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Induction of aromatic catabolic activity in *Sphingomonas aromaticivorans* strain F199

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Enzyme induction studies with *Sphingomonas aromaticivorans* F199 demonstrated that both toluene and naphthalene induced expression of both naphthalene and toluene catabolic enzymes. However, neither aromatic compound induced expression of all the enzymes required for complete mineralization of either naphthalene or toluene. Activity measurements in combination with gene sequence analyses indicate that growth on either aromatic substrate in the absence of the other is, therefore, sub-optimal and is predicted to lead to the build-up of metabolites due to imbalance in toluene or naphthalene catabolic enzyme activities. Growth on toluene may be further inhibited by the co-expression of two toluene catabolic pathways, as predicted from gene sequence analyses. One of these pathways may potentially result in the formation of a dead-end intermediate, possibly benzaldehyde. In contrast, either *p*-cresol or benzoate can support high levels of growth. Analyses of promoter region sequences on the F199 aromatic catabolic plasmid, pNL1, suggest that additional regulatory events are modulated through the interaction of BphR with Sigma54 type promoters and through the binding of a regulator upstream of *p*-cresol catabolic genes and *xylM*. We hypothesize that the unusual gene clustering in strain F199 is optimized for simultaneous degradation of multiple aromatic compound classes, possibly in response to the heterogeneous composition of aromatic structures in the fossil organic matter present in the deep Atlantic Coastal Plain sediments from which this bacterium was isolated.

Keywords: aromatic; regulation; toluene; *p*-cresol; naphthalene; degradation

Introduction

Sphingomonas aromaticivorans F199 is capable of degrading a broad variety of aromatic compounds including toluene, all isomers of xylene, *p*-cresol, naphthalene, biphenyl, dibenzothiophene, fluorene, salicylate, and benzoate [16,17]. Two plasmids, designated pNL1 (184 kb) and pNL2 (~l480 kb), were identified in this bacterium. The sequence of pNL1 was recently determined [44], revealing a complex arrangement of genes required for catabolism of all aromatic compounds known to be metabolized by strain F199. At least 13 gene clusters are predicted to encode enzymes associated with degradation of these aromatics. While genes associated with *meta* cleavage of catechol are co-clustered, those associated with oxidation of the primary substrates to catechol intermediates are distributed on multiple gene clusters. Seven different three-component oxygenases are predicted from sequence analysis, assuming that each of the seven different pNL1-encoded oxygenase components can interact with the lone ferredoxin and ferredoxin reductase components encoded by pNL1. The oxygenase components are predicted to occur in six different gene clusters, one of which also includes the ferredoxin component. The ferredoxin reductase is part of a unique transcript. The function of only two oxygenases could be predicted by sequence analysis suggesting that the remaining oxygenases catalyze novel reactions. Genes associated with degradation of naphthalene and biphenyl to catechol are predicted to occur in at least six different gene clusters and those associated with degradation of *m*-xylene to catechol occur on a minimum of four different gene clusters. Genes for degradation of *p*-cresol to *p*-hydroxybenzoate are found in one cluster. It is not known whether additional genes required for further degradation of *p*-hydroxybenzoate are plasmid-encoded.

The DNA sequence of pNL1 regions encoding aromatic catabolic genes shows a remarkable similarity to those in *Sphingomonas* sp strain HV3 [56] and *Pseudomonas* sp strain DJ77 [34,35,49]. A 4010-bp clone from the strain HV3 plasmid, pSKY4, and a 9819-bp region from *Pseudomonas* sp strain DJ77 have 90% DNA identity to pNL1 sequences extending from within *xylJ* through *xylF* and from within *xylC* through *bphK*, respectively. A second region of homology with 92% DNA identity with strain DJ77 covers 1695 bp and extends from within *xylX* into *bphA2c*. A chromosomal region of 28 571 bp in *S. yanoikuyae* B1 [31] also has homology to pNL1 sequences beginning upstream of *bphA2e* and ending downstream of *nahD*. The homologous genes in this region are in the same order and transcriptional direction. The most prominent difference in this region in that pNL1 *orf1038* and *orf1042* are absent in strain B1. DNA identity between genes encoded by pNL1 and the chromosome of *S. yanoikuyae* B1 is between 67% and 86%. Hybridization studies with strain B1 DNA from this region indicate that similar sequences are also found in *S. yanoikuyae* Q1 and the subsurface bacteria, *S. aromaticivorans* strains B0695 and B0522, *S. subterranea* B0478 and *S. stygia* B0712 [32]. Similarities in hybridization profiles suggest that gene sequences from strain Q1 and B1 are closely related, while those from the subsurface strains are more related to each other than to strains Q1 or B1. These findings suggest that the unusual

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co-clustering of genes from different degradative pathways is typical of *Sphingomonas* species that degrade polyaromatic compounds.

Because of the unique clustering of aromatic catabolic genes, these bacteria must possess a highly complex system for regulating gene expression. Presented herein are the results of studies on the response of *S. aromaticivorans* F199 to various aromatic compounds, completed before pNL1 was sequenced. The availability of the pNL1 sequence makes it possible to better interpret our earlier findings and present a preliminary understanding of the regulation of catabolic gene expression in *S. aromaticivorans* F199.

