Isolation, characterization and nucleotide sequences of the *aroC* genes encoding chorismate synthase from *Salmonella typhi* and *Escherichia coli*

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The *aroC* genes from *Salmonella typhi* and *Escherichia coli*, encoding 5-enolpyruvylshikimate-3-phosphate phosphohyase (chorismate synthase) were cloned in *E. coli* and their DNA sequences were determined. The *aroC* gene from *S. typhi* was isolated from a cosmid gene bank by complementation of an *E. coli aroC* mutant. The corresponding *E. coli* gene was isolated from a pBR322 gene bank by colony hybridization using DNA encoding the *aroC* gene from *S. typhi* as a hybridization probe. Analysis of the nucleotide sequence revealed that both genes have an open reading frame capable of encoding proteins comprising 361 amino acids. The calculated molecular mass of the protein from *S. typhi* is 39 108 Da while that of the protein from *E. coli* is 39 138 Da. Homology is particularly strong between the coding regions of the genes: 95% when protein sequences are compared, and 83% when DNA sequences are examined. Use of a deletion variant of the *E. coli aroC* gene demonstrates that the C-terminal 36 amino acids are not essential for the correct folding or functional activity of the chorismate synthase enzyme.

Introduction

The biosynthesis of aromatic compounds including the aromatic amino acids *p*-aminobenzoic acid and 2,3-dihydroxybenzoate is of great importance for the growth and survival of bacterial cells. The only biosynthetic route for aromatic compounds possessed by micro-organisms is the shikimate pathway which leads to the synthesis of chorismic acid, a central precursor for other aromatic compounds (Giles, 1978; Pittard, 1987). The shikimate pathway is present in bacteria, yeasts and in plant cells, but is absent from mammalian cells. As a consequence, enzymes in the shikimate pathway have been identified as potential targets for chemotherapeutic agents (Kishore & Shah, 1988). Because of the importance of the shikimate pathway, attention has turned to the individual enzymes responsible for aromatic compound biosynthesis. At least seven enzymes are

required for the synthesis of chorismic acid from non-aromatic precursors. Chorismate synthase (5-enolpyruvylshikimate-3-phosphate phosphohyase; EC 4.6.1.4) is the final enzyme in the pathway and catalyses the conversion of 5-enolpyruvylshikimate 3-phosphate (EPSP) to chorismic acid. The enzyme chorismate synthase has been purified and characterized from a variety of micro-organisms (White *et al.*, 1988) and plant sources (Mousdale & Coggins, 1986).

Pathogenic bacteria carrying mutations in genes encoding enzymes in the shikimate pathway (Bacon *et al.*, 1951) such as *aroA* (Dougan *et al.*, 1987; O'Callaghan *et al.*, 1988) grow poorly *in vivo* and are of potential use as live vaccines. A long-term objective of this research is to use the deduced primary amino acid sequences of the chorismate synthase enzymes in a pharmacological study of the interaction of potential inhibitors with the protein. Significantly, the five monofunctional *aro* enzymes producing EPSP in *E. coli* have previously been shown to have conserved regions when compared with the equivalent pentafunctional *AROM* (Giles, 1978) locus of the eukaryote *Aspergillus nidulans* (Hawkins, 1987). To obtain some measure of the conservation of the protein sequences of the chorismate synthase enzymes we

Abbreviation: EPSP, 5-enolpyruvylshikimate 3-phosphate.

The nucleotide sequence data for *E. coli* and *S. typhi* reported in this paper have been submitted to GenBank and have been assigned the accession numbers M27714 and M27715, respectively.

describe the cloning and sequencing of the *aroC* genes of *S. typhi* and *E. coli*. In addition, we present initial data on the size of functional domains within the chorismate synthase enzyme using deletion variants of the *E. coli aroC* gene.

Methods

Strains, bacteriophage and plasmids. *E. coli* TG1 [K12 $\Delta(lac-pro) supE thi hsdD5/F' traD36 proA^+B^+ lacI^a lacZ \Delta M15$ (Carter *et al.*, 1985)] and λ b221 cI857 *Oam29 Pam80 rex::Tn5* (de Bruijn & Lupski, 1984) were the kind gifts of Dr T. Gibson, Laboratory of Molecular Biology, Cambridge, UK, and Dr T. Foster, Trinity College Dublin, Ireland, respectively. *E. coli* GLW40 (*aroC thi recA13*) was the kind gift of Dr I. Hunter, Institute of Genetics, Glasgow University, UK. *E. coli* HB101 and *S. typhi* Ty2 *aroA::Tn10* (Dogan *et al.*, 1987) were as previously described. BRD049 is an *E. coli* K12 strain harbouring a stable mutation in *aroC* (White *et al.*, 1988). Cosmid pH79 (Hohn & Collins, 1980) was from Amersham; plasmid pBR322 was as described previously (Bolivar *et al.*, 1977) and M13mp18 and M13mp19 (Messing & Vieira, 1982) were supplied by Pharmacia. Minicells were prepared using *E. coli* strain DS410 (Dogan & Sherratt, 1977). *E. coli* strain C600 was as previously described (Appleyard, 1954).

