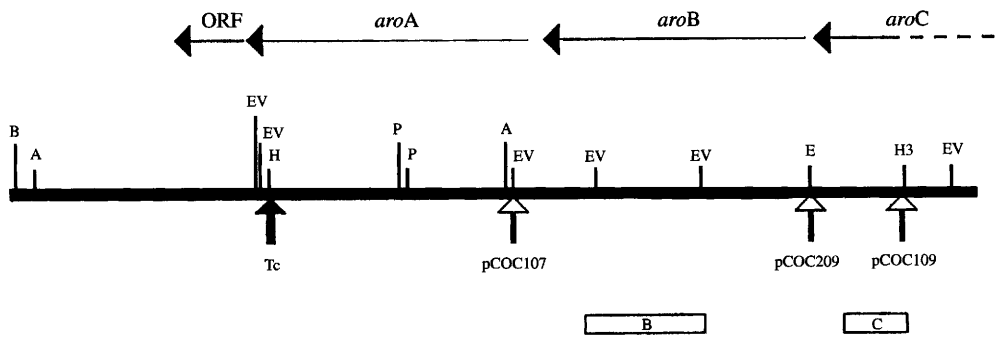
A clear bias in codon usage, with preference for A or T in the third position, was observed in the *aroA* gene. A less pronounced preference for A or T was also seen at the first and second positions. The *aroA* gene has a G+C content of 36%, which is similar to that of the *S. aureus* chromosome (33% G+C; de Ley, 1970).

Analysis of DNA 5′ to aroA

The temperature-sensitive plasmids pCOC109 and pCOC209 contain Δ*aroA*::Ka^r and Δ*aroA*::Tc^r, respectively. When cultures containing these plasmids were plated on Ka or Tc agar and incubated at 42 °C, colonies grew at frequencies of 10^{-3} and 10^{-2}, respectively. These were presumably due to integration of the plasmid into the chromosome by a single cross-over event. About 50% of the pCOC109 integrants and 30% of the pCOC209 integrants were Aro$^-$. Southern blotting (data not shown) demonstrated that integration had occurred at 1·4 kb and 1·0 kb, respectively, 5′ to the start of the *aroA* gene. In contrast, integrants of pCOC107, which contains an *aroA*-internal fragment, were all Aro$^-$.

To identify the cause of the Aro$^-$ phenotype, both of the *Eco*RV fragments located in the 5′ region of the *aroA* gene were cloned into pBluescript KS$^+$, and parts were sequenced (Fig. 5).

An ORF was identified 5′ to *aroA* which ended with a stop codon 9 nucleotides upstream of the start of *aroA*.




```
DHQ synthase      FLEELLNIHSLRDITNDQLNDM---IFKGISIKASVVQQDEKEEGIRAYL -242
                   .*  ***   ****   ** *   * ***  *  .*  **** *.* .*
region B          DIEXHLNI---RDIT-GSLNGMDKYIAKGIETKLDIVIADEKEQGVRKFL -46

                  NFGHTLGHAVEAEYGYGQITHGDAVALGMQFALYISEKTVGCEMDRKRLV -292
                  ***** *****       * ** ** .*     .           *    .
                  NFGHTFGHAVEYHH---KIPHGHAVMVGIIYQFIVANALFDSKHDINHYI -93

                  SWLKSLGYPSQIRKETETSVLLNRMMNDKKTRGGKIQFIVLNELGKVADH -342
                   .*  ****     . .   *   *  ***     .* . .   * .
                  QYLIQLGYPLYMLTDLDFETLYQYMLSDKKNDKQGVQMVLIRQFGDIVVQ -143

                  TFSRNELESWLNKWRLEETS -362
                        *       .
                  HVDQLTLQHACEQLKTYFK  -162

Chorismate synthase SINAFKGVEFGIGFEAAGRNGSEVHDEIIWDEEKGYTRATNRLGGLEGGM -300
                          ** ** ** . **.. ***..  * ** * .* ********
region C          ---------FGEGFKAAEKPGSDIQDEILYNSEIGYYRGSNHLGGLEGGM -42

                  TTGMPIVVRGVMKPIPTLYKPLKSVDIETKEPFSASIERSDSCAVPAASV -350
                  . ****.* ***    ****
                  SNGMPIIVNGVMN--NTLYKH                              -61
```

Fig. 5. Map of the chromosomal *aroC aroB aroA* locus. The site of insertion of the tetracycline resistance marker in *aroA*::Tc^r is shown by the filled arrow. The points of integration of plasmids pCOC107, pCOC109 and pCOC209 are indicated by open arrows. Boxed regions B and C indicate sequenced DNA, the translated products of which are shown below aligned with (i) the *B. subtilis aroB* gene product dehydroquinate (DHQ) synthase and (ii) the *B. subtilis aroF* (*aroC*) gene product chorismate synthase. The positions of the *aroB* and *aroC* genes were estimated from the alignments and the sizes of these genes in *B. subtilis*.

