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 *Pst*I fragment P41I (Bouché, 1982; Fig. 1) in pBR325 was kindly provided by Dr J. P. Bouché (CNRS, Toulouse, France). pUC18/19 (Norlander *et al.*, 1983) were used as vectors for DNA cloning and expression with *E. coli* K-12 strains K10 (*Hfr* PO2A *pit*-10 *relA1* *spoT1* *tonA22* T₂^R; Bachmann, 1972) and JM109 (F⁻ *recA1* *supE44* *endA1* *hsdR17* *gyrA96* *relA1* *thi* Δ (*lac*-*proAB*) [*F'* *traD36*, *proAB* *lacI*^qZ Δ M15]; Yanisch-Perron *et al.*, 1985) as hosts. *E. coli* strain C (CGSC 3121; λ^-) 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 μ g 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 OD₆₈₀ 0·6 by centrifugation

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Abbreviation: PAD, phenylacetaldehyde dehydrogenase; PEO, 2-phenylethylamine oxidase.

The SWISS-PROT accession number for the phenylacetaldehyde dehydrogenase N-terminal amino acid sequence reported in this paper is P80668. The GenBank/EMBL accession number for the nucleotide sequence reported in this paper is X99402.

Table 1. Purification of PAD

Details of the purification procedures are given in the text. One unit (U) of enzyme activity is defined as the amount of enzyme required to transform 1 μ mol substrate (min)⁻¹.

Purification step	Volume (ml)	Total protein (mg)	Total enzyme (U)	Specific activity [U (mg protein) ⁻¹]	Recovery (%)	Purification (-fold)
Ultracentrifuged crude extract	3.0	8.0	28.0	3.5	100	1
Mono Q peak fraction	1.0	1.2	19.4	16.2	69	4.6
Phenyl-Superoose peak fraction	1.0	0.3	12.2	41.0	44	11.7

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 μ m amplitude and the crude extract ultracentrifuged at 180000 *g* for 90 min at 4 °C. For purification by FPLC at 22 °C, 3.0 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-Superoose 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 carbonic 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 electroblotted 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 277009-4526). Transformations were carried out using MOPS-RbCl (Kushner, 1978).

Nucleotide sequencing and oligodeoxyribonucleotide 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 oligodeoxyribonucleotide primers were made with an Applied Biosystems model 380B DNA synthesizer using cyanoethyl-phosphoramidite chemistry.

Biochemicals and chemicals. Restriction endonucleases, calf intestinal phosphatase and T4 DNA ligase were from

Pharmacia. [³⁵S]dATP α S (1000 Ci mol⁻¹) was from Amersham. 2-Phenylethylamine, tyramine (4-hydroxyphenylethylamine), dopamine (3,4-dihydroxyphenylethylamine) and phenylacetaldehyde were from Sigma. Solutions of phenylacetaldehyde, 4-hydroxyphenylacetaldehyde and 3,4-dihydroxyphenylacetaldehyde (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 mixes 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 *Pst*I 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 *Eco*RI (1453.4 kb)-*Pvu*II (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; *feaR* encodes the positively acting regulator involved in the expression of *feaA* (PEO) and *feaB* (PAD).

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 5 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 A_{340} 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 50 ± 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 *PvuII* site gave translated amino acid sequence that matched exactly the protein N-terminal amino acid sequence reported in the preceding section. This identification of the 5' end of the PAD gene confirms the direction of transcription suggested from the expression

studies. The stem-loop system proposed to act as a transcription terminator for the *maoA* gene was 1611 bp from the *PvuII* site, close to the putative end of the PAD 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 *NcoI* and *SnaBI* 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.

Host strain	Inducer	Specific activity [U (mg protein) ⁻¹]				
		Amine oxidase			PAD	
		pUC18	pMX1	pMX2	pUC18	pMX2
JM109	None	< 0.001	0.004	0.004	ND	ND
JM109	IPTG	< 0.001	0.003	0.125	ND	ND
JM109	Phenylethylamine	0.003	0.075	0.083	ND	ND
K10	None	< 0.001	ND	0.470	< 0.001	0.320
K10	IPTG	< 0.001	ND	0.420	< 0.001	0.330

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FeaR-Ec 249 RLDFCADAI RHA .ADDEKLAGIGFHWGFSDQSHFSTVFKQRFGMTFPGEYRRKFR.....
XylS-Pp 284 KLESTRACLN DPSANVRSITEIALDYGFLHLGRFAENYRSAFGELPSDTLRQCKKEVA.....
RhaR-Ec 259 RVCHAQYLLQHSRL...LISDISTECGFEDSNYFSVVFTRETGMTPSQWRHLNSQKD.....
RhaS-Ec 224 RLMKARHLLRHSEA...SVTDIAYRCGFSDSNHFSTLFRREFNWSP...RDIRQGRDGFLQ....
AraC-Ec 231 RISQAKLLLSSTRM...PIATVGRNVGFDDQLYFSRVFKCTGASPSEFFRAGCEEKVNDVAVKLS
MelR-Ec 244 RINHVRALLSDTDK...SILDIALTAGFRSSSRFYSTFGKYVGMSPQQYRKLSQQRRTFPG...

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Fig. 2. C-terminal amino acid sequence comparison for FeaR. 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-

latory regions of all the copies of the pMX2 plasmid, thereby leading to constitutive production of the regulatory gene product due to expression via the thus unregulated *lac* promoter.

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 31.1–31.2 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, 2-phenylethylamine and tyramine can be considered to be identical, so the results reported here focusing on 2-phenylethylamine can be compared directly with those of Murooka and colleagues employing tyramine.

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 regu-

latory 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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