Media and reagents. *S. typhi* and *E. coli* strains were grown in Luria broth (LB) or on LB solidified with 1.6% (w/v) agar (Miller, 1972) (Difco). Minimal medium (MM) was made as described by Miller (1972) and was solidified with 2% (w/v) Noble Agar (Difco). Deoxy- and dideoxynucleotides, ampicillin, tetracycline, dithiothreitol and aromatic amino acids were from Sigma. Restriction endonucleases were from BRL Gibco and Pharmacia. T4 DNA ligase was from Boehringer and Klenow fragment of DNA polymerase I was from Pharmacia.

DNA isolation, Tn5 mutagenesis and minicells. Chromosomal DNA was isolated using the method of Hull *et al.* (1981) as outlined by Maskell *et al.* (1988). Tn5 mutagenesis and mapping was done with $\lambda::Tn5$ using the method described previously by de Bruijn & Lupski (1984). Minicells and SDS-PAGE were as described previously (Dogan & Sherratt, 1977).

Cloning of *S. typhi* chromosomal DNA in cosmid pH79. Cosmid vector pH79 (Hohn & Collins, 1980) was digested with *Bam*HI and ligated with *Sau*3A-digested *S. typhi* DNA in the 40–50 kb size-range, as outlined previously (Maniatis *et al.*, 1982; Morrissey & Dogan, 1986). *In vitro* packaging of ligated molecules was done with a Gigapack kit (Stratagene; Northumbria Biologicals) using the manufacturer's recommended conditions. *E. coli* HB101 recipient cells were infected with recombinant phage and plated on LB-ampicillin (50 μ g ml⁻¹). Putative recombinants, identified by their Amp^r Tet^s phenotype, were picked into microtitre plates containing, in each well, LB + 50% (v/v) glycerol + 50 μ g ampicillin ml⁻¹. Plates were incubated at 37 °C, then stored frozen at -70 °C.

DNA sequencing. DNA sequencing of the *aroC* genes from *S. typhi* and *E. coli* was done using universal primer, [α -³⁵S]dATP (deoxyadenosine 5'-[α -³⁵S]thiotriphosphate) dideoxynucleotide triphosphates, and both gradient and wedge gels (Biggin *et al.*, 1983; Sanger *et al.*, 1977).

Some clones were sequenced with modified T7 DNA polymerase (Tabor & Richardson, 1987) using the Sequenase kit (United States Biochemical Corporation; marketed by Cambridge BioScience). Gaps in the sequences were filled by using synthetic oligonucleotides as specific primers (Charles *et al.*, 1985, 1986). Some master templates for use with specific oligonucleotide primers were prepared from 100 ml cultures, the phage DNA being purified further by a CsCl gradient step (Strauss *et al.*, 1986).

Oligonucleotides. Oligonucleotides, for use as specific sequence primers, were made on a SAM I oligonucleotide synthesizer (Biolabs), or manually by the paper-disc method (Matthes *et al.*, 1984) with modifications to the wash-cycle as described by Sproat & Gait (1985).

Results and Discussion

Cloning and mapping of the *S. typhi* gene for *aroC* in *E. coli*

Cosmid recombinants containing *S. typhi* inserts were packaged *in vitro* and used to infect *E. coli* HB101. A master gene bank of 600 individual recombinants was picked into microtitre plates and frozen at -70 °C. In addition, approximately 600 individual colonies were harvested from a plate and used to prepare plasmid DNA. The resulting DNA was used to transform an *E. coli aroC* host (BRD049) and transformants were selected on MM containing ampicillin but lacking aromatic compounds. A recombinant that grew well was selected for further analysis. After demonstrating retransformation and complementation of the *aroC* lesion of BRD049, plasmid DNA was prepared and designated pWAC6. pWAC6 was subjected to restriction-enzyme mapping, transposon mutagenesis and DNA-sequence analysis.

