

A Marine Oligobacterium Harboring Genes Known To Be Part of Aromatic Hydrocarbon Degradation Pathways of Soil Pseudomonads†

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The far-ranging distribution of genes for aromatic hydrocarbon catabolism, predominantly studied in soil pseudomonads, is extended to a marine oligobacterium by finding five homologous sequences in a 5.7-kb chromosomal DNA from a new isolate, *Cycloclasticus oligotrophus* RB1. RB1 is capable of growth in unamended seawater or mineral salts media supplemented with a variety of aromatic compounds, including toluene, *o*-, *m*-, or *p*-xylenes, as sole carbon sources. The five open reading frames, designated *xylM*, *K*, *G*, *C1*, and *C2*, are 57% A+T-rich. *XylM* is predicted to be an integral membrane protein; *XylK* and *XylG* possess glutathione *S*-transferase (GST) and 2-hydroxy-5-methyl-6-oxohepta-2,4-dienoate dehydrogenase activities, respectively; *XylC1C2* are homologs of the large and small subunits of the iron sulfur protein component of the biphenyl dioxygenase (e.g., BphA1A2).

The majority of marine bacteria (ca. 99.9%) resist cultivation on standard laboratory media (3, 20). Most fall within a group called oligobacteria because of their ability to utilize nutrients at extremely small concentrations. Other properties include a small cell size (mean cell volume, ca. 0.2- μm^3), a small DNA content compared with those of most laboratory-cultured bacteria, and a large affinity for substrate to survive in the dilute pelagic environment of these bacteria (1, 2). Oligobacterium strain RB1 was isolated by dilution culture (3), which favors oligotrophs, and was propagated in unamended seawater. Affinity for acetate was low; however, substantial biomass could be produced from it, and ability to utilize other polar substrates, such as sugars and amino acids, was absent. Small aromatic hydrocarbons were used, and specific affinity for toluene was larger than that for any other organism-substrate combination known. The cells are flagellated short rods that are 0.2 μm^3 in volume and only 1.07 g cm^{-3} , which results in an unusually large surface area-to-dry mass ratio for nutrient collection (2). Recently, a new genus (*Cycloclasticus*) with the type strain *Cycloclasticus pugetii* PS-1 isolated from marine sediments in Puget Sound, Wash., has been described (4). The genus *Cycloclasticus* is a member of the γ subdivision of the *Proteobacteria*; the closest known relative of *C. pugetii* PS-1 is *Methylomonas methanica*, a methane-oxidizing bacterium (4). On the basis of nearly identical 16S rDNA sequences and nutritional properties of RB1 that are similar to those of PS-1, RB1 has been named *Cycloclasticus oligotrophus* RB1 (2). Here, we report the first molecular analysis and biochemical characterization of a gene cluster involved in aromatic hydrocarbon metabolism from RB1.

Culture conditions and growth characteristics. The basic physiological properties of *C. oligotrophus* RB1 will be described separately (2). Briefly, the organism was cultivated in synthetic seawater medium (SAS [20]) or on SAS-1.4% agar plates at 22°C. Small yellow colonies appeared after 2 to 3 weeks of incubation with *m*-xylene (0.5 $\mu\text{l/ml}$ of SAS provided

in a capped tube) as a carbon source. The yellow was likely due to the *meta*-cleavage products of the substrates, since the liquid culture with acetate was white. In SAS with acetate (3 mM) or *m*-xylene, growth of RB1 cells occurred at 5 to 30°C but not at 3 or 35°C. A number of aromatic hydrocarbons, including toluene, ethylbenzene, *o*-, *m*-, and *p*-xylenes, biphenyl (BP), naphthalene, and phenanthrene, supported growth, although toxic concentrations of toluene were easily achieved. Yellow metabolites were produced from all aromatic substrates. Incubation with 4-chlorotoluene and 4-chloro-BP also imparted yellow coloration, but these cells died after 2 to 3 days.

