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Expression, purification, crystallization and preliminary characterization of an HHED aldolase homologue from *Escherichia coli* K12

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An ORF designated b2245 (*yfaU*) in the *Escherichia coli* K12 genome sequence, identified as an HHED aldolase homologue, was cloned into the high-expression plasmid pT7-7 and overexpressed in *E. coli* B835(DE3). The enzyme was purified in three steps to 95% purity prior to crystallization. Crystals were obtained by the hanging-drop vapour-diffusion method at 277 K from a number of screening conditions. Crystals suitable for structural studies were grown from solutions containing 0.4 *M* ammonium dihydrogen phosphate and grew to a maximum dimension of approximately 0.5 mm. Diffraction data to 1.7 Å were collected using an in-house Cu $K\alpha$ radiation source at 100 K. The crystals belong to space group $C222_1$, with unit-cell parameters $a = 105.1$, $b = 136.6$, $c = 123.1$ Å. A 90% complete data set was collected to 1.78 Å from a single native crystal using in-house facilities.

1. Introduction

The benzene ring is one of the most widely distributed units of chemical structure in nature and catabolism of the aromatic nucleus is a vital function in the biosphere. The degradation of the aromatic nucleus is normally achieved by a wide range of micro-organisms, including bacteria and fungi. The contribution of such organisms to the cycling of carbon in the biosphere is crucial and the potential in the bioremediation of xenobiotic compounds is of great interest. Many herbicides, pesticides and industrial effluents contain a benzene nucleus and many instances of bacteria adapting to novel carbon sources have been documented, including *Flavobacterium* species which are able to degrade nylon oligomers (Okada *et al.*, 1983). Better understanding of the degradative process could lead to the design of biodegradable chemicals or opportunities for bioremediation using engineered organisms to break down recalcitrant chemicals (Allard & Neilson, 1997; Samanta *et al.*, 2002).

Degradation of the aromatic nucleus proceeds initially *via* dihydroxylation of the aromatic ring followed by ring fission. This can be achieved through either *ortho*-cleavage, between the two hydroxyl groups, or by *meta*-cleavage, at the side of one of the hydroxyl groups. Cleaving the benzene nucleus is the committal step of degradation. The products can then serve as substrates for other reactions leading to the production of central metabolites. Aromatic degradative pathways are abundant resources for the discovery and study of many enzymatic activities and offer the potential to understand substrate selectivity by comparative studies.

Degradation of 4-hydroxyphenylacetic acid (4-HPA) involves the longest known catabolic pathway (Cooper & Skinner, 1980), comprising of eight enzyme-catalysed steps yielding pyruvate and succinic semialdehyde and is one of the most studied in *E. coli*. Many of the enzymes have been characterized biochemically (Roper *et al.*, 1993, 1995) and crystal structures for two of the enzymes have been solved (Subramanya *et al.*, 1996; Tame *et al.*, 2002). The ability to degrade 4-HPA was first found in *Acinetobacter* and two species of *Pseudomonas*. Cooper & Skinner (1980) found *E. coli* C could grow on 3-HPA and 4-HPA, the first report that *E. coli* could grow on aromatic substances. *E. coli* B and W have since been shown to degrade HPA, but this activity is undetectable in *E. coli* K12. These results have been confirmed by Southern hybridization against chromosomal DNA (Prieto *et al.*, 1996). The genes required for 4-HPA degradation have been cloned from *E. coli* C (Jenkins & Cooper, 1988; Roper *et al.*, 1993) and *E. coli* W (Prieto *et al.*, 1996). The availability of genomic DNA sequences in recent years has enabled sequence comparisons to be made revealing the presence of 4-HPA pathways in a wide range of bacteria and the presence of 4-HPA pathway gene homologues in many others. This study focuses on the *E. coli* K12 gene b2245 designated *yfaU*, which shows 56% sequence identity (http://www.ch.embnet.org/software/LALIGN_form.html) to HHED aldolase

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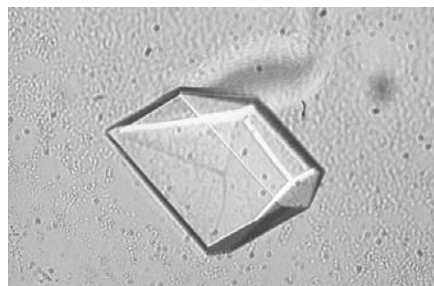
(hpcH; Stringfellow *et al.*, 1995). *E. coli* K12 does not posses any of the other 4-HPA pathway genes and therefore the presence and function of *yfaU* is an intriguing anomaly.