The recombinant pWAC6 was only 11.0 kb in size, with an insert of 4.5 kb of *S. typhi*-derived DNA, suggesting that pWAC6 had arisen by deletion of a larger cosmid. Transposon mutagenesis identified a 3.8 kb *Sph*I-*Eco*RI fragment, which was subcloned from pWAC6 into pUC8 to form the recombinant plasmid pSE7; it was also able to complement the *aroC* lesion in BRD049.

Cloning of the *E. coli* C600 *aroC* gene

Samples of chromosomal DNA from *E. coli* C600 were digested separately with the restriction endonucleases *Cla*I and *Sal*I, separated on a 0.7% (w/v) agarose gel and transferred to nitrocellulose by the method of Southern (1975). The resulting filter was probed with a *Pst*I-*Nru*I fragment from the *S. typhi aroC* gene that had been labelled with [α -³²P]dCTP (Maniatis *et al.*, 1982).

Fig. 1 (facing page). Nucleotide and deduced amino acid sequences of the *aroC* gene from *S. typhi* (upper case) and *E. coli* (lower case). The horizontal arrows indicate regions of imperfect inverted repeat; the overlined sequences at positions 292–295 and 446–451 in *S. typhi* and *E. coli*, respectively, indicate putative ribosome-binding sites. The protein sequences are shown in the single-letter code. Unique *E. coli* DNA sequence is shown in full, but where it overlaps with *S. typhi* sequence, only the nucleotide and amino acid differences are indicated.

gctgaacagaacatcgaaagcaacgcggcttgatcccaacgtcattccgatctgttccgatctgtttccgcgacttgccgaaaagtgcagtagaccctgatttgtcactaacccgcgtagtgc 120

