Chorismate synthase from *Staphylococcus aureus*

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The *aroC* gene encoding chorismate synthase and the *ndk* gene encoding nucleoside diphosphate kinase (Ndk) were cloned from *Staphylococcus aureus*. DNA sequencing suggests that *aroC* is located in an operon with *aroB* and *aroA* and encodes a protein of 388 amino acids with 61% identity to the *aroF* gene product of *Bacillus subtilis*. The *ndk* gene of *S. aureus* encodes a protein of 149 amino acids which exhibits a high degree of identity to other bacterial Ndk proteins. The 3' end of the *S. aureus gerCC* gene was also identified by sequencing and was located immediately upstream of *ndk*. The *gerCA* and *gerCB* genes were found to be located upstream of *gerCC* by Southern hybridization analysis. This observed linkage of the *gerC* genes with the *ndk*, *aroC* and *aroB* genes has been similarly observed in *B. subtilis*. The *S. aureus* chorismate synthase was overexpressed to a high level in *Escherichia coli* using a T7 promoter plasmid construct, the enzyme was purified to near homogeneity in two steps and found to be a homotetramer with a subunit molecular mass, estimated by electrospray mass spectrometry, of 43 024 Da. The properties of *S. aureus* chorismate synthase are compared with those of the *B. subtilis* and *E. coli* enzymes.

Keywords: *Staphylococcus aureus*, chorismate synthase, nucleoside diphosphate kinase

INTRODUCTION

In micro-organisms and plants, chorismic acid is a central precursor for the biosynthesis of an array of biochemically important and structurally diverse aromatic compounds. These include folic acid, vitamin K, ubiquinone and the three aromatic amino acids (Bentley, 1990; Haslam, 1993). Chorismate synthase (EC 4.6.1.4), the seventh enzyme in the pre-chorismate (shikimate) pathway, catalyses the conversion of 5-enolpyruvylshikimate 3-phosphate (EPSP) to chorismate. The reaction involves the abstraction of the C-(6*pro*-R)-hydrogen with the concomitant elimination of phosphate to generate the second double bond of the aromatic ring. Chorismate synthase has been studied to a varying extent in a number of micro-organisms and plants, including *Escherichia coli* (White *et al.*, 1988; Ramjee *et al.*, 1991; Bornemann *et al.*, 1995),

Bacillus subtilis (Hasan & Nester, 1978), *Neurospora crassa* (Gaertner & Cole, 1973; White *et al.*, 1988; Henstrand *et al.*, 1995), *Euglena gracilis* (Schaller *et al.*, 1991), *Pisum sativum* (Mousdale & Coggins, 1986) and *Corydalis sempervirens* (Schaller *et al.*, 1990). Chorismate synthase from these species has an absolute requirement for a reduced flavin cofactor, although the conversion of EPSP to chorismate is not accompanied by a net overall change in redox state. The enzymes differ, however, in their ability to generate the reduced flavin cofactor. The *N. crassa* enzyme possesses an intrinsic NADPH-dependent flavin reductase activity (Gaertner & Cole, 1973; Henstrand *et al.*, 1995), while flavin reductase forms part of a complex with chorismate synthase in both *B. subtilis* and *Eu. gracilis*.

The enzymes of the aromatic biosynthetic pathway are attractive targets for inhibitors since this pathway is absent in mammals. (6*S*)-6-Fluoroshikimic acid has been shown to be converted *in vivo* by the pre-chorismate pathway enzymes to ultimately produce inhibition of *p*-aminobenzoic acid (PABA) synthesis, and thus folic acid production (Davies *et al.*, 1994; Ewart *et al.*, 1995). Mutants defective in the pre-chorismate pathway have

Abbreviation: EPSP, 5-enolpyruvylshikimate 3-phosphate.

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vaccine potential. Aromatic-dependent (*aro*) mutants of the pathogenic bacteria *Salmonella* spp. (Hoiseth & Stocker, 1981; Jones *et al.*, 1991), *Bordetella pertussis* (Roberts *et al.*, 1990), *Yersinia enterocolitica* (Bowe *et al.*, 1989), *Bacillus anthracis* (Ivins *et al.*, 1990) and *Aeromonas salmonicida* (Vaughan *et al.*, 1993) have been shown to be avirulent and stimulate protective immunity.