Materials and methods

Bacterial strains

Sphingomonas aromaticivorans strain F199, originally isolated in this laboratory, is also maintained in the US Department of Energy's Subsurface Microbial Culture Collection at Florida State University [2]. *Pseudomonas putida* PpF1 (ATCC 700007) was used as a control in *cis*-dihydrodiol assays [21] and mutant strain 39/D (from J Spain, Tyndall AFB) was used to synthesize toluene *cis*-dihydrodiol [20]. *P. putida* PpG1901 (from IC Gunsalus, University of Illinois) is a Met^- derivative of strain PpG1, contains the naphthalene catabolic plasmid NAH7, and was used as a positive control in indigo formation assays [54]. *P. putida* mt-2 (ATCC 23973) containing the toluene catabolic plasmid pWWO was used as a control for alcohol and aldehyde dehydrogenase assays [6].

Cultural media, growth conditions, and chemicals

S. aromaticivorans F199 was routinely cultured in mineral salts broth (MSB) [51] with 20 mM lactate, or in King's Medium B (KB) [36], with the latter supporting vigorous growth of strain F199 in well-aerated cultures. Cultures were grown in MSB without shaking because previous studies indicated that strain F199 grew optimally in MSB under microaerobic conditions [17]. After incubation for 24 h at 27°C in the presence of 20 mM lactate, aromatic compounds were added directly to the culture at 1 mM to promote induction of gene expression, and incubation was continued for an additional 24–48 h before harvesting cells. For naphthalene induction, several small crystals were added directly to growth media to provide a continuous supply of this compound in solution. All cultures were harvested in mid to late log phase by centrifugation and cells were washed at least 2× in buffer to remove medium and traces of aromatic compound.

Naphthalene, catechol, 3-methyl-catechol, 4-methylcatechol, *o*-, *m*-, *p*-cresol, *p*-hydroxybenzoate, and indigo were purchased from Sigma Chemical Co (St Louis, MO, USA), *o*-, *m*-, and *p*-xylenes, and protocatechuate were from Aldrich Chemical Co (Milwaukee, WI, USA), benzoate was from Mallinckrodt Chemical Works (St Louis, MO, USA), and salicylate was from JT Baker Chemical Co (Phillipsburg, NJ, USA).

Indole conversion to indigo

Indole, prepared in methanol (50 mM), was added to induced cultures to obtain a final concentration of 1 mM. Indigo formation was measured after 24 h incubation by extracting 2 ml of culture with 1 ml of chloroform, centrifuging the mixture briefly to separate the organic and aqueous phases, and measuring the absorbance at 600 nm (A_{600}) of the organic phase. Indigo concentrations were calculated from a standard curve of authentic indigo [12].

Preparation of cell extracts and enzyme assays

Toluene dihydrodiol was obtained by growing *P. putida* 39/D in MSB at pH 7.5 with 0.2% L-arginine as the carbon and energy source and with toluene supplied in the vapor phase. After incubation for 24 h at 22°C, the pH of the culture was adjusted to 8.0 and cells were removed by centrifugation. The culture supernatant was extracted with ethyl acetate (1:1) and dried over anhydrous sodium sulfate. The solvent was removed using a rotary evaporator and toluene dihydrodiol was recrystallized from hexane/acetone.

Extracts of strain F199 cells were prepared by French pressure cell disruption (SLM Instruments, Urbana, IL, USA) of cells in 20 mM phosphate buffer at pH 7.2 with 10% acetone at 4°C. Disrupted cells were centrifuged at $33\,000 \times g$ for 60 min at 4°C to remove cellular debris. *Cis*-2, 3-dihydroxy-1-methylcyclohexa-4, 6-diene (toluene dihydrodiol) dehydrogenase activities were measured spectrophotometrically by following the increase in A_{340} readings associated with the reduction of NAD^+ to NADH in cell extracts by dihydrodiol dehydrogenase [20]. Reaction mixtures consisted of 18 μmol Tris-HCl (pH 8.1), 2.0 μmol NAD^+, 0.4 μmol diol, and cell extract (0.6–1.1 mg protein ml^{-1}) in a total volume of 1 ml. Assays were conducted under N_2 to limit re-oxidation of NADH [50].

Assays of catechol *meta* cleavage activities in strain F199 cell extracts were performed by the method of Gibson [19] as described previously [44]. The substrates tested include catechol, $\lambda_{max} = 375$ nm and $\epsilon = 33\,400$ cm^{-1} M^{-1}; 3-methylcatechol, $\lambda_{max} = 388$ nm and $\epsilon = 13\,400$ cm^{-1} M^{-1}; and 4-methylcatechol, $\lambda_{max} = 382$ nm: $\epsilon = 28\,100$ cm^{-1} M^{-1} [3]. Catechol 1,2-dioxygenase (C12O) activities in cell extracts were determined by measuring the *ortho* ring cleavage product of catechol, $\lambda_{max} = 260$ nm $\epsilon = 16\,900$ cm^{-1} M^{-1} [19]. Protein concentrations were determined using the Pierce (Pierce Chemical Co, Rockford, IL, USA) Protein Assay Reagent.

