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Overlapping Substrate Specificities of Benzaldehyde Dehydrogenase (the *xylC* Gene Product) and 2-Hydroxymuconic Semialdehyde Dehydrogenase (the *xylG* Gene Product) Encoded by TOL Plasmid pWW0 of *Pseudomonas putida*

JUN INOUE,[1] JEFFREY P. SHAW,[2]† MONIQUE REKIK,[2] AND SHIGEAKI HARAYAMA[1,2]*

Marine Biotechnology Institute, Kamaishi Laboratories, 3-75-1 Heita, Kamaishi City, 026 Iwate, Japan,[1] and Département de Biochimie Médicale, Centre Médicale Universitaire, 1211 Geneva 4, Switzerland[2]

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Two aldehyde dehydrogenases involved in the degradation of toluene and xylenes, namely, benzaldehyde dehydrogenase and 2-hydroxymuconic semialdehyde dehydrogenase, are encoded by the *xylC* and *xylG* genes, respectively, on TOL plasmid pWW0 of *Pseudomonas putida*. The nucleotide sequence of *xylC* was determined in this study. A protein exhibiting benzaldehyde dehydrogenase activity had been purified from cells of *P. putida*(pWW0) (J. P. Shaw and S. Harayama, Eur. J. Biochem. 191:705–714, 1990); however, the amino-terminal sequence of this protein does not correspond to that predicted from the *xylC* sequence but does correspond to that predicted from the *xylG* sequence. The protein purified in the earlier work was therefore 2-hydroxymuconic semialdehyde dehydrogenase (the *xylG* gene product). This conclusion was confirmed by the fact that this protein oxidized 2-hydroxymuconic semialdehyde ($k_{cat}/K_m = 1.6 \times 10^6\ s^{-1}\ M^{-1}$) more efficiently than benzaldehyde ($k_{cat}/K_m = 3.2 \times 10^4\ s^{-1}\ M^{-1}$). The *xylC* product, the genuine benzaldehyde dehydrogenase, was purified from extracts of *P. putida*(pWW0−161Δ*xylG*) which does not synthesize 2-hydroxymuconic semialdehyde dehydrogenase. The amino-terminal sequence of the purified protein corresponds to the amino-terminal sequence deduced from the *xylC* sequence. This enzyme efficiently oxidized benzaldehyde ($k_{cat}/K_m = 1.7 \times 10^7\ s^{-1}\ M^{-1}$) and its analogs but did not oxidize 2-hydroxymuconic semialdehyde or its analogs.

The TOL plasmid from a soil bacterium *Pseudomonas putida* encodes a metabolic pathway for the degradation of toluene, xylenes, and their alcohol and carboxylate derivatives (1). The genes encoding the enzymes of this pathway are clustered in two operons on TOL plasmid pWW0. The upper operon encodes the first three enzymes of this pathway, namely, xylene monooxygenase, benzyl alcohol dehydrogenase, and benzaldehyde dehydrogenase, which together transform the initial substrates (toluene or xylenes) into benzoate or toluates. The *meta* operon contains the genes responsible for the degradation of benzoate and toluates. In this pathway, two dehydrogenases which oxidize aldehydes to carboxylates are present. These two dehydrogenases are benzaldehyde dehydrogenase encoded by the *xylC* gene (upper operon) and 2-hydroxymuconic semialdehyde dehydrogenase encoded by the *xylG* gene (*meta* operon; Fig. 1).

We have previously purified and characterized a 57-kDa protein from *P. putida*(pWW0) which exhibits the benzaldehyde dehydrogenase activity (19, 21). In this study, the amino-terminal sequence of this protein and the nucleotide sequence of the *xylC* gene, the structural gene for benzaldehyde dehydrogenase, were determined. The amino-terminal sequence of the purified 57-kDa protein does not correspond to the amino-terminal sequence of benzaldehyde dehydrogenase deduced from the nucleotide sequence of *xylC* but did correspond to that of 2-hydroxymuconic semialdehyde dehydrogenase (the *xylG* gene product). The *xylC* gene product, the genuine benzaldehyde dehydrogenase, was purified from extracts of *P. putida*(pWW0Δ*xylG*), which does not synthesize 2-hydroxymuconic semialdehyde dehydrogenase, and characterized.

* Corresponding author. Mailing address: Marine Biotechnology Institute, 3-75-1 Heita, Kamaishi City, 026 Iwate, Japan. Phone: 81-193-26-6544. Fax: 81-193-26-6592. Electronic mail address: sharayam@ddbj.nig.ac.jp.