Its deduced amino acid sequence was found to be homologous with the C-terminal end of the *aroB* gene product of *B. subtilis* (Fig. 5). Plasmids pCOC102 and pCOC201 were introduced into *E. coli* AB2847 *aroB*. Both plasmids were able to complement the *aro* mutation in this strain, confirming the presence of the *aroB* gene 5′ to *aroA*. The *aroB* gene in both *B. subtilis* and *E. coli* encodes a protein of 362 amino acids (Millar & Coggins, 1986; Henner, 1991). There is sufficient DNA within the cloned *Eco*RI fragment to code for a protein of this size.

Some of the 400 bp lying between the right-hand *Eco*RI and *Hin*dIII sites in pCOC102 was sequenced (Fig. 5). An ORF was identified, the translated product of which has significant homology with the C-terminus of the *aroF* gene product of *B. subtilis* (Fig. 5). This gene encodes chorismate synthase and is designated *aroC* in *E. coli*. This suggests that the *aroC* gene of *S. aureus* lies immediately 5′ to *aroB*. The position and direction of transcription of *aroB* and *aroC* with respect to *aroA* were deduced (Fig. 5).

Isolation of aroA::Tc^r

A temperature-sensitive shuttle plasmid pCOC204 was constructed, which carried an *aroA*::Tc^r insertion mutation in the *Hin*dIII site in the 3′ part of *aroA*. This was electroporated into *S. aureus* 8325-4 selecting for Tc^r at 42 °C. At this temperature the plasmid will not replicate, and should therefore behave as a suicide vector. Ten Tc^r colonies were recovered and screened for loss of Cm resistance associated with the shuttle plasmid. One colony was Cm^s and was subsequently shown to require tryptophan and tyrosine for growth in DSM. The replacement of the wild-type allele by the mutated gene

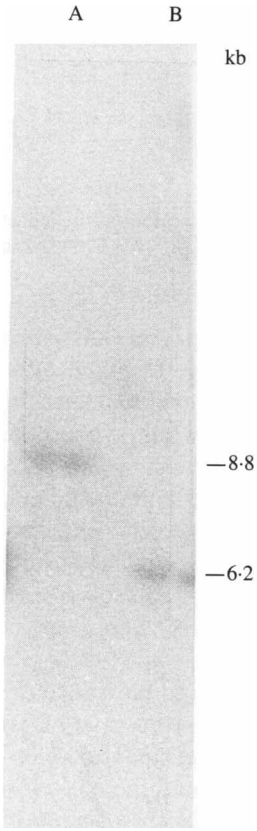


Fig. 6. Southern hybridization analysis of DU5859. Chromosomal DNA from *S. aureus* DU5859 (lane A) and 8325-4 (lane B) was cleaved with *Eco*RI, separated on a 1% (w/v) agarose gel and transferred to a nitrocellulose filter. This was probed with ^{32}P-labelled pCOC201 DNA carrying the wild-type *aroA* gene.

was confirmed by Southern hybridization (Fig. 6). Genomic DNA of the mutant and of the wild type was cut with *Eco*RI and probed with ^{32}P-labelled pCOC201 DNA. The 6·2 kb *Eco*RI fragment in the wild-type strain was replaced with a fragment of about 8·8 kb in the mutant, confirming that allelic replacement had occurred as predicted. Strain 8325-4 *aroA*::Tc^r was unable to grow on DSM unless supplemented with Trp and Tyr. A modified form of DSM was also prepared which lacked nucleotides and methionine. These are the end-products of folate metabolism, and their exclusion from the medium permitted the detection of PAB auxotrophy. Strain 8325-4 grew slowly on this medium. However, 8325-4 *aroA*::Tc^r was capable of growing on this medium without PAB supplementation, provided Trp and Tyr were present. This novel phenotype was designated AAA$^-$ PAB$^+$. *E. coli* strain AB1321 containing pCOC204 had the same phenotype, whereas without the plasmid it required PAB as well (AAA$^-$ PAB$^-$). This indicates that the AAA$^-$ PAB$^+$ phenotype was associated with the *aroA*::Tc^r mutation on the plasmid. The integration of plasmids pCOC107, pCOC109 and pCOC209 into the

