**xyLUW**, two genes at the start of the upper pathway operon of TOL plasmid pWW0, appear to play no essential part in determining its catabolic phenotype

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The upper pathway operon of the toluene catabolic pathway of TOL plasmid pWW0 was shown to carry two open reading frames between the start of transcription and *xylC* (encoding benzaldehyde dehydrogenase), the first previously reported gene of the operon. These were designated *xyLUW*: *xylU* encoded a protein of 131 amino acid residues (*M*, 14244) which bore no relationship with any protein in the databases, and *xylW* encoded a protein of 348 residues (*M*, 36 992) which was strongly homologous to other long-chain Zn-containing alcohol dehydrogenases. Extracts of *Escherichia coli* carrying *xyLUW* in expression vector pTrc99A contained a novel protein corresponding to *XyIW*, but no NAD+-dependent dehydrogenase activity against benzyl alcohol, mandelate or benzylamine. A mini-Tn5 transposon carrying the meta pathway operon was constructed and from it two strains of *Pseudomonas putida* were constructed with the normally plasmid-encoded catabolic operons integrated into the chromosome. Three derivatives of plasmid pKNG101 containing modified *xyLUW* genes were constructed, two of which had frameshifts in *xylU* and *xylW*, respectively, and a third with a deletion from the 3′ end of *xylU* into the 5′ end of *xylW*. The wild-type genes of the two *Pseudomonas* strains were substituted by the mutant alleles by reverse genetics. The ability of the constructed mutant strains to utilize the aromatic substrates of the TOL pathway was not significantly affected.

Keywords: TOL plasmid pWW0, *xyl* genes, toluene/xylene catabolism, *Pseudomonas putida* strain mt-2

**INTRODUCTION**

The TOL plasmids encode the catabolism of toluene and some alkyltoluenes (Williams & Murray, 1974; Worsey & Williams, 1975; Kunz & Chapman, 1981) to benzoates and the alkylbenzoates, which are then further metabolized via catechols and meta (extradiol) ring cleavage to metabolites of the tricarboxylic acid cycle (Murray et al., 1972). The genes are organized in two regulons, the 'upper pathway' operon encoding the enzymes for conversion of the hydrocarbons to benzoates (Harayama et al., 1986, 1989) and the 'meta pathway' operon *xylXYZLTEGFQKZJH* (Harayama & Rekik, 1990) encoding the enzymes for further conversion of the benzoates. Until recently the first operon was reported to consist of the genes *xylC* (for benzaldehyde dehydrogenase) (Lebens & Williams, 1985; Shaw & Harayama, 1990; Inoue et al., 1995), *xylMA* (for the two components of xylene monooxygenase) (Suzuki et al., 1991; Shaw & Harayama, 1995), *xylB* (for benzyl alcohol dehydrogenase, BADH) (Inouye et al., 1981; Shaw & Harayama, 1990; Shaw et al., 1993) and *xylN*, an open reading frame encoding a protein of
unknown function (Harayama et al., 1989). The xyl-CMAB region apparently encodes all the enzymic apparatus necessary for the conversion of the hydrocarbons to benzoates. However, the regulatory signals for expression of the operon and the start of transcription (Inouye et al., 1983, 1984, 1990) were recognized as being considerably (1-2 kbp) upstream of the start codon of xylC (Lebens & Williams, 1985) but no function had been assigned to the intervening DNA. In this paper we report its nucleotide sequence and describe experiments designed to determine the role of the open reading frames in the catabolism of toluene and its metabolites.

METHODS

Bacterial strains and plasmids. Escherichia coli strains were grown in liquid culture on Luria broth (LB) and on plates of Isosensitest agar to which antibiotics were added, as appropriate, at the following concentrations (µg ml⁻¹): ampicillin (100), kanamycin (75). pUC plasmids were maintained in E. coli strain NM322. Expression plasmid pTrc99A was purchased from Pharmacia. Pseudomonas strains were all derivatives of P. putida mt-2, the original host of TOL plasmid pWW0, and were grown on minimal salts medium with carbon sources added at 5 mM for aromatics and 10 mM for succinate. Growth on toluene, m-xylene and p-xylene was chased from Pharmacia.