† Present address: Department of Structural Biology, Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA 02154-9110.

MATERIALS AND METHODS

Chemicals. The coenzymes were purchased from Sigma (St. Louis, Mo.). Other chemicals were purchased from either Aldrich (St. Louis, Mo.) or Wako Pure Chemical Industries (Tokyo, Japan) and were of the highest quality available. The ring cleavage products of catechol (2-hydroxymuconic semialdehyde) and 4-methylcatechol (2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate) were prepared enzymatically with purified catechol 2,3-dioxygenase as described previously (8).

Bacterium, plasmids, and DNA manipulation. KT2440, a plasmid-free strain of *P. putida*, was used for the host bacterium. The pWW0-161 plasmid is a Tn*401* insertion derivative of the TOL plasmid pWW0 and carries a complete set of the structural and regulatory genes for the degradation of toluene and xylenes (7). The pWW0-161Δ*xylG* plasmid, carrying a deletion in the structural gene for 2-hydroxymuconic semialdehyde dehydrogenase, has been described previously (10). Various pUC18- and pUC19-based plasmids containing the whole or partial sequence of *xylC*, the structural gene for benzaldehyde dehydrogenase, were constructed either in our previous studies (9, 12) or in this study. All the techniques for DNA manipulation have been described previously (2).

DNA sequencing. DNA sequencing with double-stranded DNA was performed with the T7 DNA polymerase kit from Pharmacia (Uppsala, Sweden). Nucleotide sequencing of plasmid DNA around Tn*1000* insertions was carried out with Tn*1000*-specific oligonucleotides as primers (22). The computer-assisted sequence analysis was done with the PC/GENE software package (IntelliGenetics, Mountain View, Calif.). The alignment of amino acid sequences was performed with a computer program, CLUSTAL (14).

Cultural conditions. KT2440 containing either pWW0 or pWW0-161Δ*xylG* was cultivated in L broth containing 10 mM benzyl alcohol. During the cultivation, *m*-xylene was added as a vapor. Benzyl alcohol and *m*-xylene are inducers of TOL-catabolic enzymes (18).

Enzyme assays. In the standard conditions, the benzaldehyde dehydrogenase activity was assayed at 25°C in 100 mM glycine–NaOH (pH 9.2) containing 40 μM benzaldehyde and 1 mM NAD^+ by measuring A_{340} to monitor the formation of NADH. In the assay for 2-hydroxymuconic semialdehyde dehydrogenase, the

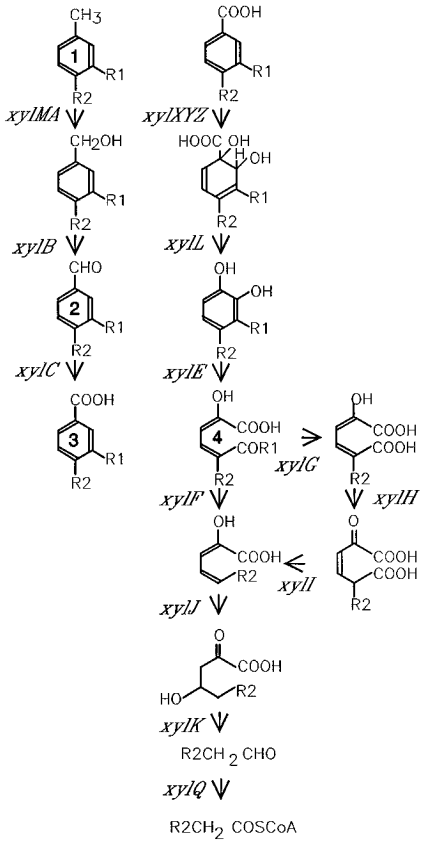

FIG. 1. Catabolic pathway for the degradation of toluene and xylenes encoded by the TOL plasmid pWW0. The genes and enzymes for the transformation of toluene and xylenes to benzoate and toluates (upper pathway) have been described by Harayama et al. (12) and Shaw et al. (20), while the genes and enzymes for the transformation of toluates to precursors of Krebs cycle intermediates (*meta* pathway) have been summarized by Harayama and Rekik (11). Compound 1, toluene (R1 = H; R2 = H), *m*-xylene (R1 = CH_3; R2 = H), *p*-xylene (R1 = H; R2 = CH_3); compound 2, benzaldehyde (R1 = H; R2 = H), *m*-methylbenzaldehyde (R1 = CH_3; R2 = H), *p*-methylbenzaldehyde (R1 = H; R2 = CH_3); compound 3, benzoate (R1 = H; R2 = H), *m*-toluate (R1 = CH_3; R2 = H), *p*-toluate (R1 = H; R2 = CH_3); compound 4, 2-hydroxymuconic semialdehyde (R1 = H; R2 = H), 2-hydroxy-6-oxohepta-2,4-dienoate (R1 = CH_3; R2 = H), 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate (R1 = H; R2 = CH_3). CoA, coenzyme A.

