APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1992, p. 2820–2826 Vol. 58, No. 9
0099-2240/92/092820-07$02.00/0

Immunological Demonstration of a Unique 3,4-Dihydroxyphenylacetate 2,3-Dioxygenase in Soil *Arthrobacter* Strains

PATRICIA E. OLSON,[1] BO QI,[1] LAWRENCE QUE, JR.,[2] AND LAWRENCE P. WACKETT[1]*

Department of Biochemistry and Gray Freshwater Biological Institute, University of Minnesota, P.O. Box 100, Navarre, Minnesota 55392,[1] and Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455[2]

Received 29 January 1992/Accepted 8 June 1992

Many bacteria biosynthesize 3,4-dihydroxyphenylacetate 2,3-dioxygenases for growth on aromatic acids, but gram-negative organisms have been most extensively studied. A gram-positive strain containing 2,3-dioxygenase activity was identified as *Arthrobacter* strain Mn-1. The 2,3-dioxygenase from strain Mn-1 was purified to homogeneity by fast protein liquid chromatography with a Mono Q anion-exchange column. Rabbit polyclonal antidioxygenase antibodies were prepared. Ouchterlony double-diffusion and Western blotting (immunoblotting) protocols were used to probe the distribution of the Mn-1 dioxygenase antigen in soil bacteria. Fourteen 2,3-dioxygenase-containing *Bacillus* and *Pseudomonas* strains did not contain immunologically cross-reactive proteins. Six of eight *Arthrobacter* strains contained 2,3-dioxygenase activity, and all of them produced cross-reactive proteins. The data presented here suggest that a unique type of dioxygenase is geographically widespread but is taxonomically confined to *Arthrobacter* soil bacteria.

Aromatic compounds are widespread in the environment. They originate from biosynthesis, natural diagenesis, and human activity (22). Important biological sources of aromatic ring structures are the amino acids phenylalanine and tyrosine and the rigid biopolymer of trees, lignin. Organic matter containing these compounds decay, with the production of aromatic acids. Typically, aerobic bacteria in soils and waters use molecular oxygen and oxygenase enzymes in the further metabolism of these compounds (17). A reaction common to these pathways is the ring cleavage of vicinal dihydroxy aromatic acids with the concomitant incorporation of two atoms of molecular oxygen into the substrate (40). These widespread dioxygenases catalyze reactions cleaving the aromatic ring between the two hydroxyl groups (*ortho* fission) or adjacent to one of the hydroxyl groups (*meta* fission). 4-Hydroxyphenylacetate is derived from aromatic amino acids and lignin decomposition. It is known to be oxidatively metabolized to homogentisate or 3,4-dihydroxyphenylacetate (homoprotocatechuate), and the latter typically undergoes a *meta* ring fission reaction catalyzed by a 2,3-dioxygenase.

Although aromatic ring cleavage dioxygenases are widespread in both gram-negative and gram-positive bacteria (19, 32, 46, 49), most studies have involved gram-negative bacteria. *Pseudomonas* spp. have been most frequently investigated (21, 23, 32). In previous immunological experiments, antibodies prepared against protocatechuate 3,4-dioxygenase from gram-negative *Azotobacter vinelandii* reacted strongly against two isofunctional *Pseudomonas* enzymes and weakly with the dioxygenase from *Acinetobacter calcoaceticus* (19). Similar comparative studies with gram-positive bacteria are needed to better understand biodegradative potential and evolutionary relationships in this group. This is particularly important given that *Arthrobacter* spp. are, numerically, the most prevalent bacteria in most soils (38). Furthermore, *Arthrobacter* spp. have demonstrated diverse catabolic activities with aromatic acids (7, 34), 2-hydroxypyridine (29), nicotine (10), phenanthrene (26), xanthone (12), and phenolic amines (30).

The present study was conducted to develop further insights into aromatic ring cleavage enzymes in gram-positive bacteria. A bacterial strain was taxonomically identified as an *Arthrobacter* sp., and it was denoted strain Mn-1. It metabolized aromatic acids exclusively via *meta* ring cleavage catalyzed by 3,4-dihydroxyphenylacetate 2,3-dioxygenase. The *Arthrobacter* strain Mn-1 dioxygenase was purified to homogeneity, and polyclonal antibodies were prepared. Antibodies were used in Ouchterlony and Western blotting (immunoblotting) protocols to screen diverse, largely gram-positive, soil bacteria. 3,4-Dihydroxyphenylacetate 2,3-dioxygenase activity was found in more than 20 gram-positive bacteria, but only *Arthrobacter* spp. contained antigenically related proteins, most of which were immunologically identical to the Mn-1 dioxygenase. These data suggest an important role for this unique *Arthrobacter* 2,3-dioxygenase in the global metabolism of aromatic acids in soils.

MATERIALS AND METHODS

Bacterial strains and growth. The strains used in this study are listed in Table 1. Unless indicated otherwise, bacteria were grown at 30°C with shaking at 200 rpm in mineral medium at pH 7.0 containing 0.1% (wt/vol) 4-hydroxyphenylacetate as the carbon source (41).

Soil enrichment cultures were inoculated with 1% (wt/vol) garden soil. The soil suspensions were heated at 80°C for 10 min prior to conducting enrichment protocols (15) with minimal medium (41) containing 0.1% (wt/vol) 4-hydroxyphenylacetate as the carbon source.