O_2 uptake measurements

Induced and non-induced cultures were washed twice in 10 mM phosphate buffer at pH 6.8, and O_2 consumption of resting cells was determined using a Yellow Springs Model 5300 Biological Oxygen Monitor (Yellow Springs, OH, USA) at 25°C. For lysates, cells were lysed by pressure cell disruption, as described earlier, but were not centrifuged. Lysates were evaluated for the presence of viable cells by plating 100 μl on KB agar. The total reaction mixture volume was 3 ml and contained 10 mM phosphate buffer at pH 6.8, 3.3 μmol substrate, and cell suspension or lysate (0.1–0.4 mg protein). 0.01 M substrate stocks were prepared in water. For naphthalene, a saturated solution

(~200 μg naphthalene L^{-1}) prepared in buffer was used as the stock solution for O_2 consumption studies. Two milliliters of this stock solution were used in a final volume of 4 ml for polarographic measurements. We experienced considerable variation in O_2 consumption between different cultures induced with the same organic compound although the relative difference in O_2 consumption with different substrates was similar. Due to this between-culture variation, all reported values are for a single measurement corrected for endogenous cell or lysate respiration. For those few analyses where two or more measurements were made on the same culture using the same substrate, the standard error varied between 5 and 30%.

Sequence analysis

Gene clusters shown in Figure 1 were identified as a set of contiguous genes encoded on the same DNA strand that are separated by no less than 50 nucleotides. Prediction of the localization of proteins encoded by genes was computed using the PSORT WWW server (http://psort.nibb.ac.jp:8800/). Predictions of Sigma 54 and Integration Host Factor binding sites were made using Seqscan (http://www.bmb.psu.edu/seqscan/seqform1.htm).

Results

Induction of oxygen consumption by aromatic compounds

Lactate was supplied as the principal carbon and energy source in experiments to obtain cultures at approximately the same stage of growth and cell density. When cultures were grown with *p*-cresol, *m*-xylene, or *p*-xylene alone, the substrate oxidation rates were 2–10 times higher than observed with cells grown with both 20 mM lactate and 1 mM of the aromatic compound as an inducer. When cells were grown with lactate alone, no significant O_2 consumption was observed with the aromatic substrates tested (Table 1).

The highest oxidation rates with toluene, all isomers of xylene, naphthalene, and salicylate were achieved in cells induced with naphthalene. Benzoate oxidation was highest in benzoate-induced cells, but significant levels of oxidation were also seen with naphthalene- and salicylate-induced cells. Oxidation of all cresol isomers was highest in *p*-cresol-induced cells, again with significant induction of oxidation also seen in naphthalene- and salicylate-induced cells. By contrast, oxidation of naphthalene and salicylate by benzoate- and *p*-cresol-induced cells was low. Although