disappearance of a substrate, 2-hydroxymuconic semialdehyde or 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate, was monitored by measuring A_{375} or A_{382}, respectively. In the standard assay conditions, the reaction was carried out at 25°C in 100 mM Tris-HCl (pH 8.5) containing either 33 μM 2-hydroxymuconic semialdehyde or 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate and 1 mM NAD^+ as described previously (8). Other buffers used for the assays are described in Results. The Michaelis-Menten kinetic parameters were obtained by measuring the initial velocities determined under these conditions except that various concentrations of the substrates were used. The K_m and V_{max} values and their standard errors were calculated on the Lineweaver-Burk double reciprocal plot using computerized least-squares-fit methods.

Protein purification. The *xylG* gene product (2-hydroxymuconic semialdehyde dehydrogenase) was purified by the method described previously (19, 21). This enzyme has erroneously been called benzaldehyde dehydrogenase in previous publications (see below). The *xylC* gene product (benzaldehyde dehydrogenase) was purified from a culture of KT2440(pWW0-161Δ*xylG*) by the following method. The strain was cultivated overnight in eight flasks each containing 250 ml of L broth supplemented with 10 mM benzyl alcohol and vapor of *m*-xylene. The culture was centrifuged at 7,700 × *g* for 20 min at 4°C, and the pellet was resuspended in 50 ml of 20 mM potassium phosphate buffer (pH 7.4) and centrifuged at 7,700 × *g* for 20 min at 4°C. The pellet was resuspended in 50 ml of 20 mM potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol (buffer A), and the cells were lysed by passage through a precooled French press (Ohtake Works, Tokyo, Japan) with a pressure difference of 76 MPa. The resulting extract was centrifuged for 20 min at 37,000 × *g* at 4°C; the supernatant was then centrifuged at 165,000 × *g* for 60 min. The resulting supernatant was filtered through a Nalgen filter (0.45-μm pore size), and the filtrate was loaded onto an anion-exchange high-pressure liquid chromatography column (TSKgel DEAE-5PW; 55-mm inner diameter [ID] 20 cm; Tosoh, Tokyo, Japan) preequilibrated in buffer A. Proteins were eluted after a 60-ml wash, by a gradient of 0 to 0.25 M Na_2SO_4 in 500 ml of buffer A at a flow rate of 5 ml per min. The eluant was collected in 5-ml fractions. The fractions containing benzaldehyde dehydrogenase activity (30 ml) were pooled and brought to a concentration of 0.5 M in $(NH_4)_2SO_4$ at 4°C; the resulting suspension was centrifuged at 7,700 × *g* for 20 min. Benzaldehyde dehydrogenase activity was recovered in the supernatant. The resulting supernatant was filtered through a Nalgen filter and loaded onto a hydrophobic-interaction chromatography column (TSKgel Phenyl-5PW; 21.5-mm ID by 15 cm; Tosoh) preequilibrated in buffer A containing 0.5 M $(NH_4)_2SO_4$. After the proteins were washed with 40 ml of the same buffer, they were eluted at a flow rate of 5 ml per min from the column by using a linear gradient in which the $(NH_4)_2SO_4$ concentration was reduced from 0.5 M to zero over 60 min. The eluant was collected in 5-ml fractions; the fractions containing activity (32 ml) were pooled and dialyzed overnight against 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol. The dialyzed sample was loaded onto a hydroxyapatite chromatography column (HCA; 7.6-mm ID by 10 cm; Mitsui-Toatsu, Tokyo, Japan) preequilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol; after a 50-ml wash, proteins were eluted by a gradient of 10 to 350 mM potassium phosphate (pH 7.4) in 100 ml at a flow rate of 1 ml per min. The eluant was collected in 5-ml fractions. The active fractions were pooled (83 ml) and concentrated by hydrophobic-interaction chromatography with a TSKgel Phenyl-5PW column.