This paper reports the molecular cloning, sequencing and expression of the *aroC* and *ndk* genes and the purification and properties of chorismate synthase from *Staphylococcus aureus*.

METHODS

Bacterial strains, plasmids and media. *E. coli* K-12 GLW40 (*aroC* *thi* *recA13*) (AB2849 made *recA*) and *E. coli* AB1321 (*proA2* *aroA2* *his-4* *thi-1* *lacY1*) were used as hosts for *aroC* and *aroA* complementation experiments, respectively (Pittard & Wallace, 1966). *E. coli* K-12 DH5 α (*endA1* *hsdR17* *supE44* *recA1* *gyrA96* *relA1* ϕ 80/*lacZ*AM15 *thi*) was used as a host for pBluescript and pUC vectors. *E. coli* BL21(DE3) (*hsdS* *gal* [λ imm²¹ *Sam7* *nin5* *lacUV5*-T7 *gene1*]) and GLW40(DE3) were used for expression of genes cloned under T7 promoter control. GLW40(DE3) was prepared using a commercially available lysogenization kit (Novagen Inc.). *S. aureus* 601055 was a clinical isolate obtained from Zeneca Pharmaceuticals culture collection. Plasmids pBluescript SKII (Short *et al.*, 1988) and pUC18 (Yanisch-Perron *et al.*, 1985) were used as general cloning vectors. Plasmid pCOC102 contains the *aroA* *aroB* genes of *S. aureus* 8325-4 in pUC18 (O'Connell *et al.*, 1993). Plasmid pMAY1 contains the *gerC* *AgerCBgerCC* genes of *B. subtilis* 1604 in pUC119 (Yazdi, 1991). The expression vector pTB361 contains the T7 promoter (Brockbank & Barth, 1993). *E. coli* and *S. aureus* strains were grown in LB broth or on LB-agar at 37 °C. Antibiotics were incorporated at the following concentrations: ampicillin, 100 μ g ml⁻¹; chloramphenicol, 17 μ g ml⁻¹; tetracycline, 12.5 μ g ml⁻¹. Screening for complementation of the aromatic deficiencies of *E. coli* strains GLW40 and AB1321 was performed on M9 minimal medium agar supplemented with amino acids and vitamins as appropriate.

Molecular cloning and DNA manipulation. Chromosomal DNA was purified from *S. aureus* after cell lysis with lysostaphin (100 μ g ml⁻¹) followed by phenol and chloroform extractions (Sambrook *et al.*, 1989). Plasmid DNA was isolated by the method of Holmes & Quigley (1981) except if required for DNA sequencing when Qiagen columns were used. DNA was restricted and analysed using standard procedures (Sambrook *et al.*, 1989). Restriction enzymes were from New England Biolabs or Promega. DNA blotted onto Hybond-N (Amersham) was hybridized against radiolabelled DNA using standard methods. DNA probes were prepared using a Megaprime kit (Amersham) incorporating [α -³²P]dATP. Homologous probes were washed with 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl, 0.015 M Na citrate) at 65 °C following hybridization. Reduced stringency conditions for heterologous probing with the *gerC* genes from *B. subtilis* was achieved by probing at 50 °C in 6 $\times$ SSC followed by washes at 50 °C in 2 $\times$ SSC. The *gerC* fragments used as heterologous probes were obtained from digestion of pMAY1 and included a 2252 bp *Hind*III *gerC* *AgerCBgerCC* fragment, a 1215 bp *Hind*III-*Ssp*I *gerC* *AgerCB* fragment, a 452 bp *Hind*III-*Nsi*I *gerCA* fragment and an 843 bp *Dra*I-*Hind*III *gerCC* fragment.

