2,4'-Dihydroxyacetophenone dioxygenase (EC 1.13.11.41) from *Alcaligenes* sp. 4HAP: a novel enzyme with an atypical dioxygenase sequence

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2,4'-Dihydroxyacetophenone dioxygenase (EC 1.13.11.41) was purified to homogeneity from *Alcaligenes* sp. 4HAP grown on 4-hydroxyacetophenone. Measurements of the M_r of the native enzyme ranged from 81 600 to 87 000, whereas values of 21 000 and 20 379 were given by SDS/PAGE and electrospray MS respectively. The enzyme is a homotetramer and contains one atom of iron per molecule of enzyme. From C- and N-terminal analyses, primers for PCR were designed and the *dad* gene cloned and sequenced. The predicted amino acid sequence of *dad*, deduced from the nucleotide sequence, confirms the N-terminal

amino acid sequencing data and contains the sequence of an internal tryptic peptide. It gave a calculated M_r of 20 364. The gene was expressed in *Escherichia coli* and yielded active enzyme. The derived amino acid sequence does not show significant similarity to other dioxygenases or any strong similarity to protein sequences presently available in the databases.

Key words: *Escherichia coli*, heterologous expression, iron oxygenase.

INTRODUCTION

The aerobic catabolism of aromatic compounds by bacteria is highly dependent on the activity of oxygenases. They are involved both in the preparation of aromatic compounds for ring cleavage by metabolism of ring substituents, or ring hydroxylation, and in the ring-fission reactions themselves. The enzymes for these latter reactions are dioxygenases, most of which are iron-containing, although some require manganese. Dioxygenases are also used in some pathways to catalyse the insertion of two hydroxy groups into the aromatic ring for the production of a suitable ring-fission substrate. This phase of catabolism, where compounds are prepared for ring fission, may sometimes involve mono-oxygenases. These are usually involved in hydroxylation reactions, but, in the case of the metabolism of aromatic ketones such as acetophenone or 4-hydroxyacetophenone by strains of *Pseudomonas*, *Arthrobacter* and *Nocardia*, mono-oxygenases were shown to insert oxygen into the substrate to give an ester, which was then hydrolysed [1,2]. Such enzymes catalyse the equivalent of the Baeyer–Villiger reaction and are often referred to as Baeyer–Villiger mono-oxygenases. In contrast, *Alcaligenes*

sp. 4HAP uses a different pathway for 4-hydroxyacetophenone metabolism, which was shown to proceed by hydroxylation of the methyl group to give 2,4'-dihydroxyacetophenone (referred to in previous papers as '4-hydroxybenzoylmethanol') [3]. This is then cleaved in an oxygen-requiring reaction to 4-hydroxybenzoate and formate (Scheme 1) [4]. Incubations of reaction mixtures using partially purified enzyme with $^{18}\text{O}_2$ and H_2^{18}O showed that oxygen from O_2 was incorporated into both products and that the enzyme concerned is, therefore, a dioxygenase [5]. This represents an unusual reaction for an oxygenase and raises the question of the relatedness of this enzyme to other dioxygenases, particularly ring-fission dioxygenases, which are also involved in C—C bond cleavage. Here we report the complete purification of 2,4'-dihydroxyacetophenone dioxygenase (EC 1.13.11.41) and some of its characteristics, including its sequence, which differs markedly from that of any other enzymes in this class.

EXPERIMENTAL

Bacterial strains and plasmids

The enzyme was purified from the *Alcaligenes* sp. 4HAP isolated for growth on 4-hydroxyacetophenone [3]. The gene cloning and expression studies used the following strains of *Escherichia coli*: XL1-Blue, TB1, RR1 and N-4830. The plasmids used were: pBluescriptII SK⁺ (Stratagene Europe, Amsterdam, The Netherlands), pGEM-T Easy (Promega, Southampton, U.K.) and pλ-1cvt [6] (Table 1).

Chemicals and enzymes

The procedure of Robertson and Robinson [7] was used for the preparation of 2,4'-dihydroxyacetophenone. 4-Hydroxyacetophenone was from Lancaster Synthesis Ltd., Eastgate, Lancs., U.K. For DNA manipulations, unless specified, all chemicals were from Sigma. Restriction enzymes and other DNA-modifying enzymes were from New England Biolabs. Agarose was from Boehringer.