DNA manipulations. RB1 cells were grown in SAS with *m*-xylene for 3 to 4 days. The medium was amended with acetate (3 mM) during the final day to increase the yield of cell mass. The cells were harvested by centrifugation at 6,000 $\times g$ for 10 min. Chromosomal DNA was isolated by the method described by Marmur (12). No plasmid DNA, large or small, was detected by conventional isolation methods and gel electrophoresis (19).

Oligonucleotide primers were derived from two regions of the *Pseudomonas putida* F1 toluene dioxygenase *TodC1* subunit corresponding to peptide positions 214-QFCSDMYHA and 373-EQDDGENWV (24); these primers amplified a PCR product with the expected size (500 bp) from RB1 genomic DNA. Direct sequencing of the PCR product found it to be homologous to the equivalent portion of *todC1*. Both DNA amplification and sequencing were carried out as described previously (22). With this PCR product as a probe, a 5.7-kb *SalI* fragment and a 7-kb *PstI* fragment by a Southern hybridization of RB1 genomic DNA were identified (not shown). The *SalI* fragment was cloned in pBluescript II KS⁻ (Stratagene, La Jolla, Calif.) at the same site with *Escherichia coli* XL1-Blue MRF' (Stratagene) as the host. The recombinant plasmid was named pRB1 and was mapped with various restriction enzymes (Fig. 1A).

Sequence features. The insert of pRB1 was sequenced on both strands (Fig. 2). This DNA contains 5,729 bp, with a G+C content of 42.24%. This value is close to the genomic 41.6% G+C content determined by high-performance liquid chroma-

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† This publication is issued as NRCC number 34521.

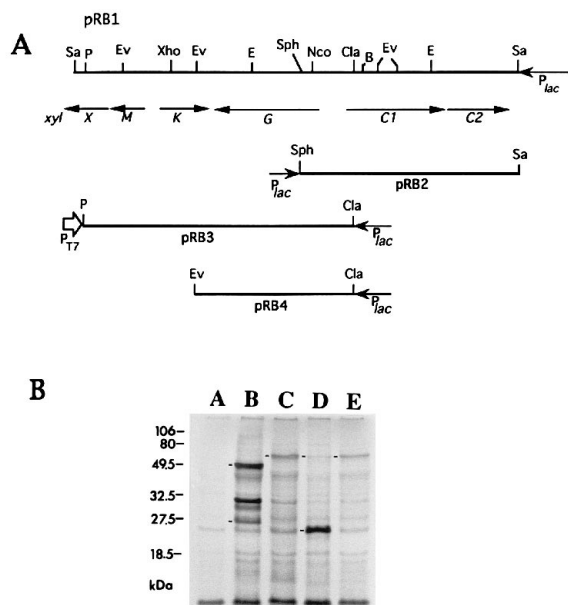


FIG. 1. (A) Restriction map of plasmid pRB1 and its derivatives. Restriction sites: B, *Bam*HI; Cla, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; Nco, *Nco*I; P, *Pst*I; Sa, *Sa*II; Sph, *Sph*I; and Xho, *Xho*I. The *Sph*I-*Sa*II fragment containing *xylC1C2* was subcloned in pUC19 at the same sites to produce pRB2. pRB3 was derived from *Cla*I and *Pst*I deletions of pRB1; pRB4 was an *Eco*RV deletion derivative of pRB3. The orientations of the *lacZ* promoter and T7 promoter from vector pBluescript are indicated. (B) In vitro syntheses of XylKGC1C2. Short bars indicate the expected products. Lanes: A, no DNA added; B, pRB2, which encodes XylC1 (calculated molecular mass, 50 kDa) and XylC2 (22 kDa); C, pRB3, in which the XylG product (53 kDa) was identified; D, pRB3 with additional T7 RNA polymerase, in which XylK (22.5 kDa) was identified in addition to XylG; E, pRB4, which codes for XylG only.

tography analysis of RB1 DNA (14); phage λ DNA (Pharmacia) was used as standard.