Catechol 2,3-dioxygenase encoded by *xylE* on the TOL plasmid pWW0 was purified as described previously (17).

Amino-terminal sequencing. The amino-terminal sequences of purified enzymes were determined by Edman degradation with an automated protein sequencer (model 477; Applied Biosystems Japan, Tokyo, Japan).

Analytical methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a premade polyacrylamide slab gel (MultiGel 12.5; Daiichi Pure Chemicals, Tokyo, Japan). Proteins were stained for 1 h with 0.25% (wt/vol) Coomassie brilliant blue R250 in an aqueous solution containing 30% (vol/vol) ethanol and 10% (vol/vol) acetic acid and destained in a solution containing 30% (vol/vol) ethanol and 8% (wt/vol) acetic acid. Analytical gel filtration chromatography for the determination of the molecular mass of the *xylC* product was performed with a G3000 SWXL column (7.8-mm ID by 30 cm; Tosoh) with a mobile phase of 100 mM potassium phosphate buffer (pH 7.4) containing 0.1% NaN_3 at a flow rate of 0.5 ml per min. The A_{280} of the effluent was monitored, and the molecular mass of each of the enzymes was determined from the relative mobility compared with those of the molecular mass standards (Boehringer Mannheim Japan, Tokyo, Japan). The protein concentration was determined by the method of Bradford (3), using an assay kit from BioRad Japan (Tokyo, Japan) with bovine serum albumin as a standard.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to GenBank under accession number U15151.

RESULTS

Nucleotide sequence of *xylC*, the structural gene for benzaldehyde dehydrogenase. The *xylC* gene, the structural gene for benzaldehyde dehydrogenase, has previously been localized on the TOL plasmid pWW0, by transposon mutagenesis and subcloning, and a number of Tn*1000* insertions which inactivated the *xylC* activity have been isolated (9, 12, 16). The nucleotide sequence of *xylC* around the Tn*1000* insertions was determined by using two oligonucleotides, each complementary to one of the two ends of Tn*1000*, as primers. We also subcloned in pUC18 or pUC19 several restriction fragments covering the *xylC* region, and their sequences were determined. Furthermore, several oligonucleotides (21-mers) complementary to the *xylC* sequence were synthesized and used as primers for DNA sequencing. Thus, the entire *xylC* gene was sequenced on both DNA strands. There is an open reading frame preceded by a potential binding site for 16S rRNA (Fig. 2). The nucleotide sequence of *xylC* has been determined by M. Lebens (15a), and the amino acid sequence of XylC deduced from the nucleotide sequence has been quoted by Horn et al. (15). The XylC sequence of M. Lebens differed by two amino acids from the XylC sequence determined in this study. The molecular mass of the XylC protein deduced from the nucleotide sequence was 51,941 Da, and the amino-terminal sequence of the *xylC* product deduced from the nucleotide sequence was