PCR amplification of *aroC*. The coding region of the *aroC* gene was amplified from *S. aureus* genomic DNA using VENT

polymerase (New England Biolabs) and the two synthetic oligonucleotide primers CHOR 34 (5' GGCATATGAGATACCTAACATCAGGA 3') and CHOR 35 (5' GGAGATCTTA-AACTCTAATTATAAGTT 3') which contained *Nde*I and *Bgl*II restriction sites, respectively. Amplification was performed with 2.5 U polymerase, 100 pmol primers, 30 μ M each dNTP and 100 ng DNA. The amplified gene was cloned into the *Nde*I-*Bgl*II sites of pTB361 to form pMJH7EX2 and sequenced to verify the absence of errors.

DNA sequencing. DNA was sequenced on both strands using synthetic oligonucleotide primers on denatured double-stranded plasmid DNA by the method of Sanger *et al.* (1977). Synthetic oligonucleotides around 250 bp apart along the template were designed to prime sequencing reactions.

Protein overexpression and N-terminal sequencing. Protein expression from bacterial cultures of *E. coli* strains BL21(DE3) and GLW40(DE3) containing the plasmid pMJH7EX2 were examined by SDS-PAGE (Studier & Moffatt, 1986); gels were stained with Coomassie brilliant blue R-250. N-terminal protein sequencing was performed by Edman degradation (Applied Biosystems protein sequencer) after blotting the protein onto PVDF membrane (Applied Biosystems) (Matsudaira, 1987).

Western blotting of *S. aureus* chorismate synthase. Crude extracts of *E. coli* containing *S. aureus* chorismate synthase and *E. coli* chorismate synthase expressed from pMJH7EX2 and pGM605 (White *et al.*, 1988), respectively, were separated by SDS-PAGE. The gel was blotted onto nitrocellulose membrane and blocked with Tris-buffered saline and 0.5% (v/v) Tween 20. Immunodetection was performed with polyclonal rabbit anti-chorismate synthase antibody raised against denatured *E. coli* enzyme and 5% polyclonal donkey serum (SAPU). Donkey anti-rabbit immunoglobulin G horse-radish peroxidase conjugate (SAPU) was used as secondary antibody and chloronaphthol solution used for development.

Purification of *S. aureus* chorismate synthase. *S. aureus* chorismate synthase was purified from *E. coli* GLW40(DE3)-pMJH7EX2 using two chromatographic steps (DEAE-Sephacel and cellulose phosphate) essentially as described by White *et al.* (1988).

Step 1: DEAE-Sephacel. Cells (13 g wet wt) were suspended in extraction buffer (50 mM Tris/HCl, pH 7.5, 50 mM KCl, 1 mM benzamidine, 1.2 mM PMSF, 1.3 mM EDTA, 0.4 mM DTT, 20% v/v, glycerol) and broken by two passages through a French pressure cell [950 p.s.i. (6.55 MPa)]. This material was centrifuged at 28000 *g* for 30 min and the supernatant (crude extract) loaded onto a DEAE-Sephacel (Pharmacia) column (100 ml bed volume) equilibrated in buffer A (extraction buffer minus benzamidine). The column was first washed with buffer A, then eluted with a linear gradient (600 ml) of 50–500 mM KCl in buffer A. Fractions containing chorismate synthase were pooled and dialysed overnight against buffer B (10 mM potassium phosphate, pH 6.6, 0.45 mM PMSF, 1.3 mM EDTA, 0.4 mM DTT, 20% glycerol).

Step 2: Cellulose phosphate. The dialysed protein from the previous step was loaded onto a cellulose phosphate (Whatman P11) column (60 ml bed volume). The column was first washed with buffer B, then eluted with a linear gradient (400 ml) of 10–400 mM potassium phosphate in buffer B. Fractions containing chorismate synthase were precipitated with the addition of solid (NH₄)₂SO₄ and protein recovered by spinning at 28000 *g* for 30 min. The enzyme was resuspended in 50 mM Tris/HCl, pH 7.5, containing 50% glycerol and 1 mM DTT and stored at –20 °C. This preparation was stable for 6 months with no apparent loss of chorismate synthase activity.

