Molecular cloning and characterization of the *aroD* gene encoding 3-dehydroquinase from *Salmonella typhi*

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The *aroD* gene from *Salmonella typhi*, encoding 5-dehydroquinase (3-dehydroquinase), has been cloned into *Escherichia coli* and the DNA sequence determined. The *aroD* gene was isolated from a cosmid gene bank by complementation of an *S. typhimurium aroD* mutant. Analysis of the DNA sequence revealed the presence of an open reading frame capable of encoding a protein of 252 amino acids with a calculated M_r of 27706. Comparison of the deduced *S. typhi* 3-dehydroquinase protein sequence with that elucidated for *E. coli* revealed 69% homology. Alignment of the *S. typhi* sequence and equivalent *Aspergillus nidulans* and *Saccharomyces cerevisiae* sequences showed that homology was lower, at 24%, but still significant. Use of a minicell expression system demonstrated that a polyclonal antibody raised against *E. coli* 3-dehydroquinase cross-reacted with its *S. typhi* counterpart.

Introduction

The only biosynthetic route for the synthesis of aromatic compounds possessed by micro-organisms is the shikimate pathway, leading to the synthesis of chorismic acid, a central precursor for other aromatic compounds (Giles, 1978; Pittard, 1987). The products of the pathway include the aromatic amino acids *p*-aminobenzoic acid and 2,3-dihydroxybenzoate. The enzyme 3-dehydroquinase catalyses step three of the prechorismate part of the pathway and in bacteria is encoded by the gene *aroD*. In *Escherichia coli*, DNA sequence analysis of *aroD* has revealed the presence of a monocistronic gene capable of encoding a protein of 240 amino acid residues with a calculated M_r of 26377 (Duncan *et al.*, 1986). By contrast, the plant enzyme consists of 3-dehydroquinase linked with shikimate dehydrogenase as a bifunctional polypeptide (Polley, 1978). The 3-dehydroquinase activity found in *Aspergillus nidulans* (Kinghorn & Hawkins, 1982; Charles *et al.*, 1986; Hawkins, 1987), *Neurospora crassa* (Lumsden & Coggins, 1977; Catcheside *et al.*, 1985) and

Saccharomyces cerevisiae (Duncan *et al.*, 1987) has been identified as part of a pentafunctional polypeptide. In each case, the large polypeptide carries the enzyme activities associated with steps two to six of the prechorismate part of the pathway. In addition to these biosynthetic activities an inducible catabolic dehydroquinase involved in the quinic acid catabolic pathway has been characterized from *A. nidulans* (Hawkins *et al.*, 1982) and *N. crassa* (Giles *et al.*, 1985). In *A. nidulans* the quinate-inducible 3-dehydroquinase is the product of the *QuTE* gene and has an M_r of 16505. A similarly sized protein from *N. crassa*, the product of the *QA2* locus, has considerable homology to the *QuTE* gene product (Da Silva *et al.*, 1986). Neither of these two catabolic dehydroquinase genes or their deduced protein sequences shows any significant homology with their biosynthetic counterparts.

In our laboratories, interest in the shikimate pathway has come from the observation that pathogenic organisms with genetic lesions in genes encoding enzymes involved in the prechorismate pathway grow poorly *in vivo* (Dougan *et al.*, 1987; Levine *et al.*, 1987; O'Callaghan *et al.*, 1988) and are of potential use as live vaccines (Bacon *et al.*, 1951; Hoiseth & Stocker, 1981).

The nucleotide sequence data reported in this paper have been submitted to EMBL and assigned the accession number X54546.