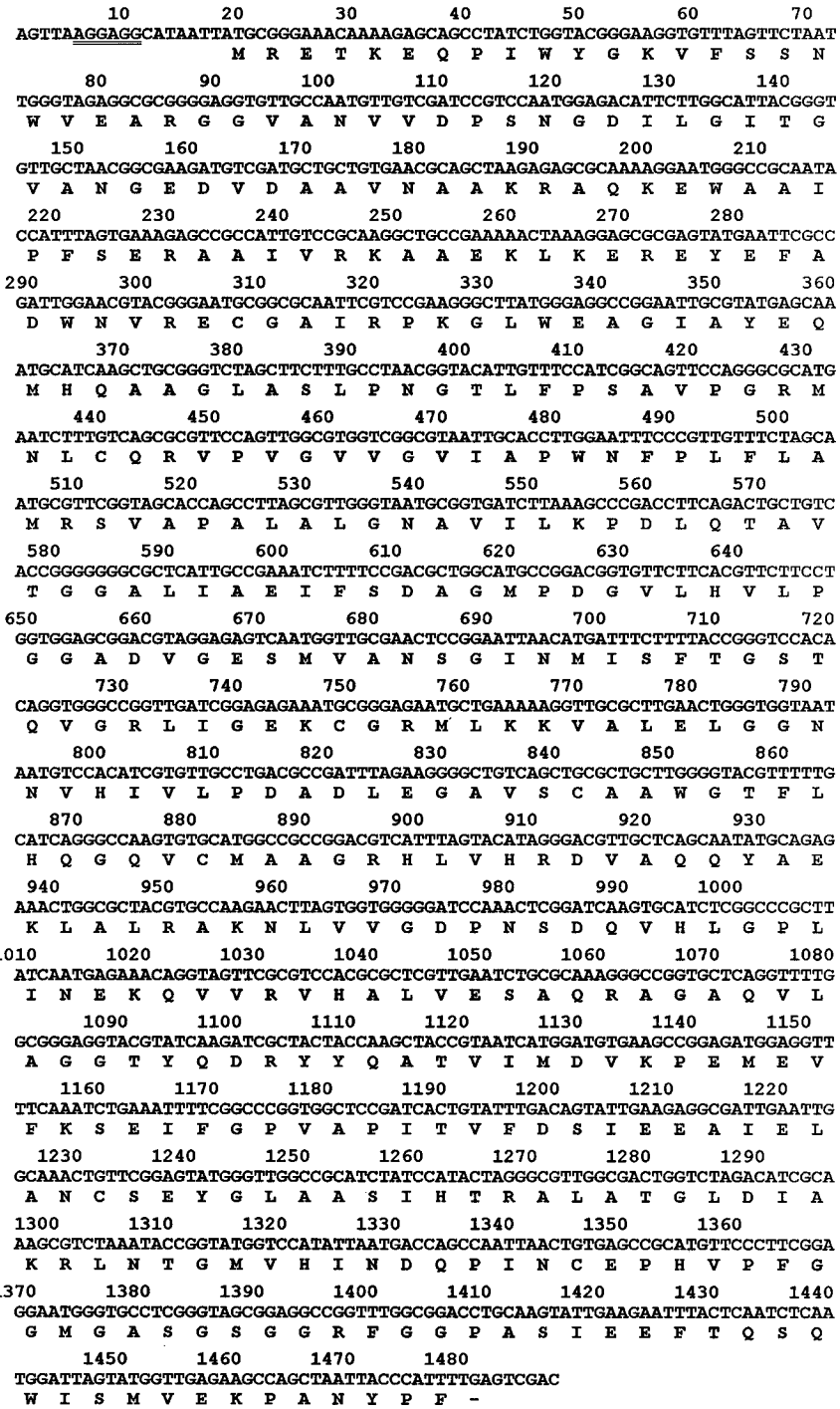

FIG. 2. Nucleotide sequence of *xylC*. The amino acid sequence of the *xylC* product is also presented. The putative Shine-Dalgarno sequence of *xylC* is underlined.

MRETKEQPIWYGKVF. The homology search between the XylC sequence and other proteins in SWISS-PROT data bank indicated that the XylC protein is closely related to the mammalian cytosolic aldehyde dehydrogenase with 33% sequence identity. XylC also showed 25% sequence identity with XylG. The highly conserved residues of the aldehyde dehydrogenase family, Cys-302 and Glu-268 (the numbering system refers to the human cytosolic aldehyde dehydrogenase), which have been implicated as essential residues for NAD^+ binding and a charge relay network (15), were conserved in the XylC sequence.

Characterization of XylG. A protein which exhibits benzaldehyde dehydrogenase activity was purified from the extract of *P. putida*(pWW0-161) in four steps: ammonium sulfate fractionation, anion-exchange chromatography, Affigel-Blue affinity chromatography, followed by hydrophobic-interaction chromatography as described previously (21). The amino-terminal sequence of this protein was determined to be MKEIKXFIS

TABLE 1. Kinetic constants of 2-hydroxymuconic semialdehyde dehydrogenase (XylG)[a]

Substrate	K_m (μM)	Apparent V_{max}[b]	Apparent k_{cat}/K_m (s^{-1} M^{-1})
Benzaldehyde	460	17	3.2×10^4
3-Methylbenzaldehyde	630	26	3.6×10^4
4-Methylbenzaldehyde	530	6.2	1.0×10^4
3-Chlorobenzaldehyde	1,300	32	2.2×10^4
3-Nitrobenzaldehyde	810	190	20×10^4
3-Methoxybenzaldehyde	940	9	0.8×10^4
3-Fluorobenzaldehyde	170	64	33×10^4
NAD^+	330 ± 13	21 ± 1.2	5.6×10^4
2-Hydroxymuconic semialdehyde	17 ± 6	27 ± 9	1.6×10^6
2-Hydroxy-5-methyl-6-oxohexa-2,4-dienoate	9.3 ± 2	22 ± 3	2.1×10^6

[a] The activity for oxidizing benzaldehyde and its analogs was measured in 100 mM glycine–NaOH (pH 9.2) containing 1 mM NAD^+ for the determination of K_m and apparent V_{max} for the aldehydes or in the same buffer containing 10 μM benzaldehyde for the determination of K_m and apparent V_{max} for NAD^+. The activity for oxidizing the ring cleavage products was measured in 100 mM Tris-HCl (pH 8.5) containing 1 mM NAD^+. Data for 2-hydroxymuconic semialdehyde and 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate were obtained in this study, while data for aldehydes were obtained from reference 21 and data for NAD^+ were obtained from reference 19.