Enzyme assay. Chorismate synthase from *S. aureus* was assayed anaerobically at 25 °C using the method of Ramjee *et al.* (1994). Assay buffer consisted of 50 mM MOPS, pH 7.0, 100 mM NaCl, 25 µM FMN and 50 µM EPSP.

Protein estimation. Protein in crude samples was determined by the method of Bradford (1976) and in purified samples by the method of Gill & von Hippel (1989) using a calculated extinction coefficient for the *S. aureus* chorismate synthase of 23 100 M⁻¹ cm⁻¹.

Gel filtration and electrospray mass spectrometry. The native molecular mass of *S. aureus* chorismate synthase was estimated using a Superose 12 gel permeation chromatography column (Pharmacia) with comparison to enzymes of known molecular mass as described by Walker *et al.* (1996). The subunit molecular mass was determined using electrospray mass spectrometry as described by Krell *et al.* (1995).

RESULTS AND DISCUSSION

Cloning of the *aroC* gene

Chromosomal DNA from *S. aureus* 601055 was digested with a number of restriction enzymes and blotted onto Hybond-N membrane (Amersham). This was probed with a 350 bp *EcoRI*–*HindIII* *aroC* fragment from pCOC102 which contains the complete *aroA*–*aroB* genes from *S. aureus* 8325-4 (O'Connell *et al.*, 1993). A 5.5 kb *ClaI* fragment was observed after hybridization. The Aro⁻ phenotype of *E. coli* strain GLW40 was then complemented using a *ClaI* plasmid library and 12 clones

were isolated. Each was found to have obvious restriction map similarities with pCOC102 and one plasmid, pMJH701, was studied further. This plasmid was also shown to complement the Aro⁻ phenotype of AB1321.

Sequencing of the *aroC* clone

A 2.5 kb *NdeI*–*ClaI* fragment from pMJH701 (Fig. 1) was cloned into *NdeI*–*AccI*-digested pUC18, forming pMJH702, and the DNA sequence of the 2.5 kb insert was determined. The DNA sequence, which has a GC content of 33 %, is shown in Fig. 2. Two complete ORFs were located by DNA sequencing. The predicted polypeptide from the larger of the two ORFs consisted of 388 amino acids and had 61 % identity with the *aroF* gene product of *B. subtilis* and 37 % identity with the *aroC* gene product of *E. coli*, both of which have chorismate synthase activity. A putative promoter and ribosome binding site were located upstream from the ATG start codon of the *aroC* gene. The second complete ORF was situated some 500 bp upstream from *aroC* and the predicted polypeptide from this consisted of 149 amino acids and had 62 % and 45 % identity with the *ndk* gene products from *B. subtilis* and *E. coli*, respectively. Contained within the 2.5 kb of DNA sequenced in pMJH702 were two partial ORFs. Situated 25 bp downstream from the TAA stop codon of *aroC* was the 5' end of an ORF which had homology with the *aroB* gene, encoding 3-dehydroquinate synthase, from *B. subtilis*. The 3' end of the second partial ORF immediately

Fig. 1. Combined schematic map of the *gerC*, *ndk*, *aro* region of the *S. aureus* chromosome. The positions of the *ndk* and *aro* genes were deduced from DNA sequencing data presented here and from previous analysis (O'Connell *et al.*, 1993). The positions of the *gerC* genes are shown by dashed arrows, indicating approximate locations based on hybridization analysis data presented here and from *B. subtilis* sequences (Henner, 1992). The map indicates the 5.5 kb *ClaI* fragment inserted in pMJH701 and the 2.5 kb *NdeI*–*ClaI* fragment subcloned to form pMJH702. The clones were mapped with a number of restriction endonucleases. A, *AccI*; C, *ClaI*; E, *EcoRI*; RV, *EcoRV*; H, *HindIII*; N, *NdeI*; P, *PstI*.