GCCACGAACTTGAGCTGGGGCTGGCGTGGCCGCCTGACGCCCTCAAATTGACCCGCCSTATCCTGGGAAATCGCCGATTAT 84

gatgcggaagatatgttcgacctgccaaacgaatac g g a t g c g t c t c t c g a c 239

CTGTCCGATGATGGCTTCTGAATTGTGAAGTCGGAACAGCATGGTACATCTGATGGAGCAGTATCCGGATGTGCCGTTCACTGGCTGGAGTTTGACAACCGCGCGATGGCTCTTT 203

tq t gt t c t a a t t t g 359

M A G N T I G Q

ATGTTGACCSAAAAGCGCAGCTGCTCGCGCCCGTAACATTTGCAATATTTATAAAGATTAACACGSCAACBACAACAACGATACGSGAGCCGATGGCAGGAACACAAATTGGACA 323

c c t a ta t c a a t c g t aa cg a ac a t 476

L F R V T T F G E S H G L A V G G I V D S V P P G I P L T E A D L Q H D L D R R

ACTCTTTCCGCTACCACCTTTTGGCGAATCACACGGCTGGCGCTGGGGGATCTGTCGATGGCTGCCGCCCGCATCCGTTGACSGAGGCCGATCTGACGACGATCTCAGACAGG 443

c g gc c ct c tt a t c a g c at c ct 596

l c

R P G T S R Y T T Q R R E P D Q V K I L S G V F D G V T T G T S I G L L I E N T

CCGCCCTGGACCTTCGCTATACTACTACGCGCCGSAACCGGACGAGTAAAAATCTCTCGGGCTGTTGATGGCGTGACGACCGGACCAAGTATGGCTGCTGATTGAAACAC 563

t t g a c c g t c tt a t c tt c 716

e

D Q R S Q D Y S A I K D V F R P G H A D Y T Y E Q K Y G L R D Y R G G G R S S A

CBATCAACGCTCGCAGGACTACAGCGCATTAAAGATGTTTTTGCCTCCGGACACGCGGATTACACCTATGAGCAGAAATACGGCTGCGGATTACCGCGCGCTGGAGCTTCTCCG 683

t c g t t t g c c t a c t c c a t t 836

R E T A M R V A A G A I A K K Y L A E K F B I E I R G C L T Q M G D I P L E I K

GGTGAAACCGCGATGCGCTAGCGGACGGGGCGATCGCCAAGAATACTTGGCGGAAAGTTTCGGCATCGAAATCCGCGCTGCTGACCGAGATGGGCGACATTCGCTGGAGATTAA 803

c c c g a t t a t c c g a t t t t t t c 956

d

D W R Q V E L N P F F C P D A D K L D A L D E L M R A L K K E G D S I G A K V T

ABACTGGCGTCAGBTGAGCTTAATCGTCTTTTGCCCCGATGCGGACAAACTTGACGCGCTGGACBAACTGATGCGCGCTGAAAAAGAGGGTGACTCCATCGGCGSAAAGTGAC 923

tcg c aa t g cc c a c ta gt t c t c 1076

q p i

V M A S G V P A G L G E P V F D R L D A T I A H A L M S I N A V K G V E I G E G

GGTATGGCGAGCGCTGCGCGAGGGCTTGGCGAACGGATTTGACCGACTGGATGCGGACATCGCCATGCGCTGATGAGCATCAATGCGGTGAAGGCGTGGAGATCGGCGAAGG 1043

c tg t c t t c a g c c t c c a t c d 1196

v

F N V V A L R G S Q N R D E I T A Q G G F Q S N H A G G I L G G I S S S Q H I V A

ATTTAAGTGGTGGCTGCGCGCAGCAGAAATCGCGATGAATCACGGCGCAGGGTTTTCAGAGCAACACGCTGGCGGATCCTCGGTGGCATGAGTAGTGGGCAACACATTTGGC 1163

c g c caaag c c t g t c t c g a cat 1316

d k i

H M A L K P T S S I T V P G R T I N R M G E E V E M I T K G R H D P C V G I R A

GCATATGGCGCTGAACCTACCTCCAGCATTACCGTGGCGGACGTACBATCAACCGGATGGGTGAAGAGGTGAGATGATCACCAGGGCGCCACBATCCGTTGTGGGATTCGCGC 1283

c g t c ct t c a t c t c c c c f 1436

V P I A E A M L A I V T M D H L L R H R A Q N A D V K T E I P R W U

AETGCCGATCGCAAGGCATGCTGGCGATCCTACTGATGGATCACCTGCTGCCCATCGGCGACAGAAATCGGGATGTAAGACAGAGATTCCACGCTGGTGAAGAAATGAAAAAACCGC 1403

g tta taga ga c tt t a t 1556

q d

BATTGCGCTGCTGGCATGGTTTGTTCAGTAGCGCCAGCTGGC16CGACGCCSTGGCAGAAAAATAACCC 1470

tct c c a a aacctatgccggtagcc 1644

Analysis of the Southern blot (data not shown) demonstrated hybridization to a 3.0 kb *Cla*I fragment, and a 4.7 kb *Sa*II fragment. Size-selected *Sa*II restriction fragments of *E. coli* C600 DNA in the range of 4–5 kb were purified from a gel by the freeze-squeeze method (Tautz & Renz, 1983) and ligated into pBR322 digested with *Sa*II.

After transformation of *E. coli* C600 approximately 2000 Amp^r colonies were recovered, lysed *in situ* on nitrocellulose, and probed with the α -³²P-labelled *Pst*I–*Nru*I fragment from the *S. typhi* *aroC* gene. A single colony showing hybridization to the probe contained a plasmid designated pAN10. This plasmid contained a 4.7 kb *Sa*II fragment, and was capable of complementing the *E. coli* *aroC* mutation following re-transformation.

DNA sequencing of the *S. typhi* and *E. coli* *aroC* genes

The DNA sequences of the two *aroC* genes were determined using universal primer, and specific oligonucleotides to extend and overlap the sequences on both strands. Analysis of the DNA sequences revealed open reading frames encoding proteins of 361 amino acids of molecular mass 39108 Da and 39138 Da for the *S. typhi* and *E. coli* genes, respectively. Fig. 1 compares the *S. typhi* and *E. coli* DNA sequences, and the two deduced protein sequences.

Polypeptide expression from *aroC* recombinants in minicells

Plasmid pWAC6 encoding the *S. typhi* *aroC* gene was transferred into the minicell-producing strain DS410. Plasmid-harboring minicells were purified, labelled with [³⁵S]methionine and analysed using SDS-PAGE and autoradiography, following immunoprecipitation with antibody raised against *E. coli* chorismate synthase (kindly supplied by J. Coggins, University of Glasgow, UK). The results are shown in Fig. 2. The *aroC*-complementing plasmid pWAC6 produced a 40 kDa polypeptide (in addition to some other non-specific proteins that were also immunoprecipitated). This polypeptide is absent from the control minicell preparation containing pHC79 vector alone (track 2) and is in good agreement with the value of 39.1 kDa for the deduced protein encoded by the *S. typhi* *aroC* gene, suggesting strongly that this polypeptide is the *S. typhi* chorismate synthase enzyme. The 40 kDa polypeptide could not be distinguished when whole-cell preparations of BRD049 harbouring *aroC*-positive recombinant plasmids were analysed using SDS-polyacrylamide gels stained with Coomassie blue (data not shown). Thus the

Fig. 2. Autoradiogram showing [³⁵S]methionine-labelled minicell preparations following immunoprecipitation with antibody raised against *E. coli* chorismate synthase. Track 1, pWAC6; track 2, pHC79. Arrow A indicates the position of a 40 kDa band in track 1 corresponding to chorismate synthase. Arrow B indicates the position of 30 kDa bands present in the negative control (track 2), as well as in track 1, that are incidental to chorismate synthase.

chorismate synthase of *S. typhi* was expressed at a relatively low level in *E. coli*.