[b] In micromoles of NADH formed per minute per milligram of protein.

GAFVGS (X represents an unidentified amino acid). This sequence did not fit the amino-terminal sequence predicted from the *xylC* nucleotide sequence but did fit the amino-terminal sequence predicted from the *xylG* nucleotide sequence (15) which encodes 2-hydroxymuconic semialdehyde dehydrogenase. It is thus likely that the protein purified was the *xylG* gene product, which exhibits the benzaldehyde dehydrogenase activity in addition to the activity of 2-hydroxymuconic semialdehyde dehydrogenase.

The substrate specificity of the purified protein (hereafter called either XylG or the *xylG* gene product) toward the ring cleavage products of catechols was examined. The ring cleavage products of catechol (2-hydroxymuconic semialdehyde) and of 4-methylcatechol (2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate) were oxidized, but the ring cleavage product of 3-methylcatechol (2-hydroxy-6-oxohepta-2,4-dienoate) possessing no aldehyde group was not oxidized by XylG. The Michaelis-Menten kinetic parameters of XylG for the ring cleavage products of catechol and 4-methylcatechol were determined (Table 1). The V_{max} values of XylG for the ring cleavage products of catechols were similar to those for benzaldehyde or its analogs, but the K_m values of XylG for the ring cleavage products of catechols were much lower than those for the benzaldehydes. It was thus concluded that the ring cleavage products of catechol and 4-methylcatechol are better substrates of XylG than benzaldehyde and its analogs, as expected from the suggested physiological role of this enzyme. The pH optima of the XylG catalysis for the oxidation of 2-hydroxymuconic semialdehyde and benzaldehyde were 8.3 and 9.6, respectively.

Purification of XylC. When benzaldehyde dehydrogenase activity was purified from cells of *P. putida*(pWW0-161) containing both XylC and XylG, the XylG protein was obtained, and it was found that the XylC protein had been lost during the purification. In order to follow the fate of XylC, this protein was purified from extracts of *P. putida* containing the pWW0-161Δ*xylG* plasmid, which synthesizes XylC but not XylG (9). Benzaldehyde dehydrogenase activity was eluted from a DEAE column at a Na_2SO_4 concentration of 0.11 M, which also eluted the XylG protein in previous purification studies

TABLE 2. Purification of the *xylC* gene product

Fraction	Volume (ml)	Protein concn (mg/ml)	Sp act[a]	Total activity[b]	Yield (%)
Cell extract	50	12	0.1	59	100
Anion exchange	30	1.2	1.1	37	62
Hydrophobic interaction	32	0.04	14	20	34
Hydroxyapatite	83	0.02	9	14	23
Hydrophobic interaction	19.5	0.02	16	5.8	9.8

[a] Benzaldehyde dehydrogenase was assayed in 100 mM glycine–NaOH (pH 9.2) containing 1 mM NAD^+ and 10 μM benzaldehyde, and its activity was expressed as micromoles of NADH formed per minute per milligram of protein.

[b] In micromoles of NADH formed per minute.

(19). We infer that if both XylC and XylG exist in cell extracts, they are copurified in the anion-exchange chromatography. When fractions containing the benzaldehyde dehydrogenase activity from *P. putida*(pWW0-161Δ*xylG*) were loaded onto an Affi-Gel Blue column, the benzaldehyde dehydrogenase activity could not be eluted either by 2 M KCl or by 20 mM NAD^+ (data not shown). This result explains why, in our previous studies in which an Affi-Gel Blue column was used in the purification (19, 21), only the XylG protein but not the XylC protein was recovered. We therefore purified the XylC product without the Affi-Gel Blue chromatography step. As shown in Table 2, the benzaldehyde dehydrogenase activity was purified 160-fold, after four chromatography steps, with a yield of 10%. The purified sample after the hydroxyapatite chromatography step gave a single protein band on a SDS-polyacrylamide gel (Fig. 3). The amino-terminal sequence of the purified protein was MRETKEQPIWYGKVF, in complete agreement with the amino-terminal sequence deduced from the nucleotide sequence of *xylC*. A single protein band corresponding to a molecular mass of 52.5 kDa was observed on a SDS-polyacrylamide gel (Fig. 3). The molecular mass of the XylC protein