Computer analysis of the nucleotide sequence revealed five complete open reading frames (ORFs) and a partial one, designated *xylXMKGC1C2* (Fig. 1 and 2). The *xyl* designation is according to the utilization of all three xylenes as growth substrates and the specific activity of XylG toward a metabolite of xylene (see below). A ribosome-binding site similar to that of *E. coli* in front of each ORF was identified. A striking feature of the sequence is the diverse orientations of the ORFs, implying the presence of multiple transcriptional units. The intergenic spaces are at least 45 bp and contain several stretches of A, T, or A+T sequences and a number of short inverted repeats (6 to 18 bp; Fig. 2), which may be involved in gene regulation, transcription termination, or genome rearrangement.

For protein expression, three pRB1 derivatives were constructed (Fig. 1A) with *E. coli* DH10B (Gibco-BRL) as the host. [³⁵S]methionine-labeled proteins agree well with the predicted sizes of XylKGC1C2 (Fig. 1B; Table 1) with the *E. coli* S30 Extract Systems for Circular DNA Templates (Promega). A band corresponding to the size of XylM was not apparent. The properties and characteristics of XylMGC1C2 are tabulated (Table 1) and elaborated as follows.

XylX and XylM. *xylM* and *xylX*, a partial ORF of 155 amino acids (aa) whose sequence was not similar to any sequence in the available databases, are possibly cotranscribed. A protein similarity search revealed that XylM (136 aa) is most related to the ORF3 in the BP-polychlorinated BP (BP/PCB) degradation pathway of *Pseudomonas pseudoalcaligenes* KF707 (21); the function of this ORF3, as well as that of a related one

(ORF4) in *Pseudomonas* sp. strain KKS102, which is also a BP/PCB degrader (9), is unknown. Our analysis showed that all three proteins are possibly integral membrane proteins. Three programs (HELIXMEM, RAOARGOS, and SOAP) of the PC/GENE package (IntelliGenetics, Inc.) consistently predict the presence of four transmembrane segments in XylM; for ORF3 and ORF4, these are three and four, respectively.

XylK. The location of *xylK* is divergently transcribed from *xylM* with a 177-bp intergenic space; *xylK* is identified as a glutathione *S*-transferase (GST)-encoding gene. This protein is most related to the recently discovered GST (BphK) found for the first time within the BP/PCB degradation pathway in *Pseudomonas* sp. strain LB400 (7) (Table 1). XylK is also 42% identical to GSTs of *E. coli* (SWISS-PROT accession no. P39100) and *Proteus mirabilis* (SWISS-PROT accession no. P15214), both of which form a subgroup of the theta class of the large family of GST isozymes found ubiquitously in nature (15).

For enzyme assays, *E. coli* cells containing recombinant plasmids were grown in Luria broth at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) was added for 2 h when necessary. Cells were harvested, resuspended in cell lysate buffer (50 mM Tris [pH 8.0], 500 mM NaCl), and sonicated. After centrifugation, the supernatant was used as a source of enzyme. XylK GST activity was assayed according to the method described by Habig et al. (6) with 1-chloro-2,4-dinitrobenzene (CDNB; Aldrich, St. Louis, Mo.) as a substrate. The amount of protein was determined with the Bradford reagent (Bio-Rad), with bovine serum albumin as the standard. Because *C. oligotrophus* is a halophile (2), enzyme activities at various concentrations of NaCl ranging from 25 to 1,000 mM were examined. In general, 400 to 700 U (micromoles of product per minute per milligram of protein) of enzyme activity toward CDNB in the cell lysate of *E. coli* BL21 (DE3)(pRB3) that had been induced with IPTG were detected. The optimal salt concentration was ~200 mM, in which the GST activity was ~600 U. GST activity was just as active at 900 mM as at 25