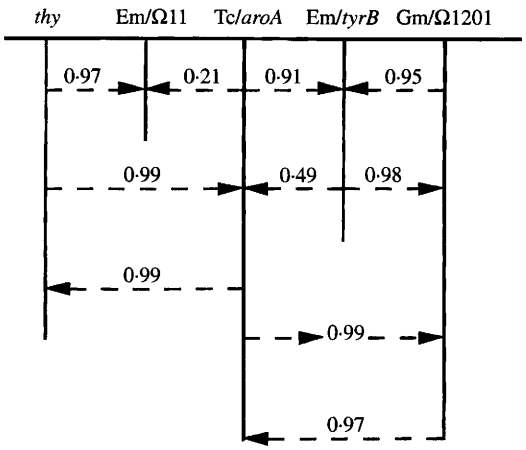


Fig. 7. Genetic mapping of the *aroA* locus. Co-transformation frequencies obtained in crosses between ISP2582 × ISP794 and ISP2504 × ISP193 are shown.

aro locus resulted in AAA$^-$ PAB$^-$ phenotypes, but these strains were very unstable (reversion frequency 10^{-3} after 48 h) and they were not suitable for *in vivo* assessment of bacterial growth or virulence. Despite numerous attempts, allele replacement mutants derived from plasmids pCOC109 or pCOC209, which would presumably have an AAA$^-$ PAB$^-$ phenotype, were never obtained.

Genetic mapping of the aroA locus

The *aroA* gene was initially located on *Sma*I fragment A in the *S. aureus* 8325-4 chromosome by PFGE and Southern hybridization using the cloned *aroA* gene as a probe (data not shown). Genetic crosses were performed by transformation to place the *aroA* gene on the *S. aureus* 8325-4 linkage map. The *aroA*::Tc^r mutation was transformed into ISP2169 which contains a Tn*551* (Em^r) insertion in *tyrB* and a closely linked silent insertion of Tn*4001* (Gm^r). DNA from this strain was transformed into the wild-type 8325 strain ISP794, selecting for each resistance marker in turn and scoring for the inheritance of unselected markers. Similarly, DNA from ISP2504 (*aroA*::Tc^r) was transformed into ISP193, selecting for Tc^r and for Thy$^+$ recombinants. The transformation data (not shown) is summarized in Fig. 7 and clearly shows that *aroA* maps between *thy* and *tyrB*.

Virulence of 8325-4 aroA::Tc^r

Strain 8325-4 *aroA*::Tc^r (AAA$^-$ PAB$^+$) had the same virulence as the wild type when injected subcutaneously or intraperitoneally in mice. In addition, the mutant and the wild type grew to the same extent in diffusion chambers implanted in the peritoneal cavity of mice. Bacteria isolated from infected animals were checked

M. tuberculosis	D	C	-	-	-	-	-	-	G	L	A	G	T	V	L	R	F	V
E. coli	E	G	A	L	E	L	F	L	G	N	A	G	T	A	M	R	P	L
S. aureus	T	P	H	Q	V	L	Y	T	G	N	S	G	T	T	T	R	L	L
B. subtilis	E	P	E	S	L	L	D	V	G	N	S	G	T	T	I	R	L	M
Petunia	K	E	E	I	Q	L	F	L	G	N	A	G	T	A	M	R	P	L
Old consensus						L	X	L	G	N	A	G	T	A	X	R	X	L
New consensus									G	X	X	G	T	X	X	R		

Fig. 8. Comparison of the consensus sequence for the PEP/glyphosate binding site of EPSP synthase derived by Padgette *et al.* (1991) with the corresponding regions in the *M. tuberculosis*, *E. coli*, *S. aureus* and *B. subtilis* and *Petunia hybrida* EPSP synthase sequences.

and shown to still be Aro$^-$ and Tc^r. These data suggest that the AAA$^-$ PAB$^+$ mutants do not lack virulence in mice.

Discussion

The aroC aroB aroA operon

Several lines of evidence suggest that the *aroA* gene of *S. aureus* is part of an operon which also includes *aroB* and *aroC*. (1) Temperature-sensitive plasmids which were forced to integrate into the chromosome 5′ to *aroA* caused an Aro$^-$ phenotype. (2) No consensus promoter sequences were observed immediately 5′ to the *aroA* coding sequence. (3) ORFs located immediately 5′ to *aroA* encode proteins which have substantial homology with the *aroB* and *aroF* gene products of *B. subtilis*. The presence of *aroB* was confirmed by complementation of *E. coli aroB* by pCOC201.