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ATCGATGAAGCTAAGGCAGTAAGTTCGAAGTATCTAAGTAAAGCTTTGGATTGTTCTGAGTTGCCAGATGGACATCCAAAATCACTACTTTTAAGTTTGACGAAAAAATGGGGTCA 120
I D E A K A V S S K Y L S K A L D L I S E L P D G H P K S L L S L T K K M G S
AGAAACACATAGTATTTATGAAAGTATTGAAAGCGCTTTACCAACCTGTTAATATATAAGTAAATATACATAAACCTATTAAACACAGGGGATACATAAAGTGGACGTACATTTTT 240
R N T *
-35 -10 rbs ndk
AATGATTAAACAGACGAGTACAAAGAAATCTAATTGGTGAAGTAATTTCAAGAATTGAAAGAAAAGGACTAAAACCTTGTGGTGGTAAATTAATGCAAGTACCAATGGAACCTGCTGA 360
M I K P D A V Q R N L I G E V I S R I E R K G L K L V G G K L M Q V P M E L A E
AACACATTATGGTGAACACCAAGGTAACCATTTTATAATGATTTAATTCATTTTATACATCAGCACCAGTGTTCGCAATGGTAGTTGAAGGTGAAGTGCAGTTAATGTATCTAGACA 480
T H Y G E H Q G K P F Y N D L I S F I T S A P V F A M V V E G E D A V N V S R H
TATTATTGGCAGCACCAATCCTTCAGAAGCTTCACCAGGATCAATTAGAGGTGATTTAGGTTTAACTGTTGGTAGAAATATCATTACGGTTGAGATTCAATAGAGTCTGCTGAACGTGA 600
I I G S T N P S E A S P G S I R G D L G L T V G R N I I H G S D S L E S A E R E
AATTAATTTATGGTTAATGAAATGAAATTAAGTCTAGCTTCCACAGTGATGCTTGGTTATACGAATAAAATATAAGCTATAAACTTTCTGATTATAAAGGAAAAAGGGTTTT 720
I N L W F N E N E I T S Y A S P R D A W L Y E *
GTTATGTGGTTAGTGATTGATGACCATTCATAACAAAGCCCTATTTTATACGGACTGATTAACTTTATAAAAAATAATCAGTCCATTTTCTTTTATTAAGAAATATAGCTTGTATAT 840
TATTTATTCGTGGTTAGCTACGATTATATGCGGTATAAGTTGTTCGAATAAAATTTAAATTTTCAAATGGTTGTTTAAACAATCTAACAATATTAGAAATTCATAACTTTTCTCGTTG 960
TGAATATATATAAGTCTTAGTTCTGTTAATATTAAGATAATCTGACATTTAAAAAGAGATATAAAATCAATTTCTTAATTGAGCTTGAAAAACAACATTTATGAATGCACAATGAA 1080
-10 -35 rbs aroC
TATGATAAGATTAACAACATATTATAATGTTATCGTGAAGTATGAAAGGAGCGAGTGTGTATGAGATACCTAACATCAGGAGAAATCACATGGACCTCAATTACAGTTATTGTTGAAGG 1200
M R Y L T S G E S H G P Q L T V I V E G
TGTACCTGCAAAATTTAGAAGTTAAGTTGAGGATTAATAAAGAAATGTTTAAAGCGTCAAGGCGGTACGGACGTGGACGTGCTATGCAAAATGAAAGAGATACAGTGGAGATTGTTTC 1320
V P A N L E V K V E D I N K E M F K R Q G Y G R G R R M Q I E K K D V E I V S
GGGTGTAAGAAATGGTTATACATTAGGTAGCCCTATTACAATTTGTTGTTACTAATGATGATTTTACACATTTGGCGAAAAATTTATGGGCCGTGGCCCAATAAGCGACGAAGAAGCAGAAAA 1440
G V R N G Y T L G S P I T I V V T N D D F T H W R K I M G R A P I S D E E R E N
TATGAAACGTACAATTACGAAGCCCAAGACCGGGACATGCAGATTAGTTGGCGGTATGAAATATAATCATCGTGACTTACGAAATGTATTAGAACGTTCTCTGCCAGAGAAACAGCAGC 1560
M K R T I T K P R P G H A D L V G G I K Y N H R D L R N V L E R S S A R E T A A
ACGTGTAGCGGTGGTGCACATGCAAAAGTTTATTAGAACAATTAGATATCGAAATATACAGTCGTGTTGTTGAGATAGGTGGCATTAAAGATAAAGATTTTATGATTTCAGAAACATT 1680
R V A V G A L C K V L L E Q L D I E I Y * S R V V E I G G I K D K D F Y D S E T F
TAAAGCAACCTTGATCGAAATGACGTCCTGTAATTGATGATGGCATCGCAACGAATGCGCGATAAAATGATGAAGCGAAAAACGATGGTGATTCAATAGGGGGCGTAGTTCAAGT 1800
K A N L D R N D V R V I D D G I A Q A M R D K I D E A K T D G D S I G G V V Q V
TGTAGTTGAAATATGCCTGTTGGTGTAGGTAGTTATGTACATTATGATCGTAAATAGATGGAAGAATAGCACAGGGTGTGCTGTTAGTATTATGCATTTAAAGGTGTAAGTTTGGAGA 1920
V V E N M P V G V G S Y V H Y D R K L D G R I A Q G V V S I N A F K G V S F G E
AGGATTAAAGCAGCTGAAAGCGCTGGTGAAGCAAAATCAAGACGAAATCTCTACAATACTGAATTTGGGCTATTATCGTGGTCAAATCACTAGGTGGTTTAGAAGGCGGTATGTCAAA 2040
G F K A A E K P G S E I Q D E I L Y N T E L G Y Y R G S N H L G G L E G G M S N
TGAATGCCAATTATCGTTAATGGTGAATGAACCAATTCACGTTATATAAACCAATTAATTCAGTAGACATTAACTACTAAGAAGACTTTAAGCAACAATTAAGCGTTCTGATAG 2160
G M P I I V N G V M K P I P T L Y K P L N S V D I N T K E D F K A T I E R S D S
TTGTGCTGTTCTGCAGCAAGTATCGTGTGTGAACATGTCGTAGCATTTGAAATTCGAAAAGCATTTATGGAAGAATTCCAATCAAATCATATTGAGCAACTTAAACAACAATTTATTGA 2280
C A V P A A S I V C E H V A F A I A K A L L E E F Q S N H I E Q L K Q Q I I E
rbs aroB
GCGCAGACAATTAATATTGAGTTTTTAAACAAGAACAATTGAGGTGTAATCATGAAATTACAAACAACATACCTTCAAACAATTATCCAATATATGTTGAACACGGTGAATCAAGT 2400
R R Q L N I E F * M K L Q T T Y P S N N Y P I Y V E H G A I K Y
ATATAGGCACATATTTAAATCAATTTGACCAAGTTTCTTGTAAATGATGAATGTAAATCAATATTTTGGCAATAAATTTGATGATATTTTATCATATG 2502
I G T Y L N Q F D Q S F L L I D E Y V N Q Y F A N K F D D I L S Y