Transfer of plasmids to P. putida from E. coli. Derivatives of pUT and pKNG101 plasmids were transformed into E. coli strains S17-1pir (De Lorenzo & Timmis, 1994) and SM10pir (Miller & Mekalanos, 1988) as respective donors and into CC118pir (Herrero et al., 1990) for maintenance. Donor and recipient cultures were grown overnight in 5 ml LB with appropriate antibiotic. Then 1 ml of each was harvested, resuspended in 1 ml sterile minimal medium and 10 µl of each was mixed on an LB agar plate and incubated overnight. The resultant patch of cells was resuspended in 1 ml sterile minimal medium and 100 µl was spread on to appropriate selection medium for single colonies of transconjugants.

Insertion of the meta pathway into chromosome of P. putida. The 17.5 kbp HindIII insert (HA) was cut from plasmid pWW53-3301 (Keil et al., 1987) with HindIII and inserted into the HindIII site of pUC1868 (Herrero et al., 1990) to form pUC1856HA. HA carries the complete meta pathway operon plus its positive regulator gene xylR from TOL plasmid pWW53 (Keil et al., 1987). Since HA has four internal Sphi sites it was necessary to remove the insert with AvrII which cuts the two Sphi sites on the pUC1856HA plasmid but does not cut within the HA fragment. Plasmid pUTKm2 is a mini-Tn5 carrier plasmid (De Lorenzo et al., 1990; De Lorenzo & Timmis, 1994) in which the kanamycin resistance gene has been inserted into the mini-transposon as an Sphi fragment and is excisable with AvrII. Plasmids pUC1856HA and pUTKm2 were both digested with AvrII and the mixture ligated to form pUTHA, in which HA was inserted within the mini-Tn5 transposition in place of the kanamycin resistance gene. This plasmid was transformed into E. coli CC118pir and, for delivery to P. putida hosts, into S17-1pir. Strain S17-1pir(pUTHA) was mated with P. putida KT2442A (a gift from V. de Lorenzo) as recipient, selecting for growth on m-toluol minimal medium: KT2442A has a mini-Tn5 insertion in its chromosome which carries both the xylUAWMABN operon and its positive regulator gene xylR. A single transconjugant, PaW140, was retained. With its chromosomal insertion of the TOL upper pathway operon, strain KT2442AΔ was only able to grow on toluene, benzyl alcohol, benzaldehyde and benzoate, which are further metabolized by the chromosomal β-ketoadiapate pathway. However, PaW140, with the additional complete meta pathway operon in its chromosome, was additionally able to grow on the 3-methyl and 4-methyl analogues, which can be metabolized via the meta pathway.

Construction of mutations within the xyl/UW region. Plasmid pWW0-7000 (Fig. 1) was cut at its unique XhoI site within xylU and the recessed 3’ ends were filled in with Klenow enzyme to produce blunt ends which were religated to obtain plasmid pWW0-7010 (Fig. 1) carrying the mutated gene designated xylU*. Plasmid pWW0-7001 was made by insertion of the 2.05 kbp Smal-BamHI fragment from pWW0-7000 into expression vector pTrc99A cut with EcoI CR1 (at its SalI site) and BamHI. The single ClaI site in xyl/W on plasmid pWW0-7001 was removed by cutting with ClaI, filling in with Klenow and religating to produce pWW0-7011 carrying the mutated allele xyl/W* (Fig. 1). xyl/W* was then removed from pWW0-7011 on a 2.6 kbp BstEII-BamHI fragment and cloned into the equivalent BstEII-BamHI sites on pWW0-7000 to generate pWW0-7012 (Fig. 1). Both pWW0-7011 and pWW0-7012 were cut with Smal and BamHI and the 2-8 kbp fragments from each were separately ligated into the Smal–BamHI sites of pKNG101, producing plasmids pWW0-7020 and pWW0-7021 respectively (Fig. 1).

A third mutation was constructed by deletion between the PsI site at 1-75 and 2-5 kbp, removing the 3’ end of xylU and the 5’ end of xyl/W. This could not be constructed directly from pWW0-7000 since a third PsI site is present in the multi-cloning site of the vector. To circumvent this problem, pUC18 (Vieira & Messing, 1982) was first modified by removing its PsI site by digestion with PsI, filling in the 3’ ends with Klenow enzyme and religating to form pUC18NP. The 3.9 kbp KpnI-BamHI fragment from pWW0-7000 was then cloned into the KpnI and BamHI sites of pUC18NP to form plasmid pWW0-7013 (not shown). This was digested with PsI and religated to give pWW0-7014 (mutated gene xylA(UW)), from which the Smal–BamHI fragment was removed and ligated into pKNG101 to form the suicide delivery plasmid pWW0-7022 (Fig. 1).