Dioxygenase mutants of *Arthrobacter* strain Mn-1 were obtained by UV light mutagenesis. Mid-exponential-phase cells were harvested and resuspended in distilled water containing 0.1 M $MgSO_4$. After UV light exposure to give

* Corresponding author.

TABLE 1. Bacteria used in this study

Bacterium	Source	Reference or description
Arthrobacter strain Mn-1	This study	Laboratory culture
Arthrobacter strain Mn-21	This study	Dioxygenase mutant of Mn-1
Arthrobacter strain Mn-21R	This study	Revertant of Mn-21
Arthrobacter strain Mn-40	This study	Dioxygenase mutant of Mn-1
Arthrobacter strain Mn-40R	This study	Revertant of Mn-40
A. globiformis CM-2	This study	Soil enrichment culture
A. globiformis	ATCC 35698	6
A. globiformis	ATCC 8010	13
A. oxydans	ATCC 14358	44
A. simplex	ATCC 6946	45
A. nicotianae	ATCC 15236	45
A. crystallopoietes	ATCC 15481	20
P. aeruginosa PAO1	Ronald Olsen, University of Michigan	16
B. brevis	ATCC 8185	18
B. brevis	ATCC 8186	18
Bacillus sp. strain WF1-1	This study	Soil enrichment culture
Bacillus sp. strain WF1-2	This study	Soil enrichment culture
Bacillus sp. strain WF1-4	This study	Soil enrichment culture
Bacillus sp. strain WF1-8	This study	Soil enrichment culture
Bacillus sp. strain WF1-1B	This study	Soil enrichment culture
Bacillus sp. strain WF1-2B	This study	Soil enrichment culture
Bacillus sp. strain WCH-1	This study	Soil enrichment culture
Bacillus sp. strain WCH-2	This study	Soil enrichment culture
Bacillus sp. strain WCH-3	This study	Soil enrichment culture
Bacillus sp. strain WCH-10	This study	Soil enrichment culture
Bacillus sp. strain WCH-11	This study	Soil enrichment culture
Bacillus sp. strain CM-1	This study	Soil enrichment culture

99% loss of cell viability, a penicillin enrichment procedure was used to elevate the percentage of mutants (11). Cells were plated onto mineral medium containing 0.1% (wt/vol) sodium succinate and 0.1% (wt/vol) 4-hydroxyphenylacetate. The dioxygenase mutants, designated Mn-21 and Mn-40, were selected by observing their accumulation of a diffusible dark-brown pigment around the colonies and their inability to produce the yellow ring fission product when sprayed with a 2% (wt/vol) solution of 4-methylcatechol in ether.

Taxonomic studies. Strain Mn-1 was an unidentified laboratory strain known to express 3,4-dihydroxyphenyl-2,3-dioxygenase. It was a nonmotile gram-positive rod showing V formations and coccoid structures in old cultures. Oxidase and catalase reactions were positive, and the strain hydrolyzed starch and casein. Cell wall extracts were prepared by published procedures (8). Amino acids in the cell walls were determined by ascending paper chromatography (5) and by high-pressure liquid chromatography at the Micro Chemical Facility of the University of Minnesota. Sugars were determined as described previously (8). API-Rapid CH metabolic tests (Analytab Products, Plainview, N.Y.) were performed according to the manufacturer's instructions.

Strain CM-2 obtained from the soil enrichment procedure showed characteristics similar to those described above for *Arthrobacter* strain Mn-1. In the API-Rapid CH test strips (Analytab), CM-2 showed identical reactions in all 49 metabolic reactions with *Arthrobacter globiformis* ATCC 35698, the type strain. The remaining 12 soil isolates were nonbranching aerobic endospore-forming gram-positive rod-shaped bacteria and were denoted as *Bacillus* spp.

In vitro enzyme assays. Cell extracts were prepared by resuspending pelleted cells at a ratio of 1 g (wet weight) of cells to 5 ml of 50 mM potassium phosphate buffer (pH 7.0) and disrupting them with a French pressure cell at 15,000 lb/in^2. The broken cell suspension was centrifuged at 20,000 $\times$ *g* for 10 min at 5°C. The supernatant fluid was used in the enzyme assays described below. Protein determinations were made by using a commercial kit (Bio-Rad Laboratories, Richmond, Calif.) and employing the Coomassie blue method (9). 3,4-Dihydroxyphenylacetate 2,3-dioxygenase activity was measured as previously described (41). One unit of activity is defined as 1 μmol/min. NADPH-dependent 4-hydroxyphenylacetate 3-hydroxylase was assayed by coupling the hydroxylase reaction to the dioxygenase reaction described above. Thus, reaction mixtures of 50 mM potassium phosphate buffer (pH 7.5) contained 120 μM NADPH and 200 μM 4-hydroxyphenylacetate, and the ring fission product was measured at 378 nm as it was for the dioxygenase reaction. $NADP^+$-dependent 5-carboxymethyl-2-hydroxymuconic semialdehyde dehydrogenase was assayed as described previously (7). Homogentisate 1,2-dioxygenase activity was assayed by the method of Sparins and Chapman (46).