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Fig. 2. Nucleotide sequence of the *aroC*–*ndk* region of *S. aureus*. The 2502 bp region shows the predicted amino acid sequences for part of *gerCC*, the entire *ndk* and *aroC* genes and part of the *aroB* gene. Putative promoters for the expression of *ndk* and *aroC* are indicated in addition to ribosome binding sites (rbs).

preceded the *ndk* gene and had homology with the *gerCC* gene product from *B. subtilis*.

Evidence for the presence of *gerCA* and *gerCB* upstream from *gerCC*

To examine the possibility that the *gerCA* and *gerCB* genes immediately preceded *gerCC* and to confirm the partial 3' ORF as *gerCC*, selected radiolabelled fragments from pMAY1 were hybridized against *S. aureus* DNA using reduced stringency conditions. The plasmid pMAY1 contains the *gerCAgerCBgerCC* operon from *B. subtilis* (Yazdi, 1991; Henner, 1992). To confirm that the partial 3' ORF upstream from *ndk* was *gerCC*, blots were probed with an 843 bp *DraI*–*HindIII* *gerCC* fragment

from pMAY1 (Fig. 3). These blots were then compared with blots and to a restriction map previously generated from probing experiments using the *aroC* gene from *S. aureus*. These blots were found to produce the expected restriction pattern for the *gerCC* gene being located immediately upstream from *ndk*. Separate *gerCA*, *gerCAB* and *gerCABC* probes were then prepared from pMAY1 and their hybridization pattern against *S. aureus* DNA was used to establish that the *gerCA* and *gerCB* genes were located directly upstream from *gerCC* (data not shown). The approximate locations of the *gerC* genes are indicated in Fig. 1.