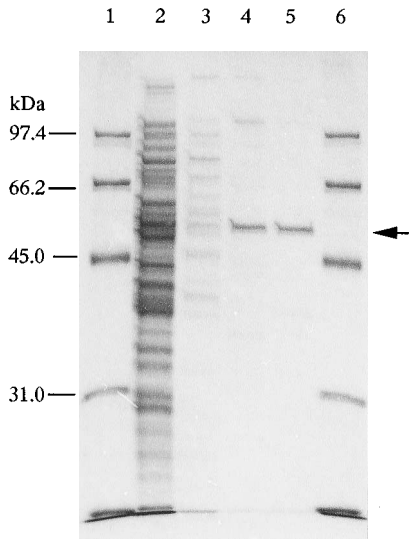

FIG. 3. SDS-PAGE of benzaldehyde dehydrogenase. Lanes 1 and 6, molecular mass markers; lane 2, cell extract; lane 3, pooled active fractions after anion-exchange chromatography; lane 4, active fractions after hydrophobic interaction chromatography; lane 5, pooled active fractions after hydroxyapatite chromatography. The arrow indicates the benzaldehyde dehydrogenase bands.

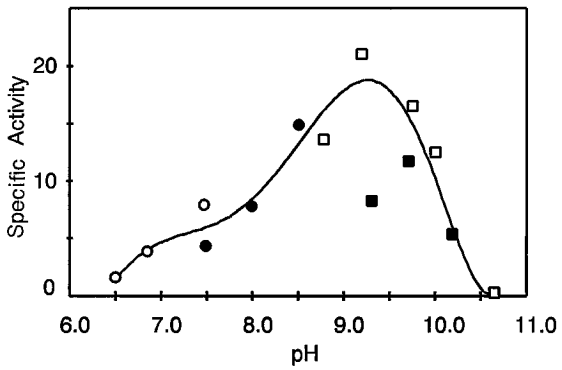

FIG. 4. pH activity profile of benzaldehyde dehydrogenase. Benzaldehyde dehydrogenase activity was measured in either 100 mM 3-(cyclohexylamino)-1-propane-sulfonic acid–NaOH buffer (■), 100 mM glycine–NaOH buffer (□), 100 mM *N,N*-bis(2-hydroxyethyl)glycine–NaOH buffer (○), or 100 mM potassium phosphate buffer (●). The activity is expressed as micromoles of NADH formed per minute per milligram of protein.

estimated by gel filtration chromatography was 208 kDa. The active XylC enzyme thus appears to consist of four identical subunits.

Characterization of XylC. Determination of the benzaldehyde dehydrogenase activity in different buffers at different pHs indicates that the XylC enzyme has an optimal pH at 9.0. The sodium 3-(cyclohexylamino)-1-propane-sulfonate buffer at a pH between 9 and 10 was inhibitory to the enzyme activity (Fig. 4). The Michaelis constants of XylC for the oxidation of benzaldehyde and its methyl, nitro, methoxy, and chloro substituents were determined (Table 3). It was found that these aldehydes at millimolar concentrations inhibit the enzyme activity. Therefore, the K_m and V_{max} values were determined by extrapolating the results of lower substrate concentrations. The V_{max} values of XylC for benzaldehyde and its substituents at position 3 were around 40 to 50 (μmol of NADH formed per min per mg of protein), with the exception of 3-nitrobenzaldehyde which gave a V_{max} almost three times higher. The K_m values of XylC for the aldehydes were lower than 3.5 μM, with the exception of that for 3-nitrobenzaldehyde which was 27 μM. The V_{max} values for the benzaldehydes of XylC were of the same magnitude as those of XylG, but the K_m values for the benzaldehydes of XylC were much lower than those of XylG (Tables 1 and 3). The XylC enzyme did not display any activity toward acetaldehyde, 2-hydroxymuconic semialdehyde, 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate, 2-hydroxy-6-oxohepta-2,4-dienoate, and benzaldehydes possessing a substitution at position 2 (2-methylbenzaldehyde, 2-nitrobenzaldehyde, 2-methoxybenzaldehyde, and 2-chlorobenzaldehyde).