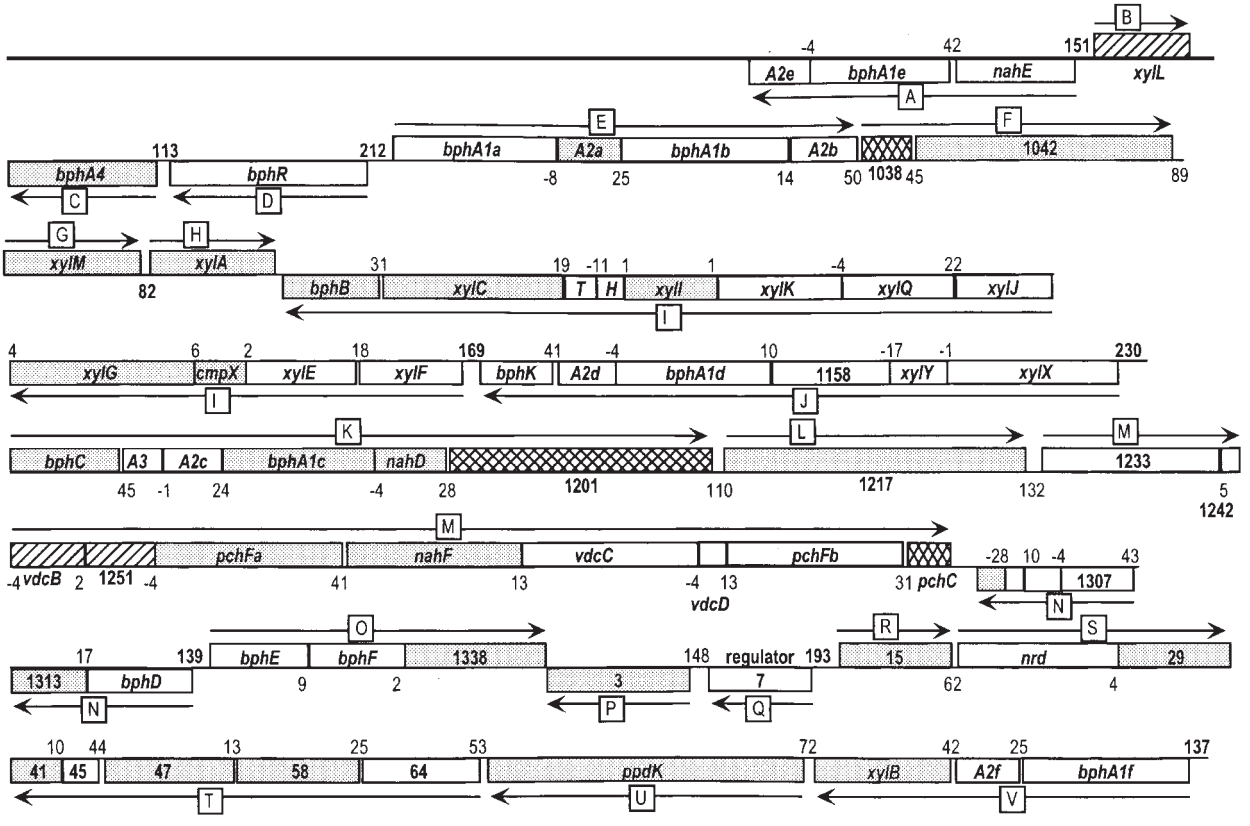

Figure 1 Graphic depiction of the portion of pNL1 that encodes aromatic catabolic genes relevant to this study. Arrows indicate the direction of transcription and predicted length of RNA transcripts. Individual putative transcripts are designated by letters within a box. Numbers shown between genes indicate the number of nucleotides that occur in the intergenic spaces. Genes that encode proteins predicted to reside in the cytoplasmic, periplasmic, or outer membrane are denoted by boxes with shading, diagonal bars, or cross hatches, respectively. Abbreviations used for genes names are *bphA2e* (*A2e*), *bphA2a* (*A2a*), *bphA2b* (*A2b*), *xylT* (*T*), *xylH* (*H*), *bphA3* (*A3*), *bphA2c* (*A2c*), and *bphA2f* (*A2f*).

Table 1 Oxygen consumption of resting cells grown with lactate alone or with various aromatic inducers

Assay substrate	Compound used as inducer[a]										
	None	Toluene	*o*-Xyl	*m*-Xyl	*p*-Xyl	Naph	Sal	Benz	*p*-Cresol	*m*-Cresol	*o*-Cresol
	(nmoles O_2 mg protein^{-1} h^{-1})										
Lactate	**5265**[b]	ND	6246	ND	ND	ND	ND	4908	ND	ND	ND
Toluene	0	**1695**	1829	2320	1383	11288	3212	178	268	ND	ND
o-Xylene	0	1205	**2052**	2097	1115	12225	4060	446	134	ND	ND
m-Xylene	0	2008	ND	**2677**	2097	9905	9503	714	178	ND	ND
p-Xylene	312	1249	1829	3569	**1785**	10574	7272	ND	312	ND	ND
Naph	0	2722	446	2632	1338	**16954**	8923	892	268	ND	ND
Salicylate	178	1026	178	1026	1160	7049	**5577**	1160	268	ND	ND
Benzoate	402	3971	178	1472	1160	6335	5354	**8254**	ND	ND	ND
p-Cresol	89	1026	446	0	178	5666	2722	ND	**15526**	14455	6157
m-Cresol	ND	ND	ND	ND	0	1338	ND	ND	4105	**2766**	402
o-Cresol	0	758	ND	ND	ND	3168	0	ND	580	535	268

[a]All values are corrected for endogenous cell respiration.
[b]Bold denotes values where inducer and substrate are the same, underlined values denote the highest oxidation rates for aromatic substrates.
ND = not determined, Xyl = xylene, Naph = naphthalene, Sal = salicylate, Benz = benzoate.

o-xylene-induced cells demonstrated inducible oxygen consumption with toluene and xylene, inducible oxygen consumption was not evident with the assay substrates naphthalene, salicylate, or benzoate. By contrast, toluene-, *m*-xylene, and *p*-xylene-induced cells demonstrated inducible oxidation of toluene, xylene, naphthalene, salicylate, and benzoate.

Inducible expression of indole oxygenase and diol dehydrogenase activity

The conversion of indole to indigo has been attributed to a variety of oxygenases that catalyze the oxidation of naphthalene [12], toluene [29], styrene [40,42], isopropylbenzene [11], *p*-cumate [9,10], *m*-xylene [8], toluene [55], or dimethyl sulfoxide [25]. A single oxygenase encoded on pNL1, comprised of the products of *bphA1f*, *bphA2f*, *bphA3*, and *bphA4*, is predicted to catalyze this reaction (see Discussion). These genes are encoded in gene clusters C, K, and V (Figure 1). Cells grown on lactate or benzoate did not produce indigo (Table 2). Levels of indigo produced by strain F199 grown on naphthalene, *m*-xylene, and *p*-xylene were similar to amounts produced by strain PpG1901 grown on naphthalene. Detectable levels of indigo were also produced by toluene-, *o*-xylene-, *p*-cresol-, and salicylate-induced cells.

Table 2 Production of indigo from indole by whole cells and levels of toluene dihydrodiol dehydrogenase activity in cell extracts

Strain	Inducer	Indigo concentration[a] (μM)	TDD specific activity (nmol NADH min^{-1} mg protein^{-1})
F199	tolune	32	14.8
	o-xylene	62	ND
	m-xylene	129	14.1
	p-xylene	125	ND
	p-cresol	11	ND
	naphthalene	184	0.03
	salicylate	34	ND
	benzoate	0	ND
	none	0	<0.01
PpG1901	naphthalene	145	ND
PpF1	toluene	ND	30.7

[a]Concentration in chloroform extracts of cultures.
TDD = toluene dihydrodiol dehydrogenase; ND = not determined.

