2-Phenylethylamine catabolism by *Escherichia coli* K-12: gene organization and expression

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**A gene encoding phenylacetaldehyde dehydrogenase (PAD), the enzyme involved together with a copper-topaquinone-containing amine oxidase in the initial steps of 2-phenylethylamine catabolism, was located at 31-1 min on the *Escherichia coli* K-12 genetic map. It was immediately adjacent to the gene encoding the amine oxidase but transcribed in the opposite direction. The purified PAD acted almost equally well on phenylacetaldehyde, 4-hydroxyphenylacetaldehyde and 3,4-dihydroxyphenylacetaldehyde. It had a subunit size of 54 kDa and its deduced amino acid sequence was approximately 40% identical to various eukaryotic and prokaryotic aldehyde dehydrogenases. A third gene encoding a positive regulatory protein required for expression of the amine oxidase and PAD genes was located next to the PAD gene. A gene previously located in this position was reported to encode a second amine oxidase but this was not confirmed. The nucleotide sequence from 1447 to 1450 kb on the *E. coli* K-12 physical map has been determined.**

**Keywords: Escherichia coli, amine oxidase, aldehyde dehydrogenase, regulator gene**

**INTRODUCTION**

We have previously described the reactions whereby *Escherichia coli* K-12 converts 2-phenylethylamine to phenylacetic acid through the sequential action of an amine oxidase and an aldehyde dehydrogenase (Parrott *et al.*, 1987). The amine oxidase is of particular interest because it is a member of the copper-topaquinone family of amine oxidases (Cooper *et al.*, 1992) whose organic co-factor is formed autocatalytically (Matsuzaki *et al.*, 1994; Hanlon *et al.*, 1995). Recently, a second amine oxidase gene was reported to be located very close to the gene encoding the 2-phenylethylamine oxidase (PEO) (Azakami *et al.*, 1994).

To see whether this putative second amine oxidase also contained copper and topaquinone, we have studied the region of the *E. coli* K-12 genome adjacent to the PEO gene. Our results show that the region reported to encode the second amine oxidase actually encodes a regulatory protein that acts positively on the genes encoding PEO and phenylacetaldehyde dehydrogenase (PAD).

**METHODS**

**Plasmids, bacterial strains and growth conditions.** Plasmid pJPB13 carrying PstI fragment P41f (Bouché, 1982; Fig. 1) in pBR325 was kindly provided by Dr J. P. Bouché (CNRS, Toulouse, France). pUC18/19 (Norrander *et al.*, 1983) were used as vectors for DNA cloning and expression with *E. coli* K-12 strains K10 (Hfr PO2A pit-10 relA1 tonA22 Tt; Bachmann, 1972) and JM109 (F recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi A(lac–proAB) [F traD36, proAB lacPZAM15]; Yanisch-Perron *et al.*, 1985) as hosts. *E. coli* strain C (CGSC 3121; i- ) was kindly provided by Dr B. J. Bachmann (CGSC, Yale University, USA). Cells were grown on M63 minimal medium (Miller, 1972) with 15 mM succinate as carbon source and supplemented with ampicillin (100 μg ml⁻¹). 2-Phenylethylamine (5 mM) or IPTG (0·5 mM) was incorporated as inducer as required.

**Enzyme assays.** Methods for the preparation of cell-free extracts and the continuous assay of amine oxidase (EC 1.4.3.6) by measurement of phenylacetaldehyde production and PAD (EC 1.2.1.39) by NADH formation were as described previously (Parrott *et al.*, 1987). Protein was measured using the Folin reagent with bovine serum albumin as standard (Layne, 1957).

**Purification of PAD.** Succinate-grown JM109(pRC1948) cells (100 ml culture) were harvested at OD₆₅₀ 0·6 by centrifugation...
at 10000 g for 5 min at 4 °C and the pellet resuspended in 4 ml 20 mM Tris/HCl buffer, pH 6-8, containing 10% glycerol and 0.5 mM DTT (extraction buffer). The cells were broken by 30 s treatment at 0 °C in a MSE Soniprep 150 ultrasonicator operating at 10 μm amplitude and the crude extract ultracentrifuged at 180000 g for 90 min at 4 °C. For purification by FPLC at 22 °C, 3 ml supernatant (8 mg protein) was applied to a Mono Q HR 5/5 anion exchange column equilibrated with the extraction buffer. A 15 ml gradient of 0-1.0 M NaCl in extraction buffer at a flow rate of 1 ml min⁻¹ was used as eluant and 1 ml fractions collected. PAD was eluted at 0.25 M NaCl. The peak fraction was applied to a Phenyl-Superose HR 5/5 column equilibrated with extraction buffer containing 1 M NaCl. The NaCl concentration was reduced to zero over a 10 ml gradient at a flow rate of 0.5 ml min⁻¹ and 1 ml fractions collected. PAD was eluted after the end of the gradient. Details of the purification are given in Table 1.