Scheme 1 Reaction catalysed by 2,4'-dihydroxyacetophenone dioxygenase

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The nucleotide sequence for the 2,4'-dihydroxyacetophenone dioxygenase gene (*dad*) has been deposited in the GenBank®/EMBL/DDBJ Nucleotide Sequence Databases under the accession number AJ133820.

Table 1 Plasmids used in the present study

Plasmid	Size (kbp)	Description	Source
pBluescript SK ⁺	3.0	Phagemid derived from pUC19; Amp ^r	Stratagene
pGEM-T Easy	3.0	PCR (A/T) cloning vector; Amp ^r	Promega
pKG-19	3.6	Regions flanking <i>dad</i> (600 bp) inserted into pGEM-Teasy; Amp ^r	The present study
pKB-10	3.6	DAD expression construct; <i>dad</i> PCR product (primers E + F with pKG-19 template cloned into pBluescript downstream of <i>lac</i> promoter); Amp ^r	The present study
pλ1-cyt	4.2	A thermo-inducible expression vector that reports expression status of a cloned foreign gene by PINK reporter system; Amp ^r	[18]
pKλ-12	4.8	Full-length <i>dad</i> dressed with S/D sequence cloned into pλ1-cyt; Amp ^r	The present study

Growth of bacteria

For enzyme purification, *Alcaligenes* sp. 4HAP was grown in a Microferm MF-114 fermenter (New Brunswick Scientific Co.) in 12 litres of medium containing (per litre): Na₂HPO₄, 4.33 g; KH₂PO₄, 2.65 g; NH₄Cl, 2.0 g; 4-hydroxyacetophenone, 0.5 g; and 4.0 ml of salts solution [8]. To increase the yield of bacteria, further additions of substrate were made during the growth period. Harvested bacteria were stored as a cell paste at -20 °C. *E. coli* was grown in Luria-Bertani broth to which was added 1.5% (w/v) agar for solid media. Where appropriate, the medium was supplemented with 75 µg ampicillin/ml.

Enzyme assay

The enzyme was assayed spectrophotometrically by monitoring the decrease in *A*₂₉₃ in a 1-cm-light-path cuvette at 25 °C containing in 3 ml of 42 mM sodium/potassium phosphate buffer, pH 7.0, 0.167 mM 2,4'-dihydroxyacetophenone and enzyme. The spectrum of the substrate changes markedly with pH, and from this change a p*K* of 7.7 was calculated, with 293 nm as an isosbestic point in the spectrum. The product has a low absorbance at this wavelength, and the Δ*ε* for the reaction, used to calculate activity, is 7.9 mM⁻¹·cm⁻¹ (derived from the absorbances of the substrate and product). The reaction was started by addition of substrate after incubation of the enzyme and buffer at 25 °C for 10 min. A unit of activity is defined as the hydrolysis of 1 µmol of substrate/min.

Enzyme purification

All purification procedures were performed at 4 °C. Frozen cells (25 g) were thawed and resuspended in three times their volume of 42 mM sodium/potassium phosphate buffer, pH 7.0, and disrupted by ultrasonic disintegration using a Soniprobe 150 sonifier (MSE, Crawley, Sussex, U.K.) using six 30 s bursts at 16 µm amplitude interspersed with cooling on ice. The suspension was then centrifuged at 28000 *g* for 20 min at 4 °C. The supernatant was taken as crude extract. This was loaded on to a DEAE-cellulose column (6 cm long × 5 cm diameter) equilibrated with 42 mM sodium/potassium phosphate buffer, pH 7.0. The column was washed with 1 vol. of this buffer and then with 2 vol. of buffer containing 0.2 M KCl. The enzyme was then eluted with a gradient from 0.2 to 0.6 M KCl in 500 ml of buffer and 7.5 ml fractions were collected. The enzyme was eluted between 0.3 and 0.4 M KCl, and active fractions were pooled and concentrated to less than 10 ml in an Amicon ultrafiltration cell fitted with a PM10 membrane. The concentrated enzyme was loaded on to a Sephacryl S200 column (80 cm long × 2.5 cm diameter) and eluted with 0.1 M Tris/HCl buffer, pH 8.0. Fractions (5 ml each) were collected, and those containing

enzyme were pooled and loaded on to a column of Q-Sepharose FF (6 cm long × 2.5 cm diameter) equilibrated with 50 mM Tris/HCl buffer, pH 8.0. The column was washed with 50 ml of this buffer and then with 50 ml of buffer containing 0.2 M KCl. Enzyme was then eluted with a gradient from 0.2 to 0.8 M KCl in 300 ml of buffer. Fractions (5 ml each) were collected and enzyme was eluted between 0.5 and 0.6 M KCl. Enzyme was stored at -20 °C after dialysis against distilled water and freeze-drying.