Induction of toluene *cis*-dihydroxydiol dehydrogenase activity was also measured in extracts from these cells before indole was added. This activity is presumably catalyzed by the product of *bphB*, which is in the *meta* catechol cleavage pathway gene cluster I. Both toluene- and *m*-xylene-induced cells catalyzed the dehydrogenation of toluene *cis*-dihydroxydiol, but only low levels of activity were evident in naphthalene-induced cells.

Induction of catechol extradiol and intradiol ring cleavage activity

Extradiol cleavage of catechol is catalyzed by the products of both *bphC* and *xylE* [44] which are encoded in gene clusters K and I, respectively. No homologs to known catechol intradiol cleavage oxygenase genes were found on pNL1; however, the presence of a novel gene whose product catalyzes this activity cannot be ruled out. Extradiol catechol cleavage activity was not detected in cells induced with benzoate or *p*-cresol (Table 3). The highest levels of activity were achieved in cells grown with either toluene or salicylate. Both benzoate and toluene induced expression of intradiol cleavage activity. *Meta* cleavage activity levels in benzoate-induced cells were lower than in non-induced cells, suggesting that benzoate repressed expression of *meta* cleavage activity. Similarly, intradiol cleavage activity was repressed in xylene- and salicylate-induced cells relative to constitutive degradation rates.

Induction of enzyme activities in toluene-grown cells

Induction of catechol extradiol cleavage (*bphC* and/or *xylE*), dihydrodiol dehydrogenase (*bphB*), and alcohol dehydrogenase (*xylB*) activity was mediated by toluene-induced cells (Table 4). No induction of benzaldehyde dehydrogenase (*xylC*) activity was observed. This result was surprising since *xylC* is clustered with *bphB* and, there-

Table 3 Extradiol and intradiol catechol dioxygenase cleavage activities in cell extracts grown with lactate alone or with various aromatic compounds

Strain	Inducer	Relative enzyme activity (nmole min^{-1} mg^{-1})			
		Extradiol[a]			Intradiol[b] Catechol
		Catechol	3-Me-Catechol	4-Me-Catechol	
F199	None	38	62	31	22
	Toluene	1474 (38.8)[c]	ND	ND	64 (2.9)
	o-xylene	588 (15.5)	710 (11.5)	552 (17.8)	9 (0.4)
	m-xylene	606 (16.0)	ND	ND	14 (0.6)
	p-xylene	220 (5.8)	314 (5.1)	176 (5.7)	5 (0.2)
	Benzoate	26 (0.7)	ND	ND	194 (8.8)
	Naphthalene	482 (12.7)	392 (6.3)	243 (7.8)	27 (1.2)
	Salicylate	1496 (39.4)	ND	ND	6 (0.3)
	p-cresol	36 (1)	59 (1)	30 (1.0)	ND
PpF1	Toluene	747	ND	ND	9

[a]Activity from BphC and XylE could not be differentiated in these assays.
[b]Extradiol activity was inhibited with H_2O_2.
[c]Values in parentheses indicate ratio of induced to noninduced activity.
ND = not determined.

Table 4 Enzyme activities in extracts from cells grown with toluene and lactate

Enzyme assayed and assay substrate	Specific activity (nmol min^{-1} mg protein^{-1})[a]
Meta cleavage dioxygenase[b]	
Catechol	57
3-Methyl catechol	9
4-Methyl catechol	24
Dihydrodiol dehydrogenase	
cis-Toluene dihydrodiol	15 (31)[c]
Alcohol dehydrogenase	
Benzyl alcohol	45 (77)[d]
Aldehyde dehydrogenase	
Benzaldehyde	<1 (30)[d]

[a]There was little or no activity for above enzymes in non-induced, lactate-grown cells.
[b]Measurements do not distinguish BphC from Xy1E activity.
[c]Value in parenthesis is for *P. putida* PpF1.
[d]Value in parenthesis is for *P. putida* mt-2 containing the TOL plasmid, pWWO.

fore, these genes are likely to be co-expressed. Possible explanations for the absence of benzaldehyde dehydrogenase activity in toluene-induced cells include: (1) benzaldehyde dehydrogenase activity is lost upon disruption of membranes; (2) benzaldehyde dehydrogenase requires a cofactor other than NAD^+, such as $NADP^+$; (3) a different aldehyde dehydrogenase, not induced by toluene, catalyzes the oxidation of benzaldehyde; or (4) benzaldehyde is not a substrate for aldehyde dehydrogenases produced by strain F199.

Five genes on pNL1: *xylC*, *xylG*, *orf1233*, *nahF*, and *xylQ*, are predicted to encode aldehyde dehydrogenases. A dendrogram depicting the evolutionary relationship to each other and to other known aldehyde dehydrogenases is shown in Figure 2. XylG and XylQ cluster closely with aldehyde dehydrogenases encoded on the toluene catabolic plasmid pWWO [22,26], while NahF clusters with salicylate and vanillin dehydrogenases. None of the aldehyde dehydrogenases cluster with the pWWO benzaldehyde dehydrogenase, XylC [27]. The pNL1 XylC clusters with NAD^+-requiring aldehyde dehydrogenases including *Xanthobacter autotrophicus* GJ10 [5] chloroacetaldehyde dehydrogenases that react with 1,2-dichloroethane aldehyde degradative intermediates and the *Rhodococcus erythropolis* [38] aldehyde dehydrogenase that reacts with dealkylated thiocarbamate degradative intermediates. ORF1233 aligns with various $NADP^+$-requiring succinate semialdehyde dehydrogenases and is the only pNL1-encoded aldehyde dehydrogenase predicted to require a cofactor other than NAD^+. Both XylG and XylC are predicted to reside in the cytoplasmic membrane, while the remaining pNL1-encoded aldehyde dehydrogenases are predicted to reside in the cytoplasm.