Molecular mass estimations. Subunit molecular mass was estimated by SDS-PAGE (Laemmli, 1970) calibrated with bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine carboxylic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa) and bovine milk α-lactalbumin (14.2 kDa).

N-terminal sequencing. The purified PAD was run on a 7.5–20% SDS-polyacrylamide gel, then electrophoretically transferred onto a PVDF membrane (Matsudaira, 1987) using 50 mM glycine/50 mM Tris, pH 10, and stained with Coomassie brilliant blue R-250. A portion of the stained 54 kDa protein was excised and loaded into an Applied Biosystems 470A gas-phase sequencer without polybrene.

DNA manipulations. Small-scale plasmid preparations were carried out as described by Sambrook et al. (1989). Restriction endonucleases, calf intestinal phosphatase and T4 DNA ligase were used according to the manufacturer’s instructions. DNA fragments were isolated from agarose gels using a JETsorb kit (GENOMED Inc., NC 27700-4526). Transformations were carried out using MOPS-RbCl (Kusher, 1978).

Nucleotide sequencing and oligodeoxiribonucleotide synthesis. Plasmid DNA was prepared using the Wizard kit (Promega). Sequencing of both DNA strands was carried out using a Sequenase version 2.0 kit (USB) employing, initially, the pUC18/19 forward and reverse primers. Sequences were extended by designing further (‘walking’) primers based on the already determined sequence. These oligodeoxiribonucleotide primers were made with an Applied Biosystems model 380B DNA synthesizer using cyanoethylphosphoramidite chemistry.

Biochemicals and chemicals. Restriction endonucleases, calf intestinal phosphatase and T4 DNA ligase were from Pharmacia. [35S]dATPaS (1000 Ci mol⁻¹) was from Amersham. 2-Phenyethylamine, tyramine (4-hydroxyphenyl-ethylamine), dopamine (3,4-dihydroxyphenyl-ethylamine) and phenylacetaldehyde were from Sigma. Solutions of phenylacetaldehyde, 4-hydroxyphenylacetaldehyde and 3,4-dihydroxyphenyl-acetaldehyde (approximately 1 mM) were prepared from the corresponding amines, using amine oxidase from the periplasm fraction of JM109(pKC13) (Hanlon et al., 1995). The formation of aldehyde was monitored using PAD and on completion the reaction mixtures were deproteinized by ultrafiltration. All other chemicals were of analytical grade.

Computer analysis. Comparisons of deduced amino acid sequences were made using the GAP program (Genetics Computer Group, 1991). International databases were searched using the basic local alignment search tool (BLAST) program (Altschul et al., 1990). The enzyme kinetic data were analysed via the Michaelis–Menten equation using a custom-written non-linear least squares-fitting algorithm.

RESULTS AND DISCUSSION

Location of the PAD and PEO genes

pJPB13 with a 9 kb PstI fragment (Fig. 1) from 1445–1454 kb on the E. coli K-12 physical map (Berlyn et al., 1996) includes the gene for PEO (Hanlon et al., 1995). A 5 kb EcoRI (1453.4 kb)-PvuII (1448.4 kb) subclone in

![Fig. 1. Partial restriction site map of pJPB13 and derived subclones. Only the cloned DNA is shown. The scale gives the corresponding region of the E. coli K-12 physical map (Berlyn et al., 1996). The locations of the 2-phenylethylamine (fea) catabolic genes are shown, with an arrow indicating the direction of transcription; feaA encodes the positively acting regulator involved in the expression of feaA (PEO) and feaB (PAD).](514)
pUC18 still expressed the amine oxidase gene in response to IPTG (specific activity 0.4 U (mg protein)^{-1}) but when the orientation with respect to the vector promoter was reversed by insertion into pUC19, the amine oxidase gene was barely expressed (specific activity 0.004 U (mg protein)^{-1}). This indicated that the direction of transcription of the amine oxidase gene was in the EcoRI→PvuII direction. When the same extracts were tested for PAD, the activity was barely detectable when pUC18 was the vector (specific activity 0.02 U (mg protein)^{-1}) but was present at very high activity for the pUC19-based construct (specific activity 3.2 U (mg protein)^{-1}). This latter construct has been designated pRC1948 (Fig. 1). Thus this 3 kb fragment encoded both enzymes for conversion of 2-phenylethylamine to phenylacetic acid but apparently the two genes were transcribed in opposing directions.