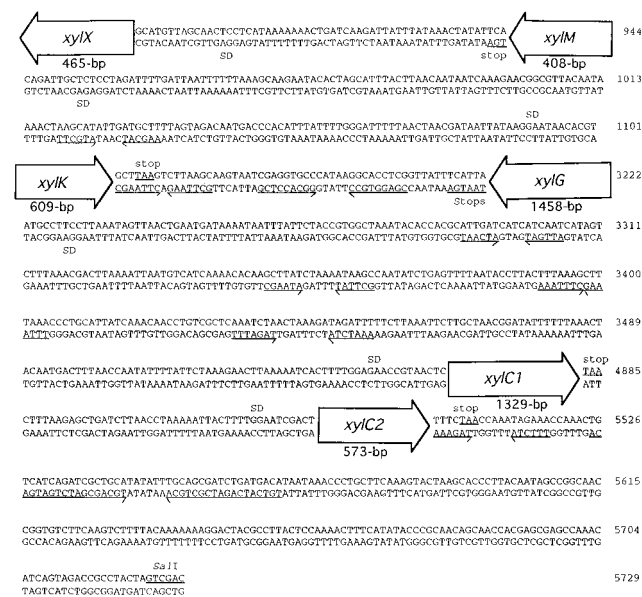


FIG. 2. Nucleotide sequence of the intergenic regions of cloned RB1 ORFs. The directions and sizes (not to scale) of the ORFs are indicated. All ORFs begin with ATG as a potential start codon. Underlining, stop codons; converging arrows, inverted repeats; SD, Shine-Dalgarno sequence.

TABLE 1. Summary of RB1 Xyl polypeptides

Name	Nucleotide position	No. of aa	Molecular mass (kDa)		Closest relative ^a	% Amino acid identity (total similarity)	Protein feature
			Predicted	Experimental			
XylM	944→517 ^b	136	15.143	ND ^c	ORF3 (KF707)	47.8 (55.4)	Membrane protein
XylK	1102→1711	203	22.510	22.5	BphK (LB400)	60.6 (69)	GST
XylG	3222→1764 ^b	486	53.075	58	DmpC (CF600)	68.3 (78.2)	Dehydrogenase
XylC1	3555→4882	443	50.330	49.5	BphA1 (KF707)	67.5 (79.2)	ISP _{RB1} large subunit ^d
XylC2	4931→5507	191	22.405	24	BphA2 (KF707)	63.3 (72.3)	ISP _{RB1} small subunit ^d

^a LB400 and CF600, *Pseudomonas* strains LB400 and CF600, respectively; KF707, *P. pseudoalcaligenes* KF707.

^b Complementary strand.

^c ND, not determined.

^d ISP, iron sulfur protein.

mM NaCl (~400 U). Uninduced cells gave a moderate activity of 30 U in 200 mM NaCl. Under the same conditions, both the plasmidless host cells and *E. coli* DH10B(pRB3) showed only ca. 2 to 3 U of activity, indicating that the RB1 *xylK* promoter was not functional in *E. coli*.

At least two bacterial GSTs are involved in dechlorination (PcpC and DcmA [11, 18]). The gene location of *bphK* in the degradation pathway of BP/PCB in *Pseudomonas* sp. strain LB400 has led to the speculation that BphK may be involved in dechlorination (7). Preliminary experiments indicated that RB1 may not have dechlorination capability.

XylG. *xylG* is a 486-aa ORF convergent with *xylK* and separated by a 45-bp intergenic space. The sequence of XylG is 68% identical to that of 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate (HMOD [Fig. 3]), which is a *meta*-cleavage product of 4-methylcatechol, and some 10-fold less with 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate, which is a cleavage product of catechol. In contrast, XylG_{TOL} oxidizes the toluene metabolite more efficiently than the *p*-xylene metabolite (8). These two chemicals used for enzyme assaying were prepared by incubation of catechol or 4-methylcatechol with cell lysate of *E. coli* containing a *xylE*-related gene cloned from *Sphingomonas paucimobilis* EPA505 (10, 16). XylG activity was assayed according to the method described by Inoue et al. (8) in the

E. coli HB101 containing pRB3 or pRB4 produced the same level of enzyme activity toward 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate (HMOD [Fig. 3]), which is a *meta*-cleavage product of 4-methylcatechol, and some 10-fold less with 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate, which is a cleavage product of catechol. In contrast, XylG_{TOL} oxidizes the toluene metabolite more efficiently than the *p*-xylene metabolite (8). These two chemicals used for enzyme assaying were prepared by incubation of catechol or 4-methylcatechol with cell lysate of *E. coli* containing a *xylE*-related gene cloned from *Sphingomonas paucimobilis* EPA505 (10, 16). XylG activity was assayed according to the method described by Inoue et al. (8) in the