Dioxygenase purification. The dioxygenase was purified from crude cell extracts by fast protein liquid chromatography (FPLC) with a Mono Q HR 16/10 anion-exchange column (Pharmacia, Piscataway, N.J.). Crude cell extract was loaded onto the column preequilibrated with 50 mM potassium phosphate buffer (pH 8.0). The bound dioxygenase was eluted with a 2-h linear 0 to 1.0 M KCl gradient at a flow rate of 3.0 ml min^{-1}. Fractions containing dioxygenase activity were desalted by ultrafiltration and loaded onto the same column preequilibrated with 50 mM potassium phosphate buffer (pH 6.3). The dioxygenase was eluted with a linear KCl gradient as described for the first step. The pooled dioxygenase fractions contained a single polypeptide with a molecular weight of 43,000 as determined by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS–12% PAGE) (31, 39).

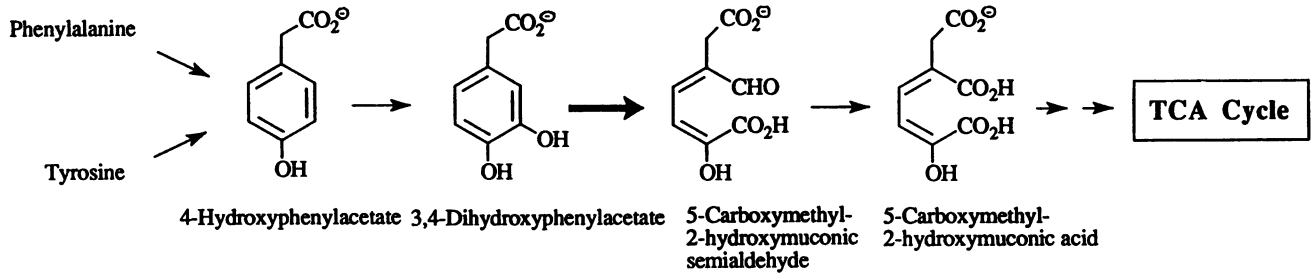

FIG. 1. Proposed pathway for the oxidation of 4-hydroxyphenylacetic acid by *Arthrobacter* strain Mn-1. TCA, tricarboxylic acid.

Immunological methods. Polyclonal antibodies were raised in a New Zealand White rabbit by using purified 2,3-dioxygenase prepared from *Arthrobacter* strain Mn-1. The protein antigen (1 mg in 2 ml of 10 mM potassium phosphate buffer [pH 7.0]) was emulsified in 2 ml of Freund's incomplete adjuvant and injected intradermally. Booster injections were made at weekly intervals until a titer of 1:640 was attained on Ouchterlony plates. Immunoglobulin G (IgG) was purified from antisera by ammonium sulfate precipitation (3).

Ouchterlony double-diffusion analysis was performed as described previously (48). The center well contained 100 μl of a 20-mg/ml IgG solution in potassium phosphate buffer. Antigen solutions to be tested were added to outer wells in 100-μl volumes of crude extract proteins (1 to 10 mg/ml) prepared from the respective bacterial strains. Crude cell extracts were prepared as described previously.

Western blots were performed by using a modification of the general procedures described by Maniatis et al. (33). Crude extract protein (15 μg) from the various bacterial strains were analyzed by SDS-12% PAGE. Gels were blotted onto an Immobilon-P polyvinylidene difluoride (PVDF) transfer membrane (Millipore Corp., Danvers, Mass.). The PVDF membrane was prewetted in methanol for 1 to 5 s, immersed in deionized water for 1 to 2 min, and then equilibrated in transfer buffer (3.03 g of Tris, 14.4 g of glycine in deionized water [each per liter]) for several minutes. The Western blotting was carried out in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) at 26 V and 4°C overnight. The PVDF membrane was then stained with Ponceau S solution (2 g of Ponceau S, 30 g of trichloroacetic acid, 30 g of sulfosalicylic acid [each per liter]) to determine if the proteins were effectively transferred and to visualize the molecular weight standards. The primary antibody (purified IgG fractions) was diluted by $1:10^5$ in the blocking solution (5% [wt/vol] nonfat dried milk, 0.01% antifoam A, 0.02% sodium azide in phosphate-buffered saline) to prevent nonspecific protein-antibody binding interactions. The secondary antibody, goat anti-rabbit IgG (Fc) alkaline phosphatase conjugate (Promega, Madison, Wis.), was diluted by $1:10^4$ in TBST solution (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20 [vol/vol] [pH 8.0]) and reacted with the blot. A 1:2 mixture of the chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium were incubated with the antibody-treated blots for less than 30 s to avoid the appearance of some trace nonspecific reactions. Under these conditions, crude extracts from wild-type *Arthrobacter* strain Mn-1 reacted in the Western blot to yield a single stained band corresponding to the subunit molecular weight of the dioxygenase at 43,000. *Arthrobacter* strain Mn-21, which completely lacked detectable dioxygenase activity, also lacked any protein reactive in the Western blotting method. This result indicated that false positives, which can arise from impure antigen preparations, were not likely in these experiments.

Immunoprecipitation experiments were conducted by incubating 1.5-mg/ml crude extract protein solutions with purified IgG in 50 mM phosphate buffer (pH 8.0) at 25°C for 1 h. Control incubations were run with crude extract protein and buffer alone. After 1 h, the mixtures were centrifuged at 12,000 × *g* for 5 min and assayed for dioxygenase activity.

Materials. Pyridine nucleotides were purchased from Sigma Chemical Co. (St. Louis, Mo.). Other chemicals were obtained from Aldrich Chemical Co. (Milwaukee, Wis.).