Reverse genetics with pKNG101 derivatives. The broad-host-range suicide vector pKNG101, designed for reverse genetics in Gram-negative bacteria (Kaniga et al., 1991), was used to incorporate the xyl/UW mutations described above into the appropriate Pseudomonas strains. As in the protocol of Kaniga et al. (1991), sucrose-resistant (Suc⁺) revertants were selected from sucrose-sensitive (Suc⁻) streptomycin-resistant (Str⁺) transconjugants in which the complete plasmid had integrated into the chromosome by a single homologous recombination at the wild-type xyl/UW locus. A proportion of these revertants should have the wild-type gene replaced by the mutated gene.

DNA manipulations and electrophoretic analysis of recombinant plasmids. Plasmid DNA was prepared from E. coli hosts using commercial Qiagen or Pharmacia plasmid preparation kits. Plasmid DNA from pWW0 was prepared from P. putida by the sucrose density method (Wheatcroft & Williams, 1981). Digestion of DNA with restriction enzymes, ligations, preparation of competent cells, transformations, agarose gel electrophoresis of digested plasmid DNA and PCR were carried out according to the suppliers’ instructions and/or by standard procedures.
**Fig. 1.** Restriction maps of recombinant plasmids derived from the 5' end of the upper pathway operon of TOL plasmid pWW0: the plasmid designations are on the right. Plasmids pWW0-7012 and -7020 carry the xyl\(U^W\) allele, pWW0-7014 and -7021 the xyl\(U^A(UW)\) allele, and pWW0-7010 and -7022 the xyl\(U^W\) allele. The grey boxes denote the termini of the vectors, which are named on the left, and the solid lines represent the inserts of pWW0 DNA. The restriction sites from the multicloning sites of each vector into which the pWW0 DNA is inserted are above the ends of each terminus. The direction of transcription from the promoter of expression vector pTrc99A in pWW0-7001 and pWW0-7011 is denoted by the arrows. The box surrounded by dashed lines spanning plasmids pWW0-7012, -7014 and -7010 delineates the three Smal-BamHI fragments cloned into pKNG101 to form pWW0-7020, -7021 and -7022 respectively. Abbreviations for restriction sites: Bam, BamHI; Bst, BstEII; Cla, Clal; Eco, EcoRl; ECISma, the hybrid site formed from blunt-end ligation of a Smal site on the insert into an EcoCRl site on the vector; Kpn, Kpnl; Pst, Pstl; Sal, Salt; Pvu, PvuII; Xho, Xhol. Note that the Pstl site normally to the right of the BamHI multicloning site in pUC18 is absent in pWW0-7014, having been eliminated in the formation of pUC18NP.

**RESULTS**

**Analysis of nucleotide sequence**

Two open reading frames were identified between the promoter region Pu and the start codon of xyl\(C\) for benzaldehyde dehydrogenase (Fig. 2). The ATG start codon of the first, designated xyl\(U\), was originally shown to be the translational start of the operon but was wrongly identified as the reading frame for xylene monooxygenase (Inouye et al., 1984). The structural genes for all the enzymic steps of the conversion of toluene to benzoate (xyl\(CMAB\)) were subsequently shown to be > 1 kbp downstream of this codon (Lebens & Williams, 1985; Harayama et al., 1986). The ORF designated xyl\(U\) appears to encode a small protein of...
131 amino acid residues (M, 14244) with no pronounced hydrophilic character. It shared significant homology with only one protein in the databanks, TmbU (GenBank U14301). Downstream of xylU was xylW, a second ORF of 348 codons (M, 36992). This terminated just upstream of the start of xylC and shows significant homology with the family of group 1 long-chain Zn-containing alcohol dehydrogenases (ADHs). Apart from TmbU (see Discussion) the two proteins most similar to it are the 341-residue threonine dehydrogenase (TDH) from E. coli (GenBank P07913) and the sorbitol dehydrogenase from Bacillus subtilis (GenBank Q60004). Alignment of the amino acid sequences of XylW and TDH introduced only five gaps of a single residue, and showed 37% identity and 62.9% similarity. XylW had only limited homology (28% identity and 53% similarity) with benzyl alcohol dehydrogenase (BADH; XylB), another Group 1 ADH (Shaw et al., 1993), but negligible homology with benzaldehyde dehydrogenase (XylC), the adjacent downstream gene product (Inoue et al., 1995).