Electrophoresis of enzyme

Purified enzyme was electrophoresed under non-denaturing conditions and with SDS on Bio-Rad Mini Protean II Ready Gels (4–20% gradient). Denaturing gels were calibrated with Sigma 4000–700000- and 30000–200000-*M_r* markers. Proteins were detected by staining with Coomassie Brilliant Blue R-250.

Terminal analysis of enzyme

The N-terminal analysis of the enzyme and of a peptide obtained by tryptic digest was performed by automated Edman degradation on an Applied Biosystems model 473A sequencer at the sequencing service of the University of Nottingham, Nottingham, U.K. The tryptic peptide was prepared by incubation of enzyme dissolved in 250 mM ammonium bicarbonate with sequencing-grade trypsin (Promega Ltd.) for 16 h at 37 °C. The resulting peptides were separated by HPLC on a Jupiter C₁₈ (250 cm long × 2.0 mm diameter) column (Phenomenex, Torrance, CA, U.S.A.) using solvent A (0.1% trifluoroacetic acid in water) and a gradient from 0 to 100% of solvent B [0.07% trifluoroacetic acid and 70% (v/v) acetonitrile in water] run over 80 min at a flow rate of 0.15 ml/min. C-terminal analysis was performed at the Mayo Protein Core Facility (Mayo Clinic, Rochester, MN, U.S.A.) using an Applied Biosystems Procise C chemical C-terminal sequencer.

Determination of the *M_r* of the enzyme

The *M_r* of the native enzyme was determined by gel filtration on two types of column. A calibrated Sephacryl S-200 column (77 cm long × 2.5 cm diameter) was used with 0.1 M Tris/HCl buffer, pH 8.0, as eluent. A calibrated Bio-Rad Bio-Sil TSK-125 gel-filtration column (300 mm long × 7.5 mm diameter) was used for HPLC of the enzyme with an eluting buffer of 42 mM sodium/potassium phosphate, pH 7.0, containing 0.15 M NaCl.

Analytical ultracentrifugation was also used to measure the *M_r*. This was done using a Beckman Spinco model E analytical ultracentrifuge equipped with Raleigh interference optics by the meniscus-depletion method of Yphantis [9]. A speed of 19017

Table 2 Primers used for PCR

The primers are shown with the corresponding amino acid sequence, from which they were derived, given underneath. The underlined sequences in E and F are the sites for *Pst*I and *Eco*RI respectively. The boxed sequence in E is the Shine-Dalgarno sequence.

A	5'-GAR TTY TGG MGN GAY AT-3' E F W R D I
B	3'-CAN TGN RAN AAR GC-5' V T L F R
C	5'-ATG GTN GCN AAY GCN ATY WS-3' M V A N A I S
D	3'-CAN TGN RAN AAR TCY A-5' V T L F R
E	5'-ATCTGCAGATAAAGGAGAACACAACA ATG GTC GCT AAC M V A N GCA ATC AGC GAG TTC TGG AGG GAC ATC CG-3' A I S E F W R D I R
F	3'-CAG CAA TGA GAA AAG GCG ATC CTTAAGAT-5' V V T L F R *

rev./min at 20 °C was used and a value of 0.725 assumed for the partial specific volume.

The M_r was also measured by electrospray MS using a LCT oa-TOF mass spectrometer (Micromass U.K. Ltd., Wythenshaw, Manchester, U.K.) in the electrospray mode. The capillary voltage was 3.2 kV and the cone voltage 50 V. The protein was dissolved in 0.2 % (v/v) formic acid to give 200 ng/ μ l, mixed with an equal volume of acetonitrile and injected at a rate of 5 μ l/min with nebulizer gas at 100 litres/h. The instrument was calibrated with myoglobin.

Determination of iron

The iron content of the enzyme was determined by the colorimetric method of Fish [10].