In summary, toluene-induced cells do not express a NAD^+-dependent benzaldehyde dehydrogenase with properties similar to the *P. putida* PpF1 benzaldehyde dehydrogenase. Although PSORT analysis of XylC from mt-2 suggests that it resides in the inner membrane, benzaldehyde dehydrogenase activity was detected in our assays. Therefore, the predicted inner membrane localization of strain F199 XylC and XylG is poor evidence for suggesting that cell disruption might destroy aldehyde dehydrogenase activity. Comparisons of the sequences of the three cluster I aldehyde dehydrogenases, XylC, XylG, and XylQ do not support the hypothesis that $NADP^+$ is used as a cofactor by these enzymes. Only the cluster M aldehyde dehydrogenase (ORF1233) is predicted to use $NADP^+$ as a cofactor. Therefore, there is a good possibility that benzaldehyde is not a substrate for pNL1-encoded XylC, XylG, or XylQ. However, further experimentation is needed to test this prediction.

Measurements of oxygen consumption in whole cells vs lysates

Membrane disruption resulted in substantial loss of oxidative activity in *p*-cresol-induced cells (Table 5). This is not surprising in light of the fact that *p*-cresol methylhydroxylase is localized in the periplasm in some Gram-negative bacteria such as *P. putida* [33]. The cytochrome *c* component of the methylhydroxylase (PchC) is predicted to occur in the membrane in both *S. aromaticivorans* F199 (Figure 1) and *P. putida*. Homologs of the methylhydroxylase flavin component, PchFa and PchFb (pNL1), PchF (*P. putida*), and VaoA (*Penicillium simplicissimum*), do not possess a typical N-terminal leader sequence. Analysis of C-terminal residues in PchF suggests that it is translocated across the membrane by a sec-independent pathway [33]. Similarly, C-terminal residues in VaoA suggest that this protein is translocated to the peroxisome [15]. Although similar C-terminal signaling domains are not obvious in PchFa and PchFb, a *sec*-independent secretory pathway may also be responsible for translocation of these proteins across the cell membrane (Figure 3).

Interestingly, O_2 consumption by whole cells with 4-methylcatechol as the substrate was approximately 5-fold