RESULTS

Taxonomic studies on *Arthrobacter* strain Mn-1. Strain Mn-1 showed V-shaped cell formation and a rod-turning-to-coccus growth cycle in complex media, and spores were never observed. These structural features are described for members of *Arthrobacter*, *Brevibacterium*, and related genera (25). Cell wall extracts contained lysine, alanine, glutamate, and glycine, but not diaminopimelic acid. The cell wall sugars were determined to be glucose and galactose. These data are all consistent with the identification of strain Mn-1 as an *Arthrobacter* sp. It performed similarly, but not identically, in metabolic tests conducted in parallel with six *Arthrobacter* spp. obtained from the American Type Culture Collection (Table 1). The strain was denoted *Arthrobacter* strain Mn-1.

Metabolism of aromatic acids by *Arthrobacter* strain Mn-1 and derivative mutants. *Arthrobacter* strain Mn-1 grew on various aromatic acids, including L-tyrosine, L-phenylalanine, phenylpyruvate, salicylate, 4-hydroxybenzoate, and 4-hydroxyphenylacetate. The metabolism of 4-hydroxyphenylacetate was examined in greater detail. During growth on this carbon source, NADPH-dependent 4-hydroxyphenylacetate 3-hydroxylase, 3,4-dihydroxyphenylacetate 2,3-dioxygenase, and $NADP^+$-dependent 5-carboxymethyl-2-hydroxymuconic semialdehyde dehydrogenase activities were detected consistent with the metabolic pathway shown in Fig. 1. Homogentisate 1,2-dioxygenase activity could not be detected. Two mutants defective in 3,4-dihydroxyphenyl 2,3-dioxygenase activity were obtained by UV mutagenesis of *Arthrobacter* strain Mn-1. They failed to grow on 4-hydroxyphenylacetate or tyrosine, suggesting that this dioxygenase is essential for the metabolism of these compounds. One mutant, Mn-21, contained no detectable activity, while the other, Mn-40, showed very low activity compared with that of the wild type under various growth regimes (Table 2). Revertants of both strains simultaneously regained the ability to grow on 4-hydroxyphenylacetate or tyrosine.

It was also of interest that significant 2,3-dioxygenase

TABLE 2. 3,4-Dihydroxyphenylacetate 2,3-dioxygenase activity in crude cell extracts of *Arthrobacter* strains Mn-1, Mn-21B, and Mn-40

Growth substrate	Sp act[a] (U/mg)		
	Wild type	Mn-40	Mn-21
4-Hydroxyphenylacetate	1.22	NG[b]	NG
Tyrosine	0.34	NG	NG
Glucose	0.31	0.02	<0.01
Succinate	0.24	<0.01	<0.01
Tyrosine + glucose	0.44	0.04	<0.01
4-Hydroxyphenylacetate + succinate	1.23	0.03	<0.01
Tyrosine + succinate	0.28	0.02	<0.01

[a] Specific activity in crude cell extracts prepared from the wild type and the two mutant strains. Assay described in Materials and Methods.
[b] NG, no growth.

TABLE 3. Immunological characterization of cell extracts containing 3,4-dihydroxyphenylacetate 2,3-dioxygenase activity prepared from soil isolates

Isolate	Strain identity	Immunological data[a]	
		Ouchterlony	Western blot
CM-2	*A. globiformis*	+, Id[b]	+, 43K[c]
WF1-1	*Bacillus* sp.	–	–
WF1-2	*Bacillus* sp.	–	–
WF1-4	*Bacillus* sp.	–	–
WF1-8	*Bacillus* sp.	–	–
WF1-1B	*Bacillus* sp.	–	–
WF1-2B	*Bacillus* sp.	–	–
WCH-1	*Bacillus* sp.	–	–
WCH-2	*Bacillus* sp.	–	–
WCH-3	*Bacillus* sp.	–	–
WCH-10	*Bacillus* sp.	–	–
WCH-11	*Bacillus* sp.	–	–
CM-1	*Bacillus* sp.	–	–

[a] Experiments using rabbit antibody against the *Arthrobacter* strain Mn-1 manganese-containing 3,4-dihydroxyphenylacetate 2,3-dioxygenase.
[b] Id, immunologically identical to the *Arthrobacter* strain Mn-1 dioxygenase.
[c] 43K indicates the presence of a single positive band corresponding to a subunit molecular weight of 43,000.

activity was observed in *Arthrobacter* strain Mn-1 grown on nonaromatic carbon sources. The dioxygenase levels after growth on glucose or succinate were 25 and 20%, respectively, of those observed with cell growth on 4-hydroxyphenylacetate (Table 2). These data differ from those of previous studies with *Pseudomonas* strains in which negligible dioxygenase activities were present in the absence of an aromatic substrate (4).

***Arthrobacter* strain Mn-1 dioxygenase reactions with polyclonal antibodies.** The 2,3-dioxygenase from wild-type *Arthrobacter* strain Mn-1 was purified and used to raise polyclonal antibodies in a rabbit as described in Materials and Methods. The antibodies showed reactions with purified enzyme and crude extracts from *Arthrobacter* strain Mn-1 in Ouchterlony double-diffusion assays and via Western blotting (Fig. 2, lane B). With crude cell extracts, only a band at a molecular weight of 43,000 was observed, consistent with the subunit molecular weight determined for the enzyme.