The organization of the *aro* gene cluster in *S. aureus* is different from that of *B. subtilis*, where the *aroA* gene is part of a 'supraoperon' of thirteen genes involved in the biosynthesis and transport of aromatic amino acids. This includes the *trp* operon, which is located between the *aroF aroB aroH* genes and the *hisH tyrA aroE* genes. The genetic map of *S. aureus* shows that the *trp* operon is linked to *tyrA* (Pattee *et al.*, 1990). However, data presented here show that *aroA* (and thus *aroB* and *aroC*) are linked to *tyrB* which maps some distance from the *trp* operon. The map position of *aroJ*, the *tyrB* equivalent of *B. subtilis*, is unknown.

Comparison of EPSP synthases

There is considerable interest in elucidating the active sites of EPSP synthases because of the importance of the enzyme as a target for the herbicide glyphosate. EPSP is believed to interact with the substrate PEP only when complexed with shikimate-3-phosphate (S3P). The S3P binding site is in the N-terminal portion of the enzyme, around Leu^{22} in the *E. coli* sequence. A glyphosate-tolerant mutant of *S. typhimurium* contains the single amino acid substitution Pro^{101}Ser (Stalker *et al.*, 1985). Glyphosate tolerance in *E. coli* B is associated with the substitution of Gly^{96} for Ala (Kishore *et al.*, unpublished results, cited by Padgette *et al.*, 1991). Alignment of EPSP synthase amino acid sequences allowed Padgette *et al.* (1991) to identify a highly conserved region between residues 90 and 102 from which a consensus sequence (Fig. 8) was derived. The importance of this region was confirmed by site-directed mutagenesis and kinetic analysis of the mutant enzymes (Padgette *et al.*, 1991). The corresponding regions of the EPSP synthases of Gram-positive bacteria were not considered in the consensus of Padgette *et al.* (1991). These sequences have allowed the consensus to be refined (Fig. 8). Leu^{92} is not conserved in the Gram-positive bacterial proteins. The conserved Ala of the tetrapeptide motif GNAG has been substituted by Ser in both the *B. subtilis* and the *S. aureus* enzymes and by Leu in *M. tuberculosis*, while the Ala in the TAXRXL motif is converted to Thr in EPSP synthases from the Gram-positive organisms. However, both the last two changes conserve amino acid function. In addition, neither of these proteins has the Pro^{101} residue associated with glyphosate tolerance in the *S. typhimurium* enzyme, and the *B. subtilis* enzyme has Met instead of Leu at position 102. The new consensus is GXXGTXXR. Possibly only these highly conserved residues are involved in binding to PEP and glyphosate. *B. subtilis* has been shown to be glyphosate-sensitive (Schultz *et al.*, 1985). It should be noted that the highly conserved Arg^{100} is thought to interact with PEP via its guanidium side chain (Padgette *et al.*, 1991).

Lack of virulence of S. aureus aroA::Tc^r

Mutants with a complete block in the early aromatic biosynthetic pathway cannot make chorismate, the aromatic precursor in bacteria, yeast, fungi and plants. The loss of virulence of pathogenic bacteria carrying *aro* mutations is thought to be primarily due to the associated requirement for PAB which is a precursor of folic acid. The finding that non-leaky PAB$^-$ mutants of *S. typhimurium* are attenuated to the same degree as Aro$^-$ mutants (Stocker, 1990) is consistent with this. The lack of attenuation of *S. aureus* carrying *aroA*::Tc^r is probably because it remains PAB-independent, despite requiring aromatic amino acids. Leaky transposon

insertion mutants in the *S. typhimurium aroE* gene also retain virulence (Stocker, 1990). In *S. aureus aroA*::Tc^r, the Tc^r *Hin*dIII fragment is inserted 18 nucleotides from the termination codon of *aroA*. The mutated gene may still be able to express a partly functional EPSP synthase which can produce sufficient chorismate to allow biosynthesis of the small amount of PAB required for folate biosynthesis.

Despite several attempts, an AAA$^-$PAB$^-$ replacement mutant of *S. aureus* was never isolated, suggesting that a stable insertion mutation in the *aro* operon is lethal. This could be due to the *aro* mutation itself, or to polar effects on genes distal to *aroA*. We have identified a short ORF of unknown function just 3′ to *aroA*. It has a reasonable ribosome binding site, and might be translated. However, the *aroA*::Tc^r insertion, which would also be expected to exert polarity, was isolated with comparative ease. This leaves the unprecedented conclusion that chorismic acid biosynthesis is essential in *S. aureus*. Perhaps an as yet uncharacterized biosynthetic pathway, which is essential for growth, utilizes chorismate. In addition, if mutations that prevent chorismate biosynthesis are lethal, an explanation for the recovery of integrating plasmid mutants with the AAA$^-$PAB$^-$ phenotype is required. However, the integrants were very unstable and reverted to wild-type at a frequency of 10^{-3} or greater. Perhaps mutant bacteria could survive by cross-feeding by the revertants in the population.

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