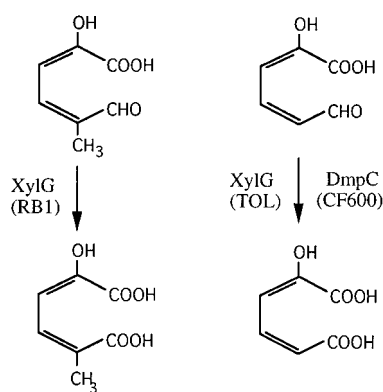


FIG. 3. Reactions carried out by XylG from RB1 and XylG from pWW0 (the TOL plasmid) or DmpC from *Pseudomonas* sp. strain CF600 toward substrate HMOD or 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate.

buffer that was used for GST assaying. The enzyme activity was stable from 50 to 200 mM NaCl (ca. 18 U) but declined at higher concentrations (9 U at 600 mM NaCl). The identification of XylG implies that a *meta*-cleavage mechanism is operational in the RB1 pathway.

XylC1 and XylC2. The C1 and C2 designations of XylC1 and XylC2 are derived from *todC1C2*, which codes for the large and small subunits of the terminal dioxygenase of the three-component toluene dioxygenase (TodC1C2BA) of *P. putida* F1 (24). Amino acid sequence comparisons of the respective subunits gave 63 and 57% identities, respectively. However, comparisons with the BP dioxygenase counterparts gave a slightly higher overall homology; the highest is obtained with BphA1A2 of KF707 (Table 1) or BphAE of LB400.

Furukawa et al. (5) showed that the large subunits of the terminal iron sulfur proteins are responsible for substrate specificity in the metabolism of toluene (*tod* operon) and BP (*bph* operon), whereas the components ferredoxin (e.g., TodB) and reductase (e.g., TodA) are interchangeable. Since the pRB1 clone lacks ferredoxin- and reductase-encoding genes, which are additional components needed for a functional dioxygenase activity, we carried out complementation experiments by introducing *xylC1C2* from pRB2 into *E. coli* HB101 containing pK-todC2BAD, which lacks *todC1* (22). Alternatively, pRB2 was introduced into a *todC1* deletion mutant of *P. putida* F1 (10). In both cases, these cells were unable to transform toluene or BP into the respective dihydrodiols as determined by gas chromatography-mass spectroscopy. The resulting cells also failed to grow on toluene or xylenes. It is not known whether the inability of XylC1 to complement TodC1 is related to the rather unusual sequence context of the Rieske-type iron sulfur center present in the former protein. This sequence is 92-C_HHRGMK_LsRDDAGNAKAFVCTY_H (underlined residues are the conserved Cys and His found in a variety of iron sulfur proteins [13]; the His and Leu-Ser at positions 93 and 99 to 100 [in small capitals] are variations from the consensus residues, which are normally Arg or Thr and Ile-Cys, respectively) (see reference 22 and references therein).

The RB1 XylC2 sequence is also unique among TodC2 and several known Bph isofunctional subunits in having a 69-RTS DLA sequence inserted between the equivalent positions 68 and 69. In *tod*-related systems, a ferredoxin-encoding gene usually immediately follows *todC2* or its equivalents. However, in RB1, this is not evident even to a distance of 221 bp. Two inverted repeats were detected downstream of *xylC2*. One of them is 18 bp long and may function as a transcriptional terminator. It is unknown at this time whether the XylC1C2 could be part of a hydroxylase system which carries out monohydroxylation instead of dihydroxylation reactions (23).