In *B. subtilis* the *aroFBH* genes form the start of an *aro*–*trp*–*aro* supraoperon of 13 genes involved in the biosynthesis and transport of aromatic amino acids. The *aroFBH* genes

Fig. 3. Southern blot of *S. aureus* 601055 DNA probed with a *gerCC* fragment from *B. subtilis*. Digested genomic DNA was probed with an 843 bp *DraI*-*HindIII* *gerCC* fragment from pMAY1. Lanes 1–8: DNA digested with *AccI*, *BamHI*, *BglII*, *Clal*, *EcoRI*, *HincII*, *HindIII* and *KpnI*, respectively.

are preceded by *cheR*, *ndk* and the *gerCABC* operon (Henner *et al.*, 1990). Physical mapping experiments with *S. aureus* have shown that *aroA* (and thus *aroB* and *aroC*) are linked to *tyrB* (O'Connell *et al.*, 1993) while the *trp* operon is linked to *tyrA* (Pattee *et al.*, 1990) which maps some distance from *tyrB*. Although no similar *aro-trp-aro* supraoperon exists in *S. aureus*, it has been demonstrated here that the *ndk* and *gerCABgerCC* genes are situated upstream from the *aroC* and *aroB* genes, indicating gene organization similarities with *B. subtilis* (Fig. 1). The *cheR* gene situated between *ndk* and *aroF* in *B. subtilis* was not observed in *S. aureus*. *CheR* encodes a receptor-methylating enzyme involved in motility and the absence of this gene in *S. aureus* is likely to be a consequence of its non-motility. Nucleoside diphosphate kinases (NDP kinases), encoded by *ndk*, are well studied enzymes that catalyse the transfer of the γ -phosphate of nucleoside triphosphates to nucleoside diphosphates. The observed linkage between the *gerC* genes and the *aro* genes in both organisms may have a functional explanation. The *gerCC* gene from *B. subtilis* shares a high degree of identity (65%) with the ORF-3 gene encoding one subunit of heptaprenyl pyrophosphate synthetase from *B. stearothermophilus*. This enzyme has been shown to have a role in the synthesis of the prenyl side-chain of the menaquinone nucleus (Koike-Takeshita *et al.*, 1995; Takahashi *et al.*, 1980). Menaquinone biosynthesis branches from chorismate and the grouping of the *aro* genes with the *gerC* genes may merely

Fig. 4. Protein expression from *E. coli* strains containing the *aroC* clones, pMJH7EX2 and pMJH701. Proteins were analysed by SDS-PAGE. Lanes: M, molecular mass markers; 1, GLW40(DE3) control; 2, GLW40(DE3)(pMJH7EX2) before induction with IPTG; 3, GLW40(DE3)(pMJH7EX2) 5 h after induction with IPTG; 4, GLW40(DE3)(pMJH7EX2pLysS) 5 h after induction with IPTG (the 28 kDa band is T7 lysozyme which is expressed from pLysS and reduces the basal level of activity of T7 RNA polymerase); 5, pMJH701 in *E. coli* DH5 α with arrow showing Ndk expression.

reflect the greater clustering of aromatic pathway genes in these organisms.