The xylUW region was cloned into vector pTrc99A to form plasmid pWW0-7001 for expression of XylU and XylW (Fig. 1). PAGE gels were run of the SDS-treated extracts of E. coli JM105 carrying pWW0-7001 with and without induction by IPTG. A unique protein band of apparent Mr ~ 38000, corresponding to XylW, was observed but no band could be detected that identified XylU (data not shown).

Formation of mutated P. putida strains and their authentication by PCR

E. coli SM10Δpir strains carrying the three plasmids pWW0-7002 (xylU*) pWW0-7021 (xylW*) and pWW0-7022 [xylA(UW)] were separately mated with P. putida strains KT2442Δ and PaW140 and transconjugants were selected on succinate/Km plates. Four transconjugants were retained in each case and their phenotypes checked: in all cases they were Km' and grew on the same range of aromatic substrates as their parent strains but failed to grow on succinate/Km plates containing 5% (w/v) sucrose. Cultures of all transconjugants were grown in LB overnight and plated on to succinate plates containing 5% sucrose. Single colonies appeared and 20 from each were retained and shown to be Strs Suc+, as expected (Kaniga et al., 1991).

The relevant genotype of the strains was determined by PCR. Two forward 19mer primers (UW1 and UW2) were designed from the 5' end of xylU (see Fig. 1) and a single 20mer reverse primer (UW3) spanning the start codon of xylC (Shaw et al., 1993); these were chosen such that the PCR products, uncut and cut with restriction enzymes, from mutant alleles of xylU and xylW could be distinguished from the wild-type alleles of the genes using both forward-reverse primer combinations. Genomic DNA from each Pseudomonas transconjugant and
its Suc\(^{-}\) revertants was subjected to PCR and the amplified fragment was subjected to digestion with the appropriate restriction enzymes. The initial transconjugants (Str\(^{-}\) Suc\(^{+}\)) with the chromosomally integrated plasmid should have contained both a wild-type and a mutant gene: a single transconjugant from each mating was retained which produced two amplified fragments with the expected patterns of digestion. The Str\(^{-}\) Suc\(^{+}\) revertants from each of these transconjugants were examined in the same way until one with a mutant gene and one with the wild-type gene were identified: one of each was retained. In no case was it necessary to examine more than eight revertants of any transconjugant before one unambiguous example of each kind was found.

All of the strains, with mutant or with wild-type genes, derived from \textit{P. putida} KT2442\(\Delta\) grew on toluene, benzy alcohol, benzy alcohol, and benzoate, and all those derived from PaW140 grew on the same four substrates as well as their 3-methyl and 4-methyl analogues (Table 1) although growth on the 4-methylated substrates of all strains was considerably slower than on the 3-methyl and unsubstituted substrates. No differences were apparent in the rates of growth on plates between any parent strain and its derivatives and no significant differences were observed in their exponential growth rates in liquid cultures.

**DISCUSSION**

The results presented show the presence of two new genes (\textit{xylUW}) at the beginning of the upper pathway operon of the TOL plasmid. \textit{XylU} joins a number of other putative ORFs found in aromatic catabolism which appear to have no counterparts in the databanks, for which functions have not yet been determined and which do not appear to be related to each other. These include \textit{XylN} at the opposite end of the same operon, which is also a Group 1 Zn-ADH (Polissi \textit{et al.,} 1995). There is one other ORF highly homologous to \textit{XylU} in the databanks, \textit{XylSi}, from its virtual identity (343/348 residues identical) from the same operon, which is also a Group 1 Zn-ADH (Polissi \textit{et al.,} 1995). There is one other ORF highly homologous to \textit{XylU} in the databanks, \textit{TmbU}. However, this is encoded by a chromosomal sequence for \textit{trimethylbenzene catabolism} (Polissi \textit{et al.,} 1990) which includes other genes also highly homologous to the \textit{pWW0} \textit{xyl} gene products: downstream of \textit{xylU} is \textit{tm6W} (= \textit{xylW}) and a partial sequence for \textit{tm6C} (= \textit{xylC}), and upstream are \textit{tm6R} (= \textit{xyrR}) and \textit{tm6S} (= \textit{xyrS}); undoubtedly this DNA is another \textit{pWW0}-like sequence, but it has a different gene order and appears to have become chromosomally located.