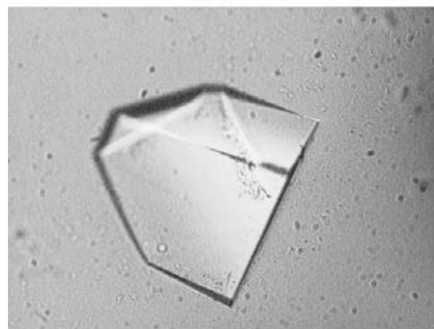
2. Materials and methods

2.1. Cloning, overexpression and purification

The gene encoding *E. coli yfaU* was amplified by PCR with primers incorporating *Nde*I and *Eco*RI sites at the 5′ and 3′ ends of the gene, respectively. The PCR fragment was digested with these enzymes and ligated into pT7-7 vector (Tabor & Richardson, 1987). After selection of recombinants containing the cloned gene, designated pCJ771, *E. coli* B834(DE3) was transformed and grown at 310 K in 1 l LB medium containing 100 µg ml^{-1} ampicillin. Induction of protein expression was obtained by the addition of IPTG to the culture to 1 m*M* and growth continued at 295 K for 16 h. The cells were harvested by centrifugation at 4000*g* for 15 min and were resuspended in 50 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl prior to lysis by sonication. The extract was clarified by centrifugation at 25 000*g* prior to initial purification by ammonium sulfate fractionation. YfaU aldolase was found to precipitate between 30 and 50% ammonium sulfate at 277 K. The pelleted protein was dissolved in 50 m*M* Tris–HCl pH 8.0, 1 *M* ammonium sulfate and applied to a phenyl Sepharose HP column (Amersham-Pharmacia) equilibrated in the same buffer. The column was developed with a linear gradient of decreasing ammonium sulfate to 0 *M*. The aldolase eluted from the column below 100 m*M* ammonium sulfate. Fractions containing the aldolase were pooled, concentrated in centrifugal concentrators and applied to a Superdex 200 HR 26/60 gel-filtration column equilibrated in 50 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl. YfaU eluting from this column was judged by SDS–PAGE to be greater than 99% pure and was dialysed into 10 m*M* Tris–HCl pH 8.0 and concentrated to 10 mg ml^{-1} prior to crystallization.



(*a*)



(*b*)

Figure 1
Photographs of YfaU crystals (*a*) obtained from Hampton Crystal Screen condition 4 (0.4 *M* ammonium dihydrogen phosphate pH 6.5) and (*b*) obtained from Clear Screen II (20% PEG 550 MME, 0.15 *M* KSCN, 0.1 *M* MES pH 6.5). The average dimensions of these crystals were 0.5 mm in each direction.

3. Crystallization

All crystallization experiments were performed using the hanging-drop vapour-diffusion method in a 24-well tissue-culture Linbro plate at 291 K. Initial crystallization trials were carried out using 0.5 ml reservoir solutions taken from the Hampton Research Crystal Screen (Jancarik & Kim, 1991) or using 1 ml solutions taken from the Clear Strategy Screen buffered at 0.1 *M* MES pH 6.5 (Brzozowski & Walton, 2000). Drops consisting of 2 µl of protein and 1 µl of reservoir solution were used throughout. Crystals appeared after 16 h from multiple conditions in the Hampton Screen I conditions, particularly solutions 2, 3, 4, 7, 14, 16, 29, 35, 37, 40, 48 and 49. In the Clear Strategy screen, screen I gave crystals in conditions 5, 10, 11, 14, 16 and 20 and screen II in conditions 4 and 16. The crystals obtained in condition 3 (Fig. 1*a*) of the Hampton Screen (0.4 *M* ammonium dihydrogen phosphate pH 6.5) and condition 16 (Fig. 1*b*) of the Clear Strategy Screen II (20% PEG 550 MME, 0.15 *M* KSCN, 0.1 *M* MES pH 6.5) were isomorphous and of a quality suitable for X-ray data collection, with dimensions in excess of 0.2 × 0.2 × 0.3 mm.

3.1. X-ray crystallographic studies

Preliminary diffraction data were collected at 100 K in-house using an Enraf–Nonius Cu *Kα* X-ray generator operating at 45 kV and 115 mA equipped with Osmic focusing mirrors and a DIP 2030b image-plate collector. The crystals from Hampton Crystal Screen I condition 3 were flash-frozen in liquid nitrogen using 30% glycerol in the mother liquor as cryoprotectant and were maintained at 100 K throughout data collection using an Oxford cryostream system. Diffraction data to 1.7 Å were observed using in-house data-collection facilities and a data set with 1° oscillations to 90% completion was collected. All intensity data were indexed, integrated and scaled using the *HKL* programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Data-collection statistics are shown in Table 1. Reflections (00*l*) with $l = 2n + 1$ are absent; therefore, the space group is $C222_1$. A self-rotation function indicated the presence of a threefold non-crystallographic axis along *y*. The combination of this and crystallographic twofold along *x* is very likely to generate a hexamer with 32 symmetry in a similar way to the structure of 2-dehydro-3-deoxygalactarate aldolase. We are currently in the process of attempting a molecular-replacement solution using the coordinates for 2-dehydro-3-deoxygalactarate aldolase (Izard & Blackwell, 2000), which shares 43% sequence identity the YfaU aldolase.

Table 1
Data-collection and processing statistics.

Values in parentheses correspond to the outer resolution shell.

Space group	$C222_1$
Unit-cell parameters (Å, °)	a = 105.1, b = 136.6, c = 123.1, $\alpha = \beta = \gamma = 90$
Matthews coefficient (Å^3 Da^{-1})	2.5 (3 mols per AU)
Solvent content (%)	43
Resolution range (Å)	30–1.9
Total observations	186908
Unique reflections	59892
Redundancy	3.1
Average $I/\sigma(I)$	16.7 (2.0)
$R_{\rm merge}$ (%)	5.2 (31.7)
Completeness (%)	85.7 (93.7)

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