TABLE 2. Codon usage for the genes cloned from *C. oligotrophus* RB1

Amino acid	Codon	No. for the following ORFs:						Total
		<i>xylC1</i>	<i>xylC2</i>	<i>xylK</i>	<i>xylG</i>	<i>xylM</i>	<i>xylX</i>	
Arg	CGA	3	4	0	3	1	2	13
	CGC	7	4	2	6	1	0	20
	CGG	0	3	0	4	0	1	8
	CGT	8	3	2	6	0	0	19
	AGA	3	3	0	2	0	0	8
	AGG	0	0	0	1	0	0	1
Ser	TCA	5	6	7	6	0	2	26
	TCC	0	1	1	5	0	0	7
	TCG	2	1	0	2	0	1	6
	TCT	8	1	4	6	0	3	22
	AGC	5	3	4	3	1	2	18
	AGT	4	3	1	2	1	2	13
Thr	ACA	3	1	4	11	2	1	22
	ACC	10	7	0	10	0	1	28
	ACG	1	1	1	8	2	1	14
	ACT	10	2	2	4	0	2	20
Ala	GCA	8	3	8	13	5	2	39
	GCC	7	2	1	7	1	1	19
	GCG	2	2	2	11	2	1	20
Val	GCT	12	5	8	12	3	5	45
	GTA	3	2	5	10	2	2	24
	GTC	5	0	0	8	3	0	16
	GTG	3	1	2	11	8	3	28
Asp	GTT	11	4	3	22	6	4	50
	GAC	9	8	3	5	0	2	27
Asn	GAT	22	7	8	21	4	8	70
	AAC	12	11	5	4	4	2	38
Gln	AAT	9	4	1	16	0	0	30
	CAA	14	3	6	8	3	3	37
Glu	CAG	5	4	3	2	0	3	17
	GAA	19	10	9	19	1	10	68
Lys	GAG	11	4	6	18	4	7	50
	AAA	19	5	7	19	3	8	61
Leu	AAG	3	1	3	9	1	3	20
	CTA	7	2	4	0	2	0	15
	CTC	2	0	1	0	0	0	3
	CTG	0	5	0	5	2	0	12
Ile	CTT	7	1	7	2	3	0	20
	TTA	11	7	14	13	5	2	52
	TTG	3	1	1	5	8	2	20
	ATA	3	0	0	3	3	1	10
His	ATC	10	3	3	0	4	1	21
	ATT	16	14	6	22	10	2	70
	CAC	8	2	2	5	0	0	17
Pro	CAT	9	3	4	8	2	4	30
	CCA	2	2	4	8	4	6	26
	CCC	5	1	1	4	0	0	11
	CCG	4	0	0	2	1	5	12
Gly	CCT	11	1	8	8	2	5	35
	GGA	5	1	2	6	0	1	15
	GGC	20	4	1	9	4	3	41
	GGG	2	0	0	7	3	2	14
Tyr	GGT	9	0	10	25	4	5	53
	TAC	4	3	5	1	2	2	17
Cys	TAT	9	4	4	8	4	4	33
	TGC	1	0	1	4	0	5	11
Phe	TGT	4	1	1	2	0	8	16
	TTC	9	3	4	3	0	0	19
Trp	TTT	12	8	7	23	4	3	57
	TGG	13	2	3	9	6	1	34
Met	ATG	14	4	2	10	5	9	44
Stop	TAA	1	1	1	1	0	NA ^a	4
	TAG	0	0	0	0	0	NA	0
	TGA	0	0	0	1	1	NA	2
G+C (%)		44	41	40	45	43	46	43
No. of codons		443	191	203	486	136	155	1,618

^a NA, not applicable.

Codon usage. The codon usage for the cloned *C. oligotrophus* RB1 ORFs (Table 2) shows a general preference for codons that have an A or T at the third variable position. Exceptions are CGC for Arg, ACC for Thr, and AAC for Asn. Leu and Ile have a marked preference for TTA and ATT codons, respectively. Rare codons for RB1 genes are likely AGG (for Arg) and CTC (for Leu). The RB1 genes are a potential source of A+T-rich probes for biomonitoring purposes, since probes of this kind are generally derived from G+C-rich genomes such as those of the pseudomonads and rhodococci.

Nucleotide sequence accession number. The sequence reported in this paper together with the coding sequences has been deposited in the GenBank data base (accession no. U51165).

We thank B. Robertson for the help with culturing *C. oligotrophus* RB1, H. Bergeron for DNA sequencing throughput, and E. X. Zhou for HPLC and GC/MS analyses.

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