The mutant strain Mn-21 did not produce any protein reactive with antidioxygenase antibodies (Fig. 2, lane D), and no dioxygenase activity was detected in crude extracts (Table 2). A spontaneous revertant strain was generated. It simultaneously regained dioxygenase activity and produced a 43,000-molecular-weight protein detectable by Western blotting (Fig. 2, lane C). These data indicated the efficacy of the Western blotting method for detecting this unique dioxygenase and suggested that an alternative dioxygenase is not biosynthesized by *Arthrobacter* strain Mn-1. This conclusion was buttressed by immunoprecipitation experiments which resulted in the loss of >95% of the 2,3-dioxygenase activity after treatment of cell extracts with antibody.

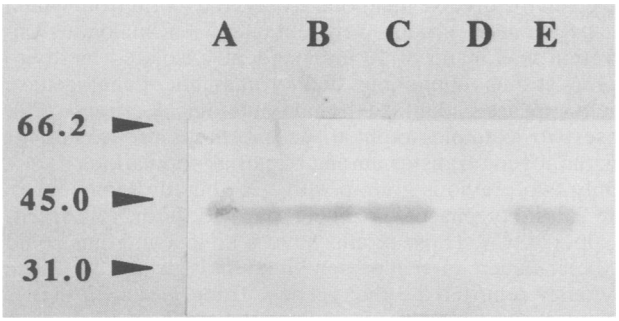

FIG. 2. Western blot analysis of proteins in cell extracts obtained from *Arthrobacter* strains. Lanes: A, *A. globiformis* CM-2; B, *Arthrobacter* strain Mn-1; C, *Arthrobacter* strain Mn-21R; D, *Arthrobacter* strain Mn-21 grown on glucose; E, *Arthrobacter* strain Mn-1 grown on glucose. Unless indicated otherwise, cells were grown on minimal medium plus 4-hydroxyphenylacetate.

Immunological reactions of proteins from soil isolates. Thirteen strains were obtained from soil by enrichment culture with 4-hydroxyphenylacetate as the sole carbon source. All of the strains were shown to contain 3,4-dihydroxyphenylacetate 2,3-dioxygenase activity. One strain was identified as *A. globiformis* CM-2, while the others were shown to be *Bacillus* spp. (Table 3). Only protein extracts obtained with *A. globiformis* CM-2 cross-reacted with polyclonal antibodies raised against the 2,3-dioxygenase from strain Mn-1. A band of identity was observed in the Ouchterlony test (Fig. 3), and the Western blot reaction showed the molecular weight to be 43,000 (Fig. 2, lane A). These data indicated that *A. globiformis* CM-2 contained a protein highly similar to the *Arthrobacter* strain Mn-1 dioxygenase. The 2,3-dioxygenase was further shown to be the antigenic protein by immunoprecipitation experiments which resulted in the loss of >95% of the dioxygenase activity.

Cross-reacting 2,3-dioxygenases in diverse bacteria. The metabolism of 3,4-dihydroxyphenylacetate via 2,3-dioxygenative cleavage has been reported in *Bacillus* spp. (24, 41, 47), various *Pseudomonas* strains (16), and *Arthrobacter* strains (7, 30). Bacterial strains from these genera were tested for immunologically related proteins by using the polyclonal antibodies. The *Bacillus* and *Pseudomonas* strains were negative (Table 4). In contrast, more than half of the *Arthrobacter* strains randomly acquired from the American Type Culture Collection contained immunologically cross-reacting proteins. *A. globiformis* strains and *Arthrobacter oxydans* showed bands of identity in the Ouchterlony analysis and bands at a molecular weight of 43,000 by Western blotting (Table 4). In contrast, *Arthrobacter crystallopoietes* cell extracts contained a significantly lower level of 2,3-dioxygenase activity and failed to yield a precipitin band in the Ouchterlony gel. However, *A. crystallopoietes* produced a protein corresponding to a subunit molecular weight of

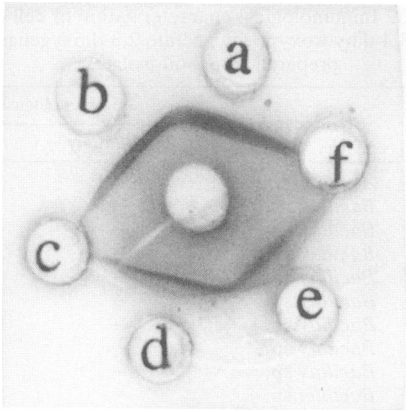

FIG. 3. Ouchterlony double-diffusion analysis of proteins in cell extracts of *Arthrobacter* strains in cross-reaction with polyclonal antibodies raised against 2,3-dioxygenase from *Arthrobacter* strain Mn-1. The letters a to f correspond to protein extracts from *Arthrobacter* strain Mn-1, *A. globiformis* CM-2, *A. simplex*, *A. globiformis*, *Arthrobacter* strain Mn-1, and *A. nicotianae*, respectively. The center well contained IgG raised against the *Arthrobacter* strain Mn-1 dioxygenase.

41,000 detectable in the Western analysis. These observations suggested that the cross-reacting antigen in *A. crystallopoietes* may be a dioxygenase of different subunit molecular weight than that expressed in *Arthrobacter* strain Mn-1. *Arthrobacter simplex* and *Arthrobacter nicotianae* did not grow on 4-hydroxyphenylacetate even when the growth medium was supplemented with 0.02% (wt/vol) yeast extract. When these strains were grown with glucose as the carbon source, no dioxygenase activity was detected and both immunological tests were negative (Fig. 3 and Table 4). When grown on glucose, *Arthrobacter* strain Mn-1 contained significant levels of 2,3-dioxygenase activity (Table 2) and produced a protein with a molecular weight of 43,000 identifiable by Western blotting (Fig. 2, lane E).