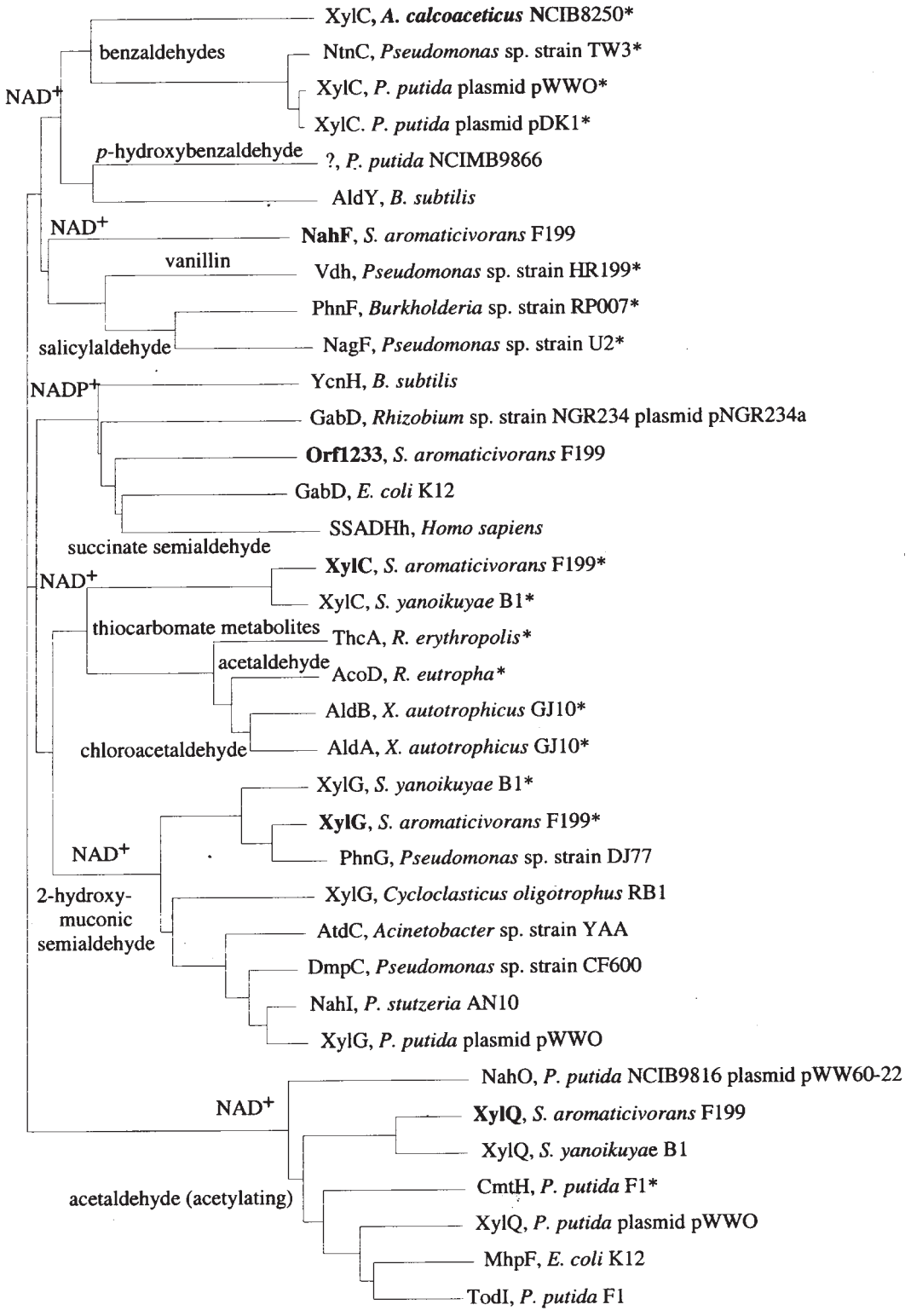

Figure 2 CLUSTALW dendrogram of representative aldehyde dehydrogenase amino acid sequence. The GenBank accession numbers associated with these sequences are as follows: *P. putida* plasmid pWWO (XylC, 1175038; XylG, 139845; XylQ, 486746), *P. putida* plasmid pDK1 (XylC, 2425079), *Pseudomonas* sp strain TW3 (NtnC, 2833676), *Acinetobacter calcoaceticus* NCIB8250 (XylC, 1408293), *P. putida* NCIMB9866 (no gene designation given, 995954), *B. subtilis* (AldY, 1783244), *S. aromaticivorans* F199 plasmid pNL1 (NahF, 3378434; ORF1233, 3378430; XylC, 3378406; XylG, 3378413; XylQ, 3378411), *Burkholderia* sp RP007 (PhnF, 3820515), *Pseudomonas* sp HR199 (Vdh, 1946288), *Pseudomonas* sp U2 (NagF, 3337416), *E. coli* K12 (GabD, 120777; MhpF, 2498557), *Homo sapiens* (SsadhH, 3766467), *Rhizobium* sp NGR234 (GabD, 2494074), *B. subtilis* (YcnH, 1805460), *Ralstonia eutropha* (AcoD, 1168307), *Xanthobacter autotrophicus* GJ10 (AldB, 2660726 and AldA, 2660722), *Rhodococcus erythropolis* (ThcA, 1174662), *Pseudomonas* sp strain DJ77 (PhnG, 2642490), *P. stutzeri* AN10 (NahI, 4104767), *Pseudomonas* sp strain CF600 (DmpC, 118689), *Acinetobacter* sp strain YAA (AtdC, 2627153), *Cycloclasticus oligotrophus* RB1 (XylG, 1354283), *P. putida* F1 (TodI, 485739, CmtH, 1263189), and *P. putida* NCIB9816 plasmid pWW60–22 (NahO, 595672). Sequences from *S. yanoikuyae* B1 were derived from the dissertation thesis of E Kim [31]. Known substrates and cofactors are shown on tree lines. The asterisks refer to proteins predicted to reside in the inner membrane by PSORT analysis.

Table 5 O_2 consumption by whole cells and lysates of cells grown with lactate and toluene or *p*-cresol

Assay substrate	Toluene induced		*p*-Cresol induced	
	Whole cells	Lysates	Whole cells	Lysates
	(nmoles O_2 mg protein^{-1} h^{-1})			
Toluene	2677	223	ND	ND
p-Cresol	ND	ND	7585	312
Catechol	6068	354	755	268
3-Methylcatechol	2989	535	982	357
4-Methylcatechol	2231	625	4461	446

ND = not determined.

higher than with catechol (Table 5). This may be due to O_2 being consumed via oxidation of the methyl group, in addition to ring cleavage, by the *p*-cresol hydroxylase (PchF-PchC) as was observed for *Aspergillus fumigatus* [30] or to induction of a different 1,2 methylcatechol dioxygenase with higher activity for 4-methylcatechol as has been observed in *Trichosporon cutaneum* [43].

Effect of ring substituent type on oxygen consumption

Cells grown with *p*-cresol oxidized only *p*-hydroxybenzoate and *p*-cresol (Table 6). Both 3-methylbenzyl alcohol and naphthalene-induced cells oxidized naphthalene. However, only naphthalene-induced cells mediated oxygen uptake with chlorinated toluene substrates, with no clear preference for substrates chlorinated at different positions. Both chloro- and methyl-benzyl alcohol and methylbenzaldehyde served as oxidation substrates for naphthalene and 3-methyl-benzyl alcohol-grown cells. A slight preference for *meta* and *para* substitutions of either substrate was evident.

Analysis of intergenic sequences of pNL1

BphR is similar to various regulators that control expression of genes involved in catabolism of toluene [7,28], phenol ([1,24,37,39,46]; Takeo, unpublished), phenanthrene, 1,2,4-trimethylbenzene, *m*- and *p*-xylene [14], naphthalene [41], and 2-hydroxy-biphenyl (Schmid and van der Meer, unpublished). Each of these regulators belongs to the NtrC family of transcriptional activators which typically activate expression of genes from promoters recognized by core RNA polymerase associated with the alternative sigma-54 factor.