DNA manipulations

Plasmid DNA was isolated either by the procedure given in [11], or by the Wizard Midiprep DNA purification system from Promega. Chromosomal DNA from the *Alcaligenes* sp. was isolated by the CTAB (cetyltrimethylammonium bromide) procedure described by Wilson [12]. The standard procedures for DNA restriction, (de)phosphorylation, agarose-gel electrophoresis, PCR and transformation of competent *E. coli* cells were performed as described by Sambrook et al. [13]; competent cells were prepared essentially as described by Hanahan [14]. PCR was performed on an Hybaid OmniGene thermal cycler, using Super Taq (Kramel Biotechnology, Cramlington, Northumberland, U.K.). Synthetic oligonucleotides (Table 2) were made by MWG Biotech (Milton Keynes, U.K.) or R & D Systems Europe Ltd. (Oxford, U.K.). PCR cycle conditions for primer pairs A and B, C and D, and E and F were as follows: 4 min at 94 °C, 15 cycles of 1 min at 92 °C, 1 min at 52 °C, and 1 min at 72 °C finishing with 4 min at 72 °C followed by 20 cycles of 2 min at 90 °C, 2 min at 54 °C, 2 min at 72 °C, finishing with 5 min at 72 °C. The PCR reaction mixture contained, in a final volume of 50 μ l, 10 mM Tris/HCl buffer, pH 9, 1.5 mM MgCl₂, 50 mM KCl, 0.1 % (v/v) Triton X-100, 0.1 % (w/v) gelatin, 0.1 mg BSA/ml, 200 μ M dNTP, 600 μ M of each primer, 25 μ g of chromosomal DNA/ml or

500 ng of plasmid DNA/ml and 10 units of Super Taq/ml. PCR products were separated by electrophoresis on agarose gels, then excised, treated with Agarase and purified with the Wizard DNA Clean-Up system (Promega). The isolated DNA was ligated with the pGEM-T Easy vector in the presence of polynucleotide kinase, introduced into *E. coli* XL1-Blue, and potential recombinants selected by 'X-Gal' (5-bromo-4-chloroindol-3-yl β -D-galactopyranoside) screening [15]. DNA sequencing was performed by use of a Li-Cor automated fluorescent sequencer (MWG Biotech, Milton Keynes, U.K.) using the primers based on T7 and SP6 sequences flanking the A/T cloning sites in pGEM-T Easy.

Heterologous expression of the dioxygenase gene

Cultures were grown at 30 °C to mid-exponential phase and then induced either by addition of 1 mM isopropyl β -D-thiogalactoside or by shifting the temperature to 39 °C, followed by growth for a further 6 h. Bacteria were harvested and extracts made by sonication for assay of enzyme activity. For electrophoretic analysis, cells from 1 ml of culture were harvested, washed with water, dissolved in 120 μ l of SDS buffer, containing 2.5 % (w/v) SDS, 25 % (w/v) glycerol, 50 mM dithiothreitol and 0.025 % (w/v) Bromophenol Blue, and heated to boiling for 5 min.

RESULTS

Purification of 2,4'-dihydroxyacetophenone dioxygenase

The enzyme was purified in three steps from *Alcaligenes* sp. 4HAP grown on 4-hydroxyacetophenone as the sole carbon source. The details are summarized in Table 3. The 45-fold purification yielded a highly purified enzyme, with a recovery of 52 %, that gave one major band when examined by PAGE in non-denaturing gels.

M_r and subunit structure

The M_r of the enzyme was determined by gel filtration on calibrated Sephacryl S200 and BioSil TSK-125 columns. These gave values of 87000 and 83000 respectively. The value from ultracentrifugal studies was 81600. SDS/PAGE of the enzyme after heating with SDS and 2-mercaptoethanol gave a single band of M_r 21000 (Figure 1) and electrospray MS of the enzyme gave a value for the M_r of 20379.

When the enzyme was examined by SDS/PAGE with prior heating in SDS but without 2-mercaptoethanol, two bands were seen (Figure 1): one at M_r 21000 as mentioned above, and another at M_r 19000. The same sample, after treatment with 2-mercaptoethanol, again gave a single band of M_r 21000, suggesting that, in the native enzyme, there might be some heterogeneity of subunits with some containing an internal disulphide bond.

UV-visible absorption spectrum

The purified enzyme was colourless with a single peak in the spectrum at 280 nm and a shoulder at about 290 nm.