Shikimate pathway enzymes are also absent from mammalian species, and consequently have been the target for chemotherapeutic agents and broad-range herbicides (Kishore & Shah, 1988). As part of our research programme to generate aromatic amino acid dependent mutants of pathogenic organisms in order to evaluate their utility as live-attenuated vaccines (Dougan *et al.*, 1987), we have cloned, sequenced and characterized a number of *aro* genes from different micro-organisms (Charles *et al.*, 1985, 1986, 1990; Maskell *et al.*, 1988). In this paper we report the cloning and characterization of the *aroD* gene from *Salmonella typhi*, and demonstrate homology between its deduced protein sequence and the protein sequences of other biosynthetic 3-dehydroquinase enzymes.

Methods

Strains, bacteriophage and plasmids. *E. coli* K12 strain TG1 [$\Delta(lac-pro) supE thi hsdD5/F' traD36 proA^+ B^+ lacI^q$ ZAM15 (Carter *et al.*, 1985)] was the kind gift of Dr T. Gibson, Laboratory of Molecular Biology, Cambridge, UK. *E. coli* SK2881 ($F^- aroD6 leuC hsdR4 recA1$) has been described by Kushner (1978). *S. typhimurium* strain S68 [*his-6165 ilv-452 metA22 metE551 trpB2 galE496 xyl-404 rpsL20 flaA6 hsdL6 hsdSA29 lamB(E. coli) zja::Tn105*] harbours the *lamB* gene from *E. coli* integrated into the chromosome and is susceptible to bacteriophage λ infection when grown on 0.2% maltose; it was the kind gift of Professor B. Stocker, Department of Medical Microbiology, Stanford University, California, USA. *S. typhimurium* CU038 (*aroD*), *S. typhi* Ty2 (*aroA::Tn10*) and bacteriophage P22 have been described previously (Dougan *et al.*, 1987; Miller *et al.*, 1989).

Cosmid pHC79 (Hohn & Collins, 1980) was obtained from Amersham. Transposon Rts1::Tn1725 has been described (Ubben & Schmitt, 1986). M13mp18 and M13mp19 (Messing & Vieira, 1982) were supplied by Pharmacia. Minicells were prepared using *E. coli* strain DS410 (Dougan & Sherratt, 1977).

Media and reagents. *S. typhimurium*, *S. typhi* and *E. coli* were grown in Luria broth (LB) or LB solidified with 1.6% (w/v) agar. For testing auxotrophic requirements, minimal medium (MM) or MM with 2% (w/v) Noble agar (Difco) was used (Miller, 1972). Restriction endonucleases were supplied by BRL Gibco and Pharmacia. The T7 and Klenow DNA sequencing kits were supplied by Pharmacia and Cambridge Bioscience. T4 DNA ligase was from Boehringer. [35 S]dATP was from Amersham.

DNA isolation and Tn1725 mutagenesis. Plasmid DNA was isolated by the alkaline-SDS lysis method (Maniatis *et al.*, 1982). DNA restriction endonuclease fragments were isolated by the method of Tautz & Renz (1983). Chromosomal DNA was prepared by the method of Hull *et al.* (1981) as modified by Maskell *et al.* (1988). Tn1725 mutagenesis and mapping was carried out by the method of Ubben & Schmitt (1986).

Construction of an *S. typhi* chromosomal gene bank in pHC79. Chromosomal DNA from *S. typhi aroA::Tn10* was partially digested with *Sau3A* and fragments in the size range 23–40 kb were size-selected as described previously (Maniatis *et al.*, 1982; Morrissey & Dougan, 1986) and ligated into *Bam*HI-digested pHC79 (Hohn & Collins, 1980). Cosmids were packaged *in vitro* with a kit supplied by Amersham using conditions recommended by the manufacturer, and phage plated out as described previously (Miller *et al.*, 1989). A high-titre stock of

Fig. 1. Sequencing strategy for the *S. typhi aroD* gene. The sites marked pCVD1005, pCVD1006, pCVD1007 and pCVD1008 identify the position of four plasmids harbouring Tn1725 insertions that were used to generate subclones for DNA sequencing.

packaged recombinant phage was mixed with *S. typhimurium* AS68 at an m.o.i. of 0.1, and recombinants selected on LB plates containing ampicillin at a concentration of 50 μ g ml $^{-1}$. Several thousand recombinants were pooled, and a P22 stock was generated by standard methods (Davis *et al.*, 1980). This P22 bank was subsequently used to complement *S. typhimurium* strain CU038 (*aroD*).