TABLE 3. Kinetic constants of benzaldehyde dehydrogenase (XylC)[a]

Substrate	K_m (μM)	Apparent V_{max}[b]	Apparent k_{cat}/K_m (s^{-1} M^{-1})
NAD^+	320 ± 59	47 ± 6	1.3×10^5
Benzaldehyde	2.5 ± 1.0	48 ± 10	1.7×10^7
3-Methylbenzaldehyde	1.9 ± 0.7	31 ± 4	1.4×10^7
4-Methylbenzaldehyde	1.5 ± 0.2	28 ± 1	1.6×10^7
3-Chlorobenzaldehyde	2.1 ± 0.5	48 ± 4	1.9×10^7
3-Nitrobenzaldehyde	22 ± 6.0	121 ± 14	0.5×10^7
3-Methoxybenzaldehyde	2.6 ± 0.5	38 ± 3	1.2×10^7
3-Fluorobenzaldehyde	2.9 ± 0.8	53 ± 6	1.6×10^7

[a] The activity of benzaldehyde dehydrogenase was measured in 100 mM glycine–NaOH (pH 9.2) containing 1 mM NAD^+ for the determination of K_m and apparent V_{max} for various aldehydes or in the same buffer containing 10 μM benzaldehyde for the determination of K_m and apparent V_{max} for NAD^+.

[b] In micromoles of NADH formed per minute per milligram of protein.

DISCUSSION

A protein exhibiting the benzaldehyde dehydrogenase activity had been purified previously (19, 21). The amino-terminal sequence of this protein, however, does not correspond to that predicted from the *xylC* sequence encoding benzaldehyde dehydrogenase but does correspond to that predicted from the *xylG* sequence. Therefore, the protein purified in the earlier work was 2-hydroxymuconic semialdehyde dehydrogenase which oxidizes not only 2-hydroxymuconic semialdehyde and its analogs but also benzaldehyde and its analogs. In this study, we purified the *xylC* product from *P. putida* (pWW0−161Δ*xylG*) which does not synthesize 2-hydroxymuconic semialdehyde dehydrogenase.

Benzaldehyde dehydrogenase synthesized from TOL plasmid pWW53 has been purified, and its amino-terminal sequence has been determined (5). The amino-terminal sequence of the benzaldehyde dehydrogenase from pWW53 is identical to that of the benzaldehyde dehydrogenase from pWW0. These two enzymes may, therefore, have very similar structures. Two benzaldehyde dehydrogenases, I and II, from *Acinetobacter calcoaceticus* have also been purified and characterized (4, 6). The K_m values of the benzaldehyde dehydrogenase from TOL plasmid pWW0 for benzaldehydes were slightly higher than those of other benzaldehyde dehydrogenases, the K_m for NAD^+ of the pWW0 enzyme was in the same range as those of other enzymes, and the V_{max} values of the pWW0 enzyme for benzaldehydes were, in general, two- to threefold lower than those of other bacterial benzaldehyde dehydrogenases.

The active form of 2-hydroxymuconic semialdehyde dehydrogenase is a dimer of identical subunits (19), while benzaldehyde dehydrogenases from pWW0 and from other bacteria are all homotetramers. The quaternary structure of the majority of eukaryotic aldehyde dehydrogenases are variable and can be either homodimers or homotetramers (13).

Substitution at position 2 of benzaldehyde greatly diminished the benzaldehyde dehydrogenase-catalyzed oxidation. Probably, the substitutions at position 2 provoked structural hindrance for the entry of substrates at an active site of this enzyme. Similarly, benzaldehyde dehydrogenase could not oxidize any ring cleavage products of catechols. On the contrary, 2-hydroxymuconic semialdehyde dehydrogenase could oxidize both 2-hydroxymuconic semialdehyde and (substituted) benzaldehydes. Furthermore, this enzyme oxidized 2-nitrobenzaldehyde, albeit its V_{max} for this compound was only 20% of that for 2-hydroxymuconic semialdehyde. Thus, the structural conformation of the active site of 2-hydroxymuconic semialdehyde dehydrogenase may accept a broader range of substrates than that of benzaldehyde dehydrogenase. To understand the structural basis which determines the broader substrate specificity of 2-hydroxymuconic semialdehyde dehydrogenase, we have recently crystallized this enzyme, and the crystals diffract to at least 2.5 Å (0.25 nm).

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