TABLE 4. Immunological characterization and 3,4-dihydroxyphenylacetate 2,3-dioxygenase activity of cell extracts from laboratory strains

Strain	Growth on 4-HPA[a]	Dioxygenase sp. act (U/mg)	Immunological data[b]	
			Ouchterlony	Western Blot
A. globiformis ATCC 8010	+	1.7	+, Id[c]	+, 43K[d]
A. globiformis ATCC 35698	+	0.5	+, Id	+, 43K
A. oxydans ATCC 14358	+	1.3	+, Id	+, 43K
A. crystallopoietes ATCC 15481	+	0.1	−	+, 41K[d]
A. simplex ATCC 6946	−	<0.001	−	−
A. nicotianae ATCC 15236	−	<0.001	−	−
P. aeruginosa	+	ND[e]	−	−
B. brevis ATCC 8185	+	0.017	−	−
B. brevis ATCC 8186	+	0.013	−	−

[a] 4-HPA, 4-Hydroxyphenylacetate. Negative strains were grown on 0.2% (wt/vol) glucose; under these conditions, *Arthrobacter* strain Mn-1 gave positive immunological results.

[b] Experiments using rabbit antibodies against the *Arthrobacter* strain Mn-1 manganese-containing 3,4-dihydroxyphenylacetate 2,3-dioxygenase.

[c] Id, band of identity; PId, band of partial identity.

[d] 43K and 41K denote deduced molecular weights of 43,000 and 41,000, respectively, of protein bands reacting with antibody.

[e] ND, not determined; previously established to produce a 2,3-dioxygenase when grown on 4-hydroxyphenylacetate.

DISCUSSION

Most information on 3,4-dihydroxyphenylacetate 2,3-dioxygenase and related enzymes derives from studies of gram-negative organisms. For example, the 2,3-dioxygenase gene from *Escherichia coli* C has recently been cloned and sequenced (42). The instability of that enzyme has precluded its detailed study. By contrast, the 2,3-dioxygenases from several *Pseudomonas* spp. have been extensively investigated (1, 27, 37, 43). Numerous other aromatic ring *meta*-cleavage dioxygenases from *Pseudomonas* spp. have been purified and characterized (2, 32, 36, 38). Recently, information on the comparative structures of these enzymes has emerged. For example, five *Pseudomonas* extradiol dioxygenases with unique substrate specificities were shown to be structurally related by DNA sequence analysis (23). Extending the comparison, intradiol dioxygenases from *Pseudomonas* and *Acinetobacter* spp. were shown to be derived from a common precursor (35).

The present study was conducted to better define 3,4-dihydroxyphenylacetate 2,3-dioxygenase structure, function, and distribution in gram-positive bacteria. The prototype dioxygenase for this investigation was obtained from *Arthrobacter* strain Mn-1. The 2,3-dioxygenase functions in a metabolic pathway reminiscent of those found in *Pseudomonas* (16), *E. coli* (14), and other *Arthrobacter* strains (7, 30). Studies with 2,3-dioxygenase mutants indicated that this is the predominant, if not the sole, aromatic ring cleavage pathway in *Arthrobacter* strain Mn-1. The dioxygenase mutants also served as negative controls for Western blotting experiments; no immunologically cross-reacting proteins were found in *Arthrobacter* strain Mn-21.

Gram-positive strains were screened for 3,4-dihydroxyphenylacetate 2,3-dioxygenase activity as a prelude to immunological investigations. In this study, soils were heated before use in enrichment culture, providing *Bacillus* and *Arthrobacter* strains with 2,3-dioxygenase activity. Culture collection strains which had been isolated from diverse locations were obtained; most of them grew on 4-hydroxyphenylacetate and produced a 2,3-dioxygenase activity. Of the 21 gram-positive wild-type strains examined, 6 of them contained immunologically cross-reacting proteins. All of those were *Arthrobacter* species. Five of the six strains gave immunological reactions indicating that an identical or highly similar protein antigen was present in each. This is remarkable given the diverse locations around the world from which the *Arthrobacter* strains were obtained. An analogous observation was made of 10 independently isolated methylotrophic strains containing dichloromethane dehalogenase which appeared identical by immunological criteria (28). These two examples point to an enormous propensity for horizontal gene transfer among bacterial populations.

Only two previous gram-positive 3,4-dihydroxyphenylacetate 2,3-dioxygenases have been purified, both from *Bacillus* spp. (24, 41). Those strains have been lost and thus could not be included in the present immunological study. The previously reported 2,3-dioxygenase from *Bacillus brevis* is particularly noteworthy since it contained two manganese atoms per holoenzyme, and this metal was implicated in the catalytic mechanism (41). The enzyme purified from *Arthrobacter* strain Mn-1 had a dissimilar subunit molecular weight but was otherwise similar in many properties to the *B. brevis* dioxygenase (39). The *Arthrobacter* strain Mn-1 dioxygenase

contains manganese, and this may be the catalytically relevant metal. The great majority of 2,3-dioxygenases, and aromatic ring cleavage dioxygenases in general, contain nonheme iron (40). The possibility that the *Arthrobacter* strain Mn-1 dioxygenase is a manganese enzyme heightens interest in its distribution. It is anticipated that the *Arthrobacter* dioxygenases will prove to contain unique structural features given that antibodies directed against it failed to react with *Pseudomonas* and *Bacillus* enzymes. Further studies on protein and gene structure should shed light on mechanistic and evolutionary relationships within the unique *Arthrobacter* class of dioxygenases.