Overexpression of chorismate synthase

The overexpression of *S. aureus* chorismate synthase was accomplished by amplifying the coding region using PCR and cloning the gene into the T7 expression plasmid, pTB361. The gene was sequenced and found to match the template sequence. The expression plasmid produced, pMJH7EX2, was transformed into the *E. coli* strains BL21(DE3) and GLW40(DE3) and expression initiated with the addition of IPTG. When expression was monitored by SDS-PAGE, two new bands of 43 and 33 kDa were observed when compared to the control (Fig. 4). The 43 kDa band had an N-terminal sequence (MRYLTSGESHGPGQLTVIVEGVANLEVK) which matched the predicted translation for the start of the *aroC* gene. The N-terminal sequence of the 33 kDa band (MKRTITKPRPGHADLVGGMK) matched an internal region of the predicted chorismate synthase sequence. The 33 kDa band is postulated to arise from a translational start event within the mRNA as this sequence corresponds

Fig. 5. Dendrogram showing relative amino acid sequence similarity among chorismate synthases from different organisms. The diagram was derived from the GCG multiple-alignment program PILEUP. This is not a precise evolutionary tree but is likely to reflect evolutionary relationships (Milkman, 1994).

to a start position downstream from a weak putative ribosome binding site (GAG).

Sequence comparison and immunoblotting of *S. aureus* chorismate synthase

Chorismate synthase from *S. aureus* shows a high degree of sequence similarity with the enzyme from *B. subtilis*. A dendrogram produced from an alignment of known chorismate synthase sequences (Fig. 5) revealed that the *S. aureus* and *B. subtilis* enzymes form a sequence group which is distinct from that formed by other bacterial, plant and fungal sequences. This difference between the *S. aureus* enzyme and other chorismate synthases was further demonstrated with immunoblotting. Antibodies raised against chorismate synthase from *E. coli* or *C. sempervirens* have previously been shown to possess strong inter-species cross-reactivity with chorismate synthases from a number of plants and micro-organisms (Schaller *et al.*, 1991). Chorismate synthase from *S. aureus*, however,

showed no detectable immunological cross-reactivity with antibodies raised against *E. coli* chorismate synthase (data not shown).

Purification and properties of chorismate synthase

S. aureus chorismate synthase was purified more than 7-fold from *E. coli* GLW40(DE3)(pMJH7EX2) to give around 100 mg of enzyme (Table 1) with a purity of $\geq 95\%$ purity as judged by SDS-PAGE. The subunit molecular mass of the enzyme was determined to be 43024 Da (in good agreement with the calculated molecular mass of 43026) by electrospray mass spectrometry and the native molecular mass was determined to be 186800 Da $\pm$ 7000 by gel filtration. The enzyme, when expressed in and isolated from *E. coli*, is therefore a homotetramer similar to the *E. coli* enzyme (White *et al.*, 1988) but different to that from *B. subtilis* which forms a heterotrimer with 3-dehydroquinate synthase and a flavin reductase (Hasan & Nester, 1978). The apparent K_m and the specific activity of the *S. aureus* enzyme were measured using the assay conditions described in Methods. The apparent K_m value for EPSP was 12.7 μ M, which is broadly similar to that of other chorismate synthases studied. The apparent K_m of FMN for the *S. aureus* enzyme was determined to be 4.8 μ M. This is similar to that for the *B. subtilis* chorismate synthase (12.7 μ M) (Hasan & Nester, 1978). However, both enzymes appear to bind FMN much less tightly than other chorismate synthases studied. The apparent K_m values for the *Eu. gracilis*, *N. crassa*, and *C. sempervirens* chorismate synthases are 76 nM, 66 nM and 37 nM, respectively (Schaller *et al.*, 1991). *S. aureus* chorismate synthase was observed to exhibit maximum specific activity (4.0 μ mol min⁻¹ mg⁻¹) with reduced FMN as cofactor. FAD and riboflavin were capable of substituting for FMN but the relative levels of activity observed (13% and 2.4%, respectively, in the standard assay) were much lower than those reported for the enzymes from *Eu. gracilis*, *N. crassa*, *C. sempervirens* and *E. coli* (73–84% for FAD and 14–39% for riboflavin) (Schaller *et al.*, 1991).

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Table 1. Purification of *S. aureus* chorismate synthase

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Yield (%)	Purification (-fold)
Crude extract	1210	416	0.34	100	1
DEAE-Sephacel	592	317	0.54	76	1.6
Cellulose phosphate	98.5	248	2.5	60	7.4

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