Iron content of enzyme

The iron content of the enzyme was measured colorimetrically by chelation with ferrozine after digestion with acid permanganate to release the iron [10]. This gave a ratio of iron to tetramer of 0.95:1, indicating the presence of a single atom of iron for each molecule of enzyme. As controls, the iron contents of myoglobin, which is known to contain 1 molecule of iron per molecule of protein, and BSA, which does not contain iron, were measured.

Table 3 Purification of 2,4'-dihydroxyacetophenone dioxygenase

This shows a typical purification starting with 25g wet weight of *Alcaligenes* sp. 4HAP grown on 4-hydroxyacetophenone.

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude extract	74	2427	156	0.064	100	—
DEAE-cellulose chromatography	100	74	97.9	1.32	63	20.7
Sephacryl S200 chromatography	39	46.8	86.3	1.84	55	28.8
Q-Sepharose FF chromatography	30.5	28.2	80.5	2.85	51.6	44.6

Figure 1 SDS/PAGE of purified 2,4'-dihydroxyacetophenone dioxygenase

A 4–10% gradient polyacrylamide gel of proteins stained with Coomassie Blue is shown. Lane a, M_r markers; lane b, purified enzyme heated with SDS and 2-mercaptoethanol; lane c, purified enzyme heated with SDS alone.

These gave ratios of iron to protein of 0.9:1 and < 0.0004:1 respectively.

Amino acid sequences

The first 20 amino acids from the N-terminus of the purified enzyme were sequenced by automated Edman degradation, giving the following result (N-terminus on the left):

VANAISEFWRDIRPIESPFK-

An internal peptide, generated by trypsin digestion, gave the sequence:

WGYLEHDWTATR

The C-terminus was sequenced by an automated chemical method, which yielded the sequence (C-terminus on the right):

-VTLFR

DNA cloning

A strategy was devised to clone the dioxygenase gene, from chromosomal DNA isolated from the *Alcaligenes* sp. 4HAP, by PCR using a combination of primers AB and AD, CB and CD

Figure 2 Sequence of the gene for 2,4'-dihydroxyacetophenone dioxygenase and derived amino acid sequence

The underlined sequences are those confirmed by protein sequencing of the purified enzyme. The nucleotides shown in **bold italics** are those added to the sequenced PCR product to enable expression of the gene as active protein. These contain an optimized translation start signal, including the Shine–Dalgarno sequence (boxed), the spacer region, the initiator methionine codon and the sequence coding for the N-terminal hexapeptide.

(Table 2) that were designed from the N- and C-termini amino acid sequences of the protein. The forward primers A and C differ in that the former was based on the amino acid sequence of the internal hexapeptide starting from the seventh residue of the N-terminal sequence, whereas the latter was based on the sequence starting from the N-terminal valine. Only primer combinations AB and AD yielded a single DNA fragment of about 600 bp, the expected length from the subunit size of the dioxygenase enzyme. For sequencing of the PCR product it was

Table 4 2,4'-Dihydroxyacetophenone dioxygenase activity in cell extracts

The gene for 2,4'-dihydroxyacetophenone dioxygenase, *dad*, was inserted into the vectors pBluescript and p λ 1-cyt to give pKB-10 and pK λ -12 respectively. These were then used to transform *E. coli* strains TB1 and N-4830. After growth and induction of the bacteria, cell extracts were prepared and assayed for dioxygenase activity.

Strain	Specific activity (units/mg)
<i>E. coli</i> N-4830 untransformed	ND*
Transformant 1 (N-4830 + p λ 1-cyt)	ND
Transformant 2 (N-4830 + pK λ -12)	0.061
Transformant 3 (N-4830 + pK λ -12)	0.089
<i>E. coli</i> TB1 untransformed	ND
Transformant 4 (TB1 + pKB-10)	0.05
Transformant 5 (TB1 + pKB-10)	0.045
<i>Alcaligenes</i> sp. 4HAP	0.064

* ND, not detected.

cloned by insertion into pGEM-T Easy, which was used to transform *E. coli* XL1-Blue. The sequence encoded a polypeptide of 170 amino acids with N- and C-termini matching the sequences on which the primers were designed and which included the sequence of the internal tryptic peptide from the enzyme (Figure 2).