ACKNOWLEDGMENTS

We thank Hsien-Chyang Tsien for assistance with the Western blotting and Clay Randall for help with the enzyme purification. We thank Ronald Olsen, University of Michigan, for kindly providing *Pseudomonas aeruginosa* PAO1. The assistance of Louise Mohn and Mary Jo Keefe in the preparation of the manuscript is gratefully acknowledged.

This research was supported by National Institutes of Health grant GM 43315 to L.P.W. and L.Q., Jr.

REFERENCES

1. **Adachi, K., Y. Takeda, S. Senoh, and H. Kita.** 1964. Metabolism of *p*-hydroxyphenylacetic acid in *Pseudomonas ovalis*. Biochim. Biophys. Acta **93**:483–493.
2. **Arciero, D. M., J. D. Lipscomb, B. H. Huynh, T. A. Kent, and E. Münck.** 1983. EPR and Mössbauer studies of protocatechuate 4,5-dioxygenase: characterization of a new Fe^{2+} environment. J. Biol. Chem. **258**:14981–14991.
3. **Ausubel, F. M., R. Brent, R. E. Kinfston, D. D. Maore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Section 11.13.1–11.13.4. *In* Current protocols in molecular biology. Green Publishing Associates, New York.
4. **Barbour, M. G., and R. C. Bayly.** 1981. Control of *meta*-cleavage degradation of 4-hydroxyphenylacetate in *Pseudomonas putida*. J. Bacteriol. **147**:844–850.
5. **Becker, B., M. P. Lechevalier, R. E. Gordon, and H. A. Lechevalier.** 1964. Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. **12**:421–423.
6. **Blakely, E. R.** 1974. The microbial degradation of cyclohexanecarboxylic acid: a pathway involving aromatization to form *p*-hydroxybenzoic acid. Can. J. Microbiol. **20**:1297–1306.
7. **Blakely, E. R.** 1977. The catabolism of L-tyrosine by an *Arthrobacter* sp. Can. J. Microbiol. **23**:1128–1139.
8. **Boone, C. J., and L. Pine.** 1968. Rapid method for characterization of actinomyces by cell wall composition. Appl. Microbiol. **16**:279–284.
9. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
10. **Brandsch, R., A. E. Hinkkanen, and K. Decker.** 1982. Plasmid-mediated nicotine degradation in *Arthrobacter oxidans*. Arch. Microbiol. **132**:26–30.
11. **Carlton, B. C., and B. J. Brown.** 1981. Gene mutation, p. 222–242. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
12. **Chen, C.-M., and P. H. Tomasek.** 1991. 3,4-Dihydroxyxanthone dioxygenase from *Arthrobacter* sp. strain GFB100. Appl. Environ. Microbiol. **57**:2217–2222.
13. **Conn, H. J., and I. Dimmick.** 1947. Soil bacteria similar in morphology to Mycobacterium and Corynebacterium. J. Bacteriol. **54**:291–303.
14. **Cooper, R. A., and M. A. Skinner.** 1980. Catabolism of 3- and 4-hydroxyphenylacetate by the 3,4-dihydroxyphenylacetate pathway in *Escherichia coli*. J. Bacteriol. **143**:302–306.
15. **Crawford, R. L., J. W. Bromley, and P. E. Perkins-Olson.** 1979. Catabolism of protocatechuate by *Bacillus macerans*. Appl. Microbiol. **37**:614–618.
16. **Cuskey, S. M., and R. H. Olsen.** 1988. Catabolism of aromatic biogenic amines by *Pseudomonas aeruginosa* PAO1 via meta cleavage of homoprotocatechuic acid. J. Bacteriol. **170**:393–399.
17. **Dagley, S.** 1975. A biochemical approach to some problems of environmental pollution, p. 81–138. *In* P. N. Campbell and W. N. Aldridge (ed.), Essays in biochemistry, vol. 2. Academic Press, Ltd., London.
18. **Dubos, R. J., and R. D. Hotchkiss.** 1941. The production of bactericidal substances by aerobic sporulating bacilli. J. Exp. Med. **73**:629–653.
19. **Durham, D. R., L. A. Stirling, L. N. Ornston, and J. J. Perry.** 1980. Intergeneric evolutionary homology revealed by the study of protocatechuate 3,4-dioxygenase from *Azotobacter vinelandii*. Biochemistry **19**:149–155.
20. **Ensign, J. C., and S. C. Rittenberg.** 1963. A crystalline pigment produced from 2-hydroxypyridine by *Arthrobacter crystallopoietes*. Arch. Mikrobiol. **47**:137–153.
21. **Furukawa, K., N. Hayase, and K. Taira.** 1990. Biphenyl/polychlorinated biphenyl catabolic gene (bph operon): organization, function, and molecular relationships in various pseudomonads, p. 111–120. *In* Pseudomonas: A. M. Chakrabarty, B. Iglewski, and S. Kaplan, and S. Silver (ed.), Pseudomonas: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
22. **Gibson, D. T.** 1980. Microbial metabolism, p. 161–192. *In* O. Hutzinger (ed.), The handbook of environmental chemistry, vol. 2, part A. Springer-Verlag, Heidelberg.
23. **Harayama, S., and M. Rekik.** 1989. Bacterial aromatic ring-cleavage enzymes are classified into two different gene families. J. Biol. Chem. **264**:15328–15333.
24. **Jamaluddin, M. P.** 1977. Purification and properties of homoprotocatechuate 2,3-dioxygenase from *Bacillus stearothermophilus*. J. Bacteriol. **129**:690–697.
25. **Jones, D., and M. D. Collins.** 1986. Irregular, nonsporing gram-positive rods, p. 1261–1434. *In* P. H. A. Sneath (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
26. **Keuthe, S., and H.-J. Rehm.** 1991. Biodegradation of phenanthrene by *Arthrobacter polychromogenes* isolated from a contaminated soil. Appl. Microbiol. Biotechnol. **34**:804–808.
27. **Kita, H.** 1965. Crystallization and some properties of 3,4-dihydroxyphenylacetate 2,3-dioxygenase from *Pseudomonas ovalis*. J. Biochem. **58**:116–122.
28. **Kohler-Staub, D., S. Hartmans, R. Gälli, F. Suter, and T. Leisinger.** 1986. Evidence for identical dichloromethane dehalogenases in different methylotrophic bacteria. J. Gen. Microbiol. **132**:2837–2843.
29. **Kolenbrander, P. E., and M. Weinberger.** 1977. 2-Hydroxypyridine metabolism and pigment formation in three *Arthrobacter* species. J. Bacteriol. **132**:51–59.
30. **Kutty, R. K., N. A. Devi, M. Veeraswamy, S. Ramesh, and P. V. Subba Rao.** 1977. Degradation of (γ)-synephrine by *Arthrobacter synephrinum*. Biochem. J. **167**:163–170.
31. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
32. **Lee, Y. T., and S. Dagley.** 1977. Comparison of two dioxygenases from *Pseudomonas putida*. J. Bacteriol. **131**:1016–1017.
33. **Maniatis, T., J. Sambrook, and E. F. Fritsch.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Marks, T. S., A. R. W. Smith, and A. V. Quirk.** 1984. Degradation of 4-chlorobenzoic acid by *Arthrobacter* sp. Appl. Environ. Microbiol. **48**:1020–1025.
35. **Neidle, E. L., C. Hartnett, S. Bonitz, and L. N. Ornston.** 1988. DNA sequence of the *Acinetobacter calcoaceticus* catechol 1,2-dioxygenase I structural gene *catA*: evidence for evolutionary divergence of intradiol dioxygenases by acquisition of DNA sequence repetitions. J. Bacteriol. **170**:4874–4880.
36. **Nozaki, M., K. Ono, T. Nakazawa, S. Kotani, and O. Hayaishi.** 1968. Metapyrocatechase. II. The role of iron and sulfhydryl