However, it is the possible function of \textit{XylW} which is arguably the most intriguing of the two new TOL ORFs. Its sequence indicates strongly that it is an ADH. Apart from its virtual identity (343/348 residues identical) with \textit{TmbW}, it has greatest similarity to TDH from \textit{E. coli} (Epperly & Dekker, 1991; Chen \textit{et al.,} 1995) and even shares a limited homology with BADH (\textit{XylB}) from the same operon, which is also a Group 1 Zn-ADH (Shaw \textit{et al.,} 1993). \textit{XylW} has many of the characters of this enzyme (343/348 residues identical) from the same operon, which is also a Group 1 Zn-ADH (Shaw \textit{et al.,} 1993). \textit{XylW} has many of the characters of this enzyme (343/348 residues identical) from the same operon, which is also a Group 1 Zn-ADH (Shaw \textit{et al.,} 1993). \textit{XylW} has many of the characters of this enzyme (343/348 residues identical) from the same operon, which is also a Group 1 Zn-ADH (Shaw \textit{et al.,} 1993). \textit{XylW} has many of the characters of this enzyme (343/348 residues identical) from the same operon, which is also a Group 1 Zn-ADH (Shaw \textit{et al.,} 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(residues 40–43), corresponding to the consensus of Cys-His-Thr-Asp (Reid & Fewson, 1994); His-64, Glu-65, Ala-185, corresponding to the archetypal horse liver ADH (HLADH) residues 67, 68, 211 respectively; and Asp-197, found in all NAD(H)-dependent enzymes but replaced by Gly in the NADP(H)-dependent enzymes (Reid & Fewson, personal communication), is the replacement Ala-185, corresponding to the archetypal horse liver ADH (HLADH) residues 67, 68, 211 respectively; and Asp-197, found in all NAD(H)-dependent enzymes but replaced by Gly in the NADP(H)-dependent enzymes (Reid & Fewson, personal communication), is the replacement residue His-64, Glu-65, Ala-185, corresponding to the archetypal horse liver ADH (HLADH) residues 67, 68, 211 respectively; and Asp-197, found in all NAD(H)-dependent enzymes but replaced by Gly in the NADP(H)-dependent enzymes (Reid & Fewson, personal communication), is the replacement His (corresponding to HLADH-51) by a hydrophobic amino acid (Leu, Val and Ile respectively).

The incorporation of mutant alleles of xylU and xylW into a chromosomally integrated copy of the xyl-UWCMABN operon produces no apparent change in the catabolic phenotype of the host strains and the mutants grow as well as wild-types on all nine of the upper pathway substrates tested. It is possible that the single restriction sites modified in two of the constructions, the XhoI site in xylU* and the ClaI site in xylW*, are both close enough to the 3' ends of the respective genes for the two modified gene products to retain activity. This cannot be true for the third mutation, the PstI deletion producing xylA(UW), as it removed the start codon of xylW and > 50% of the 5' end of the gene and fused it to the 5' end of xylU.

Two roles for XylW seem possible which do not conflict with these findings. One is that functions as a second BADH, the action of which merely supplements that of XyIB so that its loss is not a limiting factor in growth. From preliminary results (not shown) we think that this is unlikely since we have been unable to detect any such activity from an expression vector using the standard assay procedure for XyIB (Worsey & Williams, 1975). This region is also highly conserved in a number of other TOL plasmids, as seen by comparison of the restriction maps of this operon (Assinder & Williams, 1990). This implies that there is a strong pressure for its retention and that it is not simply a superfluous duplication. A second possibility is that XylW is part of a convergent branch of the pathway which converts some growth substrate, not yet identified, into one of the other recognized TOL pathway metabolites. In preliminary experiments we have tried only two which might fill this role and be converted to benzaldehyde, mandelic acid and benzylamine. Neither served as substrates for XylW in a preliminary dehydrogenase assay procedure using extracts of E. coli carrying pWW0-7001, nor did they serve as growth substrates for strains with a functional xylW gene as compared with strains with a mutated or absent xylW.

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