Further verification that this gene codes for the dioxygenase came from its expression in *E. coli*. However, the PCR product lacked the 5' proximal region that codes for the N-terminal hexapeptide and, therefore, the complete gene was re-synthesized by PCR by using primer combinations E and F with the template that had been cloned in pGEM-T Easy. The 5' proximal region of the forward primer E contained an optimized translational start signal, including the Shine-Dalgarno sequence, the space region and the initiator methionine codon, as well as a sequence encoding the missing hexapeptide. The amplified PCR product was cleaved with *Pst*I and *Eco*RI and cloned into (i) the polylinker site of pBluescript SK⁺, under the transcriptional control of the *lac* promoter and (ii) p λ -1cyt under the transcriptional control of the powerful λ_{PL} promoter (Table 1). The recombinant plasmids pKB-10 and pK λ -12 were introduced into *E. coli* strains TB1 and the *cI857*-repressor bearing N-4830-1 respectively. After induction of both of the strain lines, the cell extract contained significant dioxygenase activity (Table 4) and SDS/PAGE of the extract showed the presence of a new protein of about M_r 20 000 not present in controls.

DISCUSSION

Growth of *Alcaligenes* sp. 4HAP on 4-hydroxyacetophenone as its sole carbon source induced the enzyme 2,4'-dihydroxyacetophenone dioxygenase [2], which was purified in good yield (Table 3). It makes up about 2% of the soluble protein in the cell, as indicated by the 45-fold purification to homogeneity. A comparison of the M_r value of the native enzyme with that of the subunit suggested that the enzyme is a tetramer with four equal-sized subunits. The M_r values obtained for the native protein, by gel filtration or by ultracentrifugation, ranged from 81 600 to 87 000, whereas the value from SDS/PAGE was about 21 000. This subunit value was close to that of 20 379 given by electrospray MS, which itself is in good agreement with the value of 20 364 calculated from the gene-derived protein sequence. The findings, that the terminal sequencing of the protein gave clean sequences and that expression of the gene for the single peptide

gave active enzyme, indicate that the subunits are identical and the enzyme is a homotetramer. The two bands seen for the pure enzyme on SDS/PAGE when 2-mercaptoethanol is omitted from the denaturing buffer (Figure 1) suggest that some of the subunits have an internal disulphide bridge. The sequence reveals that there are two cysteine residues in the peptide (Figure 2), and presumably these must be close enough in the tertiary structure to form a bridge. However, it is not yet clear if this is important for the structure and activity of the enzyme.

A feature of many of the ring-fission dioxygenases is that they contain non-haem iron at the active site, and this is essential in the catalytic cycle. In some, the *ortho*-cleaving or intradiol dioxygenases, this gives a red coloration to the enzyme and the iron is present as Fe³⁺, whereas others, the *meta*-cleaving or extradiol enzymes, still contain non-haem iron, as Fe²⁺, but are colourless [16]. 2,4'-Dihydroxyacetophenone dioxygenase is colourless but, when assayed for iron, was shown to contain one atom of iron per molecule of enzyme (tetramer). This is tightly bound and is not lost during purification or on prolonged dialysis. The oxidation state of the iron has not yet been determined and it has not yet been shown to participate in catalysis. However, by analogy with other dioxygenases, it is likely to play a role in the catalytic process, either by activation of oxygen or organic substrate.

The gene for the dioxygenase was isolated by PCR using primers based on the amino acid sequences found by N- and C-terminal sequencing of the purified protein. The gene was cloned and sequenced, and several pieces of evidence identify it as the gene coding for 2,4'-dihydroxyacetophenone dioxygenase. The derived amino acid sequence contains the correct sequence for an internal tryptic peptide isolated from the purified enzyme and also corresponds with the part of the N-terminal sequence not used in primer design as well as that upon which the primers were based. The calculated M_r of the gene-derived protein was 20 367, which is close to that found by SDS/PAGE or from electrospray MS. Finally, expression of the gene in *E. coli* gave rise to dioxygenase activity with a specific activity similar to that found in *Alcaligenes* sp. 4HAP (Table 4), and an additional band, of the correct size, on SDS/PAGE of crude extract.

Although 2,4'-dihydroxyacetophenone dioxygenase resembles the ring-fission dioxygenases in that it contains iron, a comparison of its peptide sequence with those of known proteins in the databases using the BLAST program [17] did not reveal significant sequence similarity with any oxygenase enzyme. Indeed, no strong similarity with any other protein was found. It appears, therefore, that this enzyme is unusual, not only in its reaction, but also in its amino acid sequence, and it represents the first example of a novel class of protein.

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