Minicells experiments and Western blotting. Minicell experiments and SDS-PAGE were carried out as described by Dougan & Sherratt (1977). The procedure used for Western blotting was as described by Towbin *et al.* (1979).

DNA sequencing. Single-stranded DNA templates were sequenced using Klenow (Sanger *et al.*, 1977) or T7 DNA polymerase (Tabor & Richardson, 1987), using kits supplied by Pharmacia and Cambridge Bioscience. Labelled fragments were separated on buffer gradient polyacrylamide/urea gels (Biggin *et al.*, 1983). DNA sequencing was initiated from sites within the *S. typhi aroD* gene by cloning *Eco*RI fragments from four independent *aroD*-associated Tn1725 transposon insertions (Ubben & Schmitt, 1986). Gaps in the sequence were filled in using synthetic oligonucleotides as specific primers (Charles *et al.*, 1985, 1986; Strauss *et al.*, 1986).

Oligonucleotides. Oligonucleotide primers were synthesized on a SAMI oligonucleotide synthesizer (Biolabs, UK).

Results and Discussion

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

A cosmid gene bank generated in pHC79 was used to complement an *aroD* lesion in *S. typhimurium* CU038. Transformants were selected on MM containing ampicillin at a concentration of 50 μ g ml $^{-1}$, but lacking aromatic compounds. Colonies were visible after 48 h incubation at 37 °C. Of the initial six transformants that were picked on their ability to complement an *aroD* lesion, a single recombinant cosmid, pWBTYD1, that demonstrated complementation was selected for further analysis by transposon mapping, DNA sequencing and minicell-directed protein synthesis.

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CTGTGTAGTCTTTGCCGCTCTCTACGATGGGCTTTTTTTTTTACTTAAGCGGCCAGCTACCGTTATAATGGCGAGAAGAAAAAACTG
 10      20      30      40      50      60      70      80      90

M K T V T V K N L I I G E G M P K I I V S L M G R D I
ACAGGCACGCGATGAAACCGTCACCGTAAAAATCTTATCATTGGCGAAGGGATGCCCAAAATATCGTGTGTTGATGGGAAGAGACA
100      110      120      130      140      150      160      170      180

N S V K A E A L A Y R E A T F D I L E W R V D H F M D I A S
TCAATAGCGTGAAGCGGAGCGCTGGCTTACCGCAAGCTACATTCGATATCTCGAGTGGCGCGTGGATCACTTTATGGATATCGCAT
190      200      210      220      230      240      250      260      270

T D S V L T A A R V I R D A M P D I P L L F T F R S A K E G
CGACTCAATCCGTTCTTACCGCTGGCGGTGTTATCCGCGATGCGATGCCGTGACATTCGGTTACTGTTTACTTTCCGACGCGCAAGAG
280      290      300      310      320      330      340      350      360

G E Q T I T T Q H Y L T L N R A A I D S G L V D M I D L E L
GCGGCGAGCAGACAATAACCACTCAGCATTATCTCAGCTTAATCGTCCGCAATCGACAGCGGCTGGTCGATGATCGATCTTGAGC
370      380      390      400      410      420      430      440      450

F T G D A D V K A T V D Y A H A H N V Y V V M S N H D F H Q
TATTTACCGGTGATGCTGACGTTAAGCCACTGTCGATTATGCCATGCGCATAATGTTTATGTCGTGATGCTAACCACGATTTTCACC
460      470      480      490      500      510      520      530      540

T P S A E E M V S R L R K M Q A L G A D I P K I A V M P Q S
AGACGCCGTCGCGAGAGGAATGGTTTCGCGGCTACGTAAATGCAAGCACTCGGCGCGGATATCCCAAGATTGCGGTTATGCCGCAAA
550      560      570      580      590      600      610      620      630