**PAD N-terminal amino acid sequence and activity against related compounds**

The NAD-dependent PAD purified 10-fold from JM109(pRC1948) showed a single band on SDS-PAGE that corresponded to a molecular mass of 54 kDa and was at least 95% pure. The N-terminal sequence of this 54 kDa protein was found to be: Met-Thr-Glu-Pro-His-Val-Ala-Val-Leu.

The purified enzyme reacted readily with either phenylacetaldehyde, 4-hydroxyphenylacetaldehyde or 3,4-dihydroxyphenylacetaldehyde as co-substrate, but all showed inhibition at concentrations over 10 μM, as noted previously for PAD from Achromobacter eurydice (Fujioka et al., 1970). At aldehyde concentrations around 10 μM, needed to determine K_m and V_max values, the allowable ΔA_{349} changes were very small and consequently the experimental error was quite high. The kinetic values were measured using five aldehyde concentrations over the range 2–20 μM at 275 μM NAD. The K_m and V_max values, with standard errors, representative of results seen from three separate experiments were 7 ± 1 μM and 38 ± 2 U (mg protein)^{-1} for phenylacetaldehyde; 4 ± 1 μM and 30 ± 2 U (mg protein)^{-1} for 4-hydroxyphenylacetaldehyde; and 6 ± 1 μM and 32 ± 2 U (mg protein)^{-1} for 3,4-dihydroxyphenylacetaldehyde, respectively.

**Nucleotide sequence determination**

Nucleotide sequence from both ends of the 5 kb insert DNA of pRC1948 was determined to confirm the direction of transcription of the amine oxidase and aldehyde dehydrogenase genes. The amine oxidase gene (maoA) has been sequenced (Azakami et al., 1994; Parsons et al., 1995) and we located the 5' end of this sequence at the EcoRI end of the insert DNA (data not shown). Nucleotide sequence starting 130 bp from the EcoRI-PvuII direction. When the same extracts were tested for PAD, the activity was barely detectable when pUC18 was the vector (specific activity 0.02 U (mg protein)^{-1}) but was present at very high activity for the pUC19-based construct (specific activity 3.2 U (mg protein)^{-1}). This latter construct has been designated pRC1948 (Fig. 1). Thus this 3 kb fragment encoded both enzymes for conversion of 2-phenylethylamine to phenylacetic acid but apparently the two genes were transcribed in opposing directions.

The nucleotide sequence of the 1.2 kb EcoRV-PvuII region, which is immediately adjacent to the region encoding the PAD gene, was determined. This sequence showed unique NciI and SmaI restriction endonuclease sites located centrally within the cloned DNA. These were used to create a 219 bp deletion by digesting pMX2 with the two endonucleases, filling in the NcoI sticky ends and religating the DNA. This new construct, designated pMX2D, no longer led to expression of the maoA gene. It is possible that this same system, on the opposite strand, also serves as a transcription terminator for the PAD gene.

The amino acid translation of the PAD-encoding gene was compared to the databases using the BLAST procedure (Altschul et al., 1990). The evolutionary relatedness of many NAD(P)-linked aldehyde dehydrogenases has been documented (Hempel et al., 1993) and the significant amino acid sequence identity (35–40%) found between PAD and very many such aldehyde dehydrogenases from a variety of prokaryotic and eukaryotic sources indicated that it too was a member of that family of proteins. Surprisingly, 359 bp of the nucleotide sequence, which is downstream of the maoA gene, was identical to part of the untranslated sequence reported by Azakami et al. (1994) to be upstream of the maoA gene, with the match ending at a HindIII site (1449-4 kb, Fig. 1). The amino acid translation of this matching region also showed strong identity with various NAD(P)-linked aldehyde dehydrogenases.