Putative regulatory elements for the *aroC* genes

Neither gene has exact matches with the consensus –10 and –35 promoter elements (Rosenberg & Court, 1979). However, putative –10 elements from positions 201–205 (*S. typhi*) and 406–411 (*E. coli*), and –35 elements from positions 178–183 (*S. typhi*) and 383–388 (*E. coli*) were identified by eye. The sequence GGAG is found at positions 292–295 (*S. typhi*) and 446–449 (*E. coli*) and shows good agreement with the consensus *E. coli* ribosome-binding site (Shine & Dalgarno, 1974). Imperfect inverted repeats are found at 3'-positions 1408–1436 (*S. typhi*) and 1562–1590 (*E. coli*), which have calculated ΔG^0 values (Tinoco *et al.*, 1973) of -47.7 kJ mol⁻¹ (*S. typhi*), and -85.4 kJ mol⁻¹ (*E. coli*), but differ from typical *E. coli* rho-independent terminators in that they are not followed by a run of uridine residues.

Comparison of the *S. typhi* and *E. coli* aroC sequences

Alignment of the two sequences demonstrates very strong homology between the two coding regions of the genes, 95% at the protein level and 84% at the DNA level (Fig. 1). Many of the observed protein changes are conservative substitutions, both genes comprising 361 codons and using TAA as the termination codon.

Examination of the codon-usage table (data not shown) for the two genes shows a high degree of similarity, and is consistent with those *E. coli* genes that are weakly expressed (Grantham *et al.*, 1981). There are some differences in codon preference, however; the codon for valine most often used in the *E. coli* aroC is GTT, while this is infrequently used in *S. typhi*. The G + C ratio for the *S. typhi* aroC gene is 59 mol% while for the *E. coli* aroC gene the G + C ratio is 57 mol%. There is less apparent homology when the 5' and 3' non-coding regions of the two sequences are compared. Both genes exhibit a region of imperfect inverted repeat sequence 22 bp after the TAA stop codon, although there are base variations in this 22 bp region, and in the inverted repeat itself. When the 5' non-coding regions of the two genes are compared, the direct base-for-base homology observed in the coding region disappears, although both sequences have a candidate ribosome-binding site with the sequence 5'-GGAG-3'.

There is considerable divergence between the deduced C-terminal sequences of the aroC-encoded proteins reported here and a recently reported *E. coli* aroC sequence (White *et al.*, 1988). The differences arise from the fact that there is an additional A-base (at position 1457 in Fig. 1) in the sequence of White *et al.* (1988). This additional base leads to a change in the reading frame, producing a difference in the sequence of the 27 C-terminal amino acids. There are two other DNA sequence differences; at positions 126 and 151 an extra G-base is present in the sequence presented here. These differences potentially identify a polymorphism between the strain used by White *et al.* (1988) and the C600 isolate used in this study. To test whether the C-terminal amino acids are functionally critical for the correct folding and functioning of chorismate synthase, a deletion mutant was constructed. A *SalI*-*Bam*HI fragment of the *E. coli* aroC gene, lacking the C-terminal 36 amino acid residues, was inserted into suitably digested pBR322 to produce plasmid pAN11. An *E. coli* *recA* aroC strain (GLW40) transformed with this plasmid was capable of growth on minimal medium lacking aromatic amino acid supplements. This demonstrated that the *SalI*-*Bam*HI fragment can be translated to form a truncated protein that is capable of folding to give functional chorismate synthase activity. It is possible, then, that both published sequences can occur in nature and that the C-terminal 36

amino acids are not absolutely critical for the correct folding and expression of chorismate synthase.

By replacing the natural aroC promoter of *S. typhi* with a more powerful promoter it should be possible to construct an *E. coli* strain expressing high levels of chorismate synthase which would facilitate purification of the enzyme. The purified enzyme will be useful for screening *in vitro* for chemical inhibitors of enzyme activity. Eventually, we intend to use information from the DNA sequence to construct specific mutants of the chorismate synthase enzyme which will be useful for studying the interaction of potential inhibitors with the protein.

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