K H D V L T L L T A T L E M Q Q H Y A D R P V I T M S M A K
GCAAGCATGATGATTAACTTACTTACTGCGACGCTGGAGATGCAAGCAATATACGCCGACCGTCCGGTAATTACTATGTCAATGCGCA
640      650      660      670      680      690      700      710      720

E G V I S R L A G E V F G S A A T F G A V K Q A S A P G Q I
AAGAGGGTGTCTATTCACGCTTGCGCAGGGGAAGTGTGTCCTGCGCCACGTTTGGCGCGGTGAAGCAGGCTTCAGCGCGGGGCAAA
730      740      750      760      770      780      790      800      810

A V N D L R S V L M I L H N A *
TCGCCGTAATGATCTACGCAGTGTATTATGATTCTGCACAACGCCCTGATCCACAGGACGGGTGCTGGTCGCATCGATCGCGTAGTCGA
820      830      840      850      860      870      880      890      900

TAGTGGCATCCAAAGTAGCGAACGGAGCAGGACTGGGACGG
910      920      930      940

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Fig. 2. The nucleotide and derived amino acid sequence of the *aroD* gene from *S. typhi*. The overlined sequence at positions 93–96 indicates a possible ribosome-binding site. The open reading frame for *aroD* comprises 252 amino acids capable of encoding a protein of M_r 27706.

Subcloning and transposon mutagenesis of *S. typhi* *aroD*

Initial restriction endonuclease mapping of cosmid pWBTYD1 demonstrated that it carried an insert of 22 kb. In order to characterize a smaller fragment that still complemented *aroD*, *Hind*III, *Eco*RI and *Bgl*II fragments were subcloned into pUC18 and the ligation mixtures used to transform *E. coli* SK2881. Analysis of *aroD*-complementing recombinants by plasmid minipreps (Maniatis *et al.*, 1982) showed that one recombinant, pCVD1001, carried an 8.2 kb *Bgl*II fragment. This plasmid was used as a substrate for Tn1725 mapping as described previously (Ubben & Schmitt, 1986). Four pCVD1001::Tn1725 isolates were identified, three of which no longer complemented the *E. coli* *aroD* mutant SK2881, and were used in the subsequent DNA sequencing strategy (Fig. 1).

Nucleotide sequence of *S. typhi* *aroD*

A 2 kb *Hind*III fragment, identified by transposon mapping of the 8.2 kb *Bgl*II fragment as covering the

region corresponding to the *S. typhi* *aroD* gene, was cloned into M13mp18 in both orientations. These M13 templates were used as the substrates for the synthetic oligonucleotide primers. Tn1725 mutagenesis generated four plasmids, pCVD1005, pCVD1006, pCVD1007 and pCVD1008, the first three of which no longer complemented the *E. coli* *aroD* strain SK2881, presumably because of gene disruption. As a consequence of these transposon insertions, additional *Eco*RI sites were introduced into the gene for *aroD* and these new sites were used to conveniently clone *aroD*-associated fragments into M13mp18 and M13mp19 for DNA sequencing (see Fig. 1). Gaps in the sequence were filled in using synthetic oligonucleotides as specific sequencing primers. Analysis of the DNA sequence (Fig. 2) reveals the presence of an open reading frame capable of encoding a protein of 252 amino acids with a calculated M_r of 27706.