**Identification of the putative second amine oxidase gene as a regulatory gene**

It had been claimed that a gene encoding a second amine oxidase was located close to the amine-oxidase-encoding maoA (Azakami et al., 1994). To investigate this claim the EcoRV (1447-2 kb)–PvuII (1448-4 kb) region of pJPB13 was subcloned into the SmaI site of pUC18. JM109 with one such construct, pMX1, showed at least fourfold higher amine oxidase activity than JM109(pUC18). 2-Phenylethylamine caused a 20-fold increase in amine oxidase activity but IPTG had no effect (Table 2). A second construct, pMX2 (Fig. 1), with the insert DNA in the opposite orientation to that of pMX1 also showed a 20-fold increase in amine oxidase activity in response to 2-phenylethylamine. However, for pMX2, IPTG now led to a 30-fold increase in amine oxidase activity. IPTG had no effect on amine oxidase production by JM109(pUC18). These results suggested that in pMX2 a gene responsible for increased amine oxidase activity was transcribed in the same direction as the pUC18 lac promoter.

The nucleotide sequence of the 1.2 kb EcoRV–PvuII region, which is immediately adjacent to the region encoding the PAD gene, was determined. This sequence showed unique NciI and SmaI restriction endonuclease sites located centrally within the cloned DNA. These were used to create a 219 bp deletion by digesting pMX2 with the two endonucleases, filling in the NcoI sticky ends and religating the DNA. This new construct, designated pMX2D, no longer led to expression of the amine oxidase in JM109 on induction with IPTG, indicating that the gene responsible had been disrupted. Analysis of the nucleotide sequence revealed an ORF of 903 bp and the deduced amino acid sequence of this putative gene showed no significant identity with any...
Table 2. Influence of various plasmids on amine oxidase and PAD activities produced by *E. coli* K-12 strains JM109 and K10

Cells were grown at 30°C on succinate minimal medium with ampicillin and induced for two to three cell doublings. Specific activities [U (mg protein)^{-1}] are representative values from a minimum of three separate estimations. Plasmids are described in the text, ND, not determined.

<table>
<thead>
<tr>
<th>Host strain</th>
<th>Inducer</th>
<th>Specific activity [U (mg protein)^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amine oxidase</td>
<td>PAD</td>
</tr>
<tr>
<td></td>
<td>pUC18</td>
<td>pMX1</td>
</tr>
<tr>
<td>JM109</td>
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<tr>
<td>JM109</td>
<td>IPTG</td>
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<tr>
<td>JM109</td>
<td>Phenylethylamine</td>
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<tr>
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<tr>
<td>K10</td>
<td>IPTG</td>
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![Fig. 2. C-terminal amino acid sequence comparison for FeaR.](image)

The C-terminal region of the 2-phenylethylamine catabolism regulatory protein (FeaR) was matched with a selection of regulatory proteins from the AraC/XylS family. The fully conserved residues are shown in bold. The numbers indicate the residue of these approximately 300 residue proteins at which the sequence comparison starts. The alignment was carried out using the PILEUP program (Genetics Computer Group, 1991) with Gap Weight 3.0 and Gap Length Weight 0.1 settings. The sequence accession numbers are: FeaR, EMBL/GenBank X99402; XylS, SWISS-PROT P07859; RhaR, SWISS-PROT P09378; RhaS, SWISS-PROT P09377; AraC, SWISS-PROT P03021; MelR, SWISS-PROT P10411. Ec, *E. coli*; Pp, *Pseudomonas putida*.

amine oxidase sequence in the databases. However, there was some identity at its C-terminal end with members of the AraC/XylS class of transcriptional activators (Gallegos et al., 1993) as shown in Fig. 2.

**Nature of the amine oxidase elicited by pMX2 and identity of the encoded gene**

The amine oxidase produced by JM109(pMX2) was purified from the periplasmic fraction, as described previously (Cooper et al., 1992). The protein obtained was greater than 95% pure, as judged by SDS-PAGE, corresponded to a protein of molecular mass 80 kDa and was a quinoprotein, as measured by the redox cycling staining method (Paz et al., 1991).

Its N-terminal amino acid sequence was found to be His-Gly-Gly-Glu-Ala-His-Met-Val-Pro-Met which is identical to the product of the *maoA* gene (Azakami et al., 1994). Since a protein of 80 kDa could not be encoded on a 1.2 kb piece of DNA that apparently was responsible for its production and its 10 N-terminal amino acids were identical to those of PEO, it seemed that the *PvuII*-EcoRV fragment did not itself encode an amine oxidase but carried a regulatory gene whose product acted positively on PEO gene expression.