groups. J. Biol. Chem. **243:**2682–2690.
37. **Ono-Kaminoto, M.** 1973. Studies on 3,4-dihydroxyphenylacetate 2,3-dioxygenase. I. Role of iron, substrate binding and other properties. J. Biochem. **74:**1049–1059.
38. **Paul, E. A., and F. E. Clark.** 1989. Soil microbiology and biochemistry, p. 52–53. Academic Press, Inc., New York.
39. **Qi, B., C. Randall, P. E. Olson, L. Que, and L. P. Wackett.** Unpublished data.
40. **Que, L., Jr.** 1989. The catechol dioxygenases, p. 467–524. *In* T. M. Loehr (ed.), Iron carriers and iron proteins. VCH Press, New York.
41. **Que, L., J. Widom, and R. L. Crawford.** 1981. 3,4-Dihydroxyphenylacetate 2,3-dioxygenase: a manganese(II) dioxygenase from *Bacillus brevis*. J. Biol. Chem. **256:**10941–10944.
42. **Roper, D. I., and R. A. Cooper.** 1990. Subcloning and nucleotide sequence of the 3,4-dihydroxyphenylacetate (homoprotocatechuate) 2,3-dioxygenase gene from *Escherichia coli* C. FEBS Lett. **275:**53–57.
43. **Senoh, S., H. Kita, and M. Kamimoto.** 1966. The role of sulfhydryl group and iron in 3,4-dihydroxyphenylacetate-2,3-dioxygenase, p. 378–389. *In* K. Bloch and O. Hayaishi (ed.), Biological and chemical aspects of oxygenases. Maruzen Co., Ltd., Tokyo.
44. **Sguros, P. I.** 1955. Microbial transformations of the tobacco alkaloids. J. Bacteriol. **69:**28–37.
45. **Skerman, V. B. D., V. McGowan, and P. H. A. Sneath.** 1980. Approved list of bacterial names. Int. J. Syst. Bacteriol. **30:**225–240.
46. **Sparnins, V. L., and P. J. Chapman.** 1976. Catabolism of L-tyrosine by the homoprotocatechuate pathway in gram-positive bacteria. J. Bacteriol. **127:**362–366.
47. **Sparnins, V. L., P. J. Chapman, and S. Dagley.** 1974. Bacterial degradation of 4-hydroxyphenylacetic acid and homoprotocatechuic acid. J. Bacteriol. **120:**159–167.
48. **Stoller, D., and L. Levine.** 1963. Two-dimensional immunodiffusion. Methods Enzymol. **6:**845–854.
49. **Whittaker, J. W., J. D. Lipscomb, T. A. Kent, and E. Münck.** 1984. *Brevibacterium fuscum* protocatechuate 3,4-dioxygenase. J. Biol. Chem. **259:**4466–4475.