Putative regulatory elements for the *S. typhi* *aroD* gene

Examination of the DNA sequence (Fig. 2) reveals the presence of two regions with a good fit to the consensus

<i>S.cerevisiae</i>	S F S K Y I A T I T . G V R E I E I P S G R S A F V C L T F D D L
<i>A.nidulans</i>	N R F L Q V A T G Q I D S L S I I K E K E H S F F A S L T L P D L
<i>E.coli</i>	M K T V T V K D L V I G T G A P K I I . V S L M A K D I
<i>S.typhi</i>	M K T V T V K N L I I G E G M P K I I . V S L M G R D I
<i>S.cerevisiae</i>	. T E Q T E N L T P I C Y G C E A V E V R V D H L . . . A N Y . .
<i>A.nidulans</i>	. R E A G D I L E E V C V G S D A V E L R V D L L K D P A S N N D
<i>E.coli</i>	A S V K S E A L A Y R E A D F D I L E W R V D H Y A D L S
<i>S.typhi</i>	N S V K A E A L A Y R E A T F D I L E W R V D H F M D I A
<i>S.cerevisiae</i>	. . S A D F V S K Q L S I L R K A T D S I P I I F T V R T M K Q G
<i>A.nidulans</i>	I P S V D Y V V E Q L S F L R . S R V T L P I I F T I R T Q S Q G
<i>E.coli</i>	. . N V E S V M A A A K I L R E T M P E K P L L F T F R S A K E G
<i>S.typhi</i>	. . S T Q S V L T A A R V I R D A M P D I P L L F T F R S A K E G
<i>S.cerevisiae</i>	G N F P D E E F K T L R E L Y D I A L K N G . V E F L D L E L T L
<i>A.nidulans</i>	G R F P D N A H D A A L E L Y R L A F R S G . C E F V D L D I A F
<i>E.coli</i>	G E Q A I S T E A Y Y C . T H R A A I D S G L V D M I D L E L . F
<i>S.typhi</i>	G E Q T I T T Q H Y L T . L N R A A I D S G L V D M I D L E L . F
<i>S.cerevisiae</i>	P T D I Q Y E . . V . I N K R G N T K I I G S H H D F Q G L Y S W
<i>A.nidulans</i>	P E D M L R A . . V . T E M K G F S K I I A S H H D P K G E L S W
<i>E.coli</i>	T G D D Q V K E T V A Y A H A H D V K V V M S N H D F H K T P E A
<i>S.typhi</i>	T G D A D V K A T V D Y A H A H N V Y V V M S N H D F H Q T P S A
<i>S.cerevisiae</i>	D D A E W E N R F N Q A L T L D V D V V K F V G T A V N F E D N L
<i>A.nidulans</i>	A N M S W I K F Y N K A L . E Y G D I I K L V G V A R N I D D N T
<i>E.coli</i>	E E I I A R L R K M Q S F . . D A D I P K I A L M P Q S T S D V L
<i>S.typhi</i>	E E M V S R L R K M Q A L . . G A D I P K I A V M P Q S K H D V L
<i>S.cerevisiae</i>	R L E H F R D T H K N K P L I A V N M T S K G S I S R V L
<i>A.nidulans</i>	A L R K F K N W A A E A H . D V P L I A I N M G D Q G Q L S R I L
<i>E.coli</i>	T L L A A T L E M Q E Q Y A D K P I I T M S M A K T G E I S R . L
<i>S.typhi</i>	T L L T A T L E M Q Q H Y A D R P V I T M S M A K E G V I S R . L
<i>S.cerevisiae</i>	N N V L T P V T S D L L P N S . A . A P G Q L T V A Q I N K M Y T
<i>A.nidulans</i>	N G F M T P V S H P S L P F K . A . A P G Q L S A T E I R K G L S
<i>E.coli</i>	A G E V F G S G G N F W C G K K S V C A R A N L G K *
<i>S.typhi</i>	A G E V F G S A A T F G A V K Q A S A P G Q I A V N D L R S V L M
<i>S.cerevisiae</i>	S M G G I E
<i>A.nidulans</i>	L M G E I K
<i>E.coli</i>	
<i>S.typhi</i>	I L H N A *

Fig. 3. Amino acid homologies between the *S. typhi* *aroD* gene product and other published 3-dehydroquinase sequences. Sequences that are common to three or more of the species are boxed. Dots refer to regions of variable length that are introduced in the sequences to produce a best fit for regions of homology.