Further support for this view was obtained by use of *E. coli* strain C as host for pMX2. This bacterium lacks the *maoA* gene (R. A. Cooper, unpublished observation) and extracts prepared from *E. coli* C(pMX2) had no detectable amine oxidase activity.

**Control of PAD gene expression**

Both the amine oxidase and the aldehyde dehydrogenase were formed inducibly in *E. coli* K-12 strain K10 (Parrott et al., 1987). It seemed likely, therefore, that the regulatory protein that elicited amine oxidase production would also control formation of PAD. To test this possibility pMX2 was introduced into *E. coli* K10 and PAD and amine oxidase activities measured. Table 2 shows that both enzymes were present at high activities in uninduced cells and were not increased significantly by growth in the presence of IPTG. However, strain K10 with pUC18 displayed no detectable amine oxidase or PAD activities, even for cells induced with IPTG (Table 2). This indicates that the regulatory gene product was necessary for expression of the PAD gene. The apparent constitutive expression elicited by pMX2 in strain K10 is explained by the fact that K10 carries the normal *lacI* gene whose product would be unable to interact effectively with the regu-
Aromatic amine catabolism genes of E. coli K-12

Gene symbol

The gene symbol mao (monoamine oxidase) that has been used for the PEO gene (Azakami et al., 1994) is inappropriate for the PAD gene which is the second component of the E. coli K-12 system for conversion of 2-phenylethylamine to phenylacetic acid. Accordingly, we propose the gene symbol fea for 2-phenylethylamine catabolism (pea is not available) with the PEO gene being feaA, the PAD gene being feaB and the regulatory gene being feaR. These three genes are located on the E. coli K-12 physical map between the EcoRV site at coordinate 1447 kb and the HindIII site at coordinate 1453 kb (Kohara et al., 1987). This position corresponds to 311-312 min on the E. coli K-12 genetic map (Berlyn et al., 1996).

Conclusions

E. coli K-12 can use phenylacetic acid but not 3- or 4-hydroxyphenylacetic acid as carbon and energy source, whereas some other strains can grow on both aromatic acids (Burlingame & Chapman, 1983). A direct consequence of this for E. coli K-12 is that compounds such as 2-phenylethylamine that are converted to phenylacetic acid (Parrott et al., 1987) can serve as growth substrate but compounds such as tyramine that are converted to 4-hydroxyphenylacetic acid cannot. However, since the amine oxidase and aldehyde dehydrogenase that are needed for growth on 2-phenylethylamine also convert tyramine to 4-hydroxyphenylacetic acid (Parrott et al., 1987), tyramine can serve as nitrogen source for growth of E. coli K-12. Because of their interest in tyramine catabolism by Klebsiella aerogenes, Murooka and colleagues, in studying the aromatic amine oxidase of E. coli K-12, have used tyramine rather than 2-phenylethylamine as inducer and substrate (Yamashita et al., 1993). Although they stated that the precise role of the monoamine oxidase in E. coli is unknown, it has been demonstrated previously by mutant analysis that it is required for growth on 2-phenylethylamine (Parrott et al., 1987) and presumably also for use of tyramine as nitrogen source. In most, if not all, situations concerning their initial degradation, Zphenylethylamine and tyramine are converted to phenylacetic acid but compounds such as tyramine that are converted to 4-hydroxyphenylacetic acid cannot. However, since the amine oxidase and aldehyde dehydrogenase that are needed for growth on 2-phenylethylamine also convert tyramine to 4-hydroxyphenylacetic acid (Parrott et al., 1987), tyramine can serve as nitrogen source for growth of E. coli K-12.

What is described here on the identification of the putative second amine oxidase as a regulator gene is in agreement with the very recent revision of their claim (Yamashita et al., 1996). We also, importantly, show that the regulatory protein controls expression of the PAD gene. However, some differences between these two reports remain to be resolved. For instance, Yamashita et al. (1996) concluded that both the regulatory protein and an aromatic amine (tyramine) were necessary for expression of the amine oxidase gene, whereas our results with pMX2 showed that the regulator gene induced by IPTG resulted in higher amine oxidase activity than when 2-phenylethylamine was the inducer. Hence, it seemed that only the regulator gene product was needed for amine oxidase gene expression.

The sequence of the regulator gene described by Yamashita et al. (1996) was identical to that which we report here. It is not known whether the K-12 strain, W3110, the source of the DNA they analysed, has a PAD gene but the finding of some sequence upstream of the amine oxidase gene (Azakami et al., 1994) matching part of the PAD gene was unexpected.

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REFERENCES