−10 and −35 promoter elements (Rosenberg & Court, 1979). The sequence 5′-TATAAT-3′ from nucleotides 65 to 70 is an exact match with the −10 consensus sequence, while the sequence 5′-TTTACT-3′ from nucleotides 41 to 46 with a spacing of 18 bp has a 4/6 match with the consensus −35 region.

Comparison of the DNA sequence flanking the structural gene for *aroD* from *S. typhi* with the *E. coli* *aroD* flanking regions shows little homology, although both sequences have a candidate AGG ribosome-binding site (Shine & Dalgarno, 1974). In the *E. coli* *aroD*

sequence an inverted repeat has been noted 48 bp after the end of the structural gene but this feature is apparently absent from the *S. typhi* *aroD* sequence.

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

Comparison of the sequences shows a high degree of homology, 69.6% at the protein level, and 68.7% when DNA sequences were aligned. Many of the observed protein changes are, however, conservative substitutions. Interestingly, the sequence from *S. typhi* comprises

Fig. 4. Western blot showing minicell-directed proteins separated by SDS-PAGE transferred to nitrocellulose and reacted with anti-*E. coli* 3-dehydroquinase (*aroD*) antibody. Track 1, M_r markers; track 2, cosmid pH79 (negative control); track 3, cosmid pWBTD1 (expressing 3-dehydroquinase). An arrow indicates the position of a 28 kDa band in track 3 that corresponds to 3-dehydroquinase. Fainter higher- M_r bands seen in tracks 2 and 3 are cross-reacting proteins that are incidental to 3-dehydroquinase.

252 amino acid residues, while the sequence from *E. coli* has 240 residues. This sequence difference appears to be the consequence of an error in the published *E. coli* sequence for *aroD*. Resequencing the *E. coli* gene indicates that it also comprises 252 amino acid residues (J. Coggins & L. Graham, personal communication).

Comparison of the codon usage table of *S. typhi aroD* with that derived for another gene encoding a protein involved in aromatic amino acid biosynthesis from *S. typhi*, the *aroC* gene (Charles *et al.*, 1990), shows a high degree of similarity in the choice of which codons are most frequently used. The pattern is consistent for those genes that are poorly expressed in *E. coli* (Grantham *et al.*, 1981). There are some minor differences in codon preference, e.g. in the *S. typhi aroC* gene the most popular codon for alanine is GCG, while in the *S. typhi aroD* it is GCC.

Alignment of four biosynthetic dehydroquinase enzymes

Homology between four biosynthetic dehydroquinase enzymes is displayed in Fig. 3. The figure was obtained by comparing the deduced *S. typhi* 3-dehydroquinase protein sequence individually with those for *E. coli*,

Aspergillus nidulans and *Saccharomyces cerevisiae* using the program ALIGN (Staden, 1982). The four sequences were then compiled together by eye. Boxes have been drawn around sequences that are conserved in three or more of the proteins.

Minicell-directed polypeptide expression from an *aroD* recombinant

Cosmid pWBTD1 encoding the *S. typhi aroD* gene was transferred to the minicell-producing strain DS410. Plasmid-harboring minicells were purified, labelled with [35 S]methionine and analysed using SDS-PAGE followed by Western blotting. Proteins were transferred to nitrocellulose, and reacted with antibody raised against *E. coli* 3-dehydroquinase (kindly supplied by J. Coggins, University of Glasgow, UK). The results are shown in Fig. 4. The *aroD*-complementing cosmid (track 3) directs the expression of a protein of M_r approximately 28000 (marked with an arrow), which is in good agreement with the value of 27706 deduced from the DNA sequence. This polypeptide is absent from the control (track 2) sample prepared from minicells harbouring pH79 vector. Cross-reacting bands of lower mobility, present in both the negative control (track 2) and track 1, are incidental to 3-dehydroquinase expression.

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