In order to predict which gene clusters are controlled by BphR, a search for signatures typical of Sigma 54 type promoters in pNL1 was made using Seqscan. This web-based tool uses the scoring matrix described by Schwartz and Mclure [47] to search for both integration host factor (IHF) and Sigma-54 dependent promoter binding sites. The occur-

Figure 3 Alignment of C-terminal residues of pNL1 encoded PchFa and PchFb, VaoA, and PchF. The WKL peroxisomal signal peptide in VaoA is underlined as is the amphiphilic alpha helix of PchF. The double underline denotes the region having high content of hydroxylated amino acids (in bold), serine, threonine.

Table 6 Influence of ring substituent type and position on oxygen consumption by resting cells grown with lactate alone or with various aromatic hydrocarbons

Assay substrate	Aromatic inducer			
	Naphthalene[a]	*p*-Cresol	3-Methyl-benzyl alcohol	None
	(nmoles O_2 mg protein^{-1} h^{-1})			
2-Methylbenzyl alcohol	1606 (0.22)[b]	402 (0.05)	1874 (0.27)	357
2-Chlorobenzyl alcohol	3123 (0.43)	89 (0.01)	1205 (0.17)	268
3-Methylbenzyl alcohol	5398 (0.74)	312 (0.04)	**7005 (1.00)**	357
3-Chlorobenzyl alcohol	5800 (0.80)	223 (0.03)	3346 (0.48)	268
4-Methylbenzyl alcohol	2052 (0.28)	312 (0.04)	8745 (1.25)	848
4-Chlorobenzyl alcohol	7049 (0.97)	402 (0.05)	5399 (0.77)	803
2-Methylbenzyl aldehyde	937 (0.13)	535 (0.07)	2186 (0.31)	535
3-Methylbenzyl aldehyde	8075 (1.11)	1205 (0.16)	8700 (1.24)	2275
4-Methylbenzyl aldehyde	3792 (0.52)	892 (0.12)	8878 (1.27)	1562
2-Chlorotoluene	2454 (0.34)	134 (0.02)	0 (0.00)	268
3-Chlorotoluene	3569 (0.49)	223 (0.03)	893 (0.13)	0
4-Chlorotoluene	2632 (0.36)	134 (0.02)	268 (0.04)	134
Naphthalene	**7272 (1.00)**	ND	6425 (0.92)	ND
p-Cresol	ND	**7585 (1.00)**	89 (0.01)	ND
p-Hydroxybenzoate	312 (0.04)	3123 (0.41)	848 (0.12)	625
Lactate	ND	ND	5265 (0.75)	**4506 (1.00)**

[a]All O_2 consumption measurements within a given column were measured using cells from the same induced culture. Since we typically saw a higher degree of variability between experiments (each column is a different experiment), comparison of values across columns are only significant when they vary greatly (ie 100 vs 1000 nmoles O_2 mg protein^{-1} h^{-1}).
[b]Values in parentheses indicate the ratio of O_2 for cells in the presence of the assay substrate to O_2 uptake for cells with the inducer as the assay substrate. Bold values indicate that the inducer and substrate are the same.

rence of both sites in close proximity is suggested to provide a more reliable prediction of the position of potential Sigma-54 promoters. Intergenic spaces upstream of *repAb*, *tnpA*, *xylL*, *bphD*, and *orf912* contained signatures for both Sigma-54 and IHF binding sites. Binding sites for Sigma-54 alone were found in intergenic regions upstream of *orf003*, *orf574*, *bphA1a*, and *bphR*. *S. yanoikuyae* B1 genomic sequences were similarly analyzed, revealing the concurrence of Sigma-54 and IHF binding sites upstream of the bidirectionally transcribed genes *bphR* and *bphA1a*. When the cognate intergenic regions from pNL1 and strain B1 were aligned, homology was only evident between regions predicted to encode IHF binding sites (upstream of *xylL*, *bphA4*, *xylF*, *bphC*, *xylX*) and the Sigma-54 binding site upstream of *bphA1a*.

A consensus sequence, GAGGGCGGCGnnnnnn CCGCCGCCCTC, containing internal inverted repeats was found 132 bp upstream of the *xylM* start codon and 89 bp upstream of the *orf1233* start codon. This repeat was not found upstream of *xylM* in strain B1 (the region of homology with pNL1 does not extend to orf1233). The positioning and palindromic nature of these repeats suggests that it may the target of a regulatory protein.

Discussion

When studies of the aromatic catabolic properties of *S. aromaticivorans* F199 were initiated, toluene oxygenases were of considerable interest because of their ability to oxidize a broad range of environmental pollutants [48,52,53]. Four of the five currently known toluene catabolic pathways had been described and these analyses were conducted to probe which, if any, of these pathways might be used by *S. aromaticivorans* F199 during growth on toluene. The data from these studies were inconsistent with recognized pathways and difficult to interpret. We now know, based on gene sequence analysis [44], that the results were due to the unusual clustering of catabolic genes on pNL1.

Of all the aromatic compounds tested, toluene was among the poorest in terms of supporting growth of strain F199 [16]. With toluene, the highest cellular growth yield was attained when cells were grown under microaerobic conditions with toluene supplied in the vapor phase [17]. Analysis of the pNL1 sequence suggests that toluene can be degraded by toluene side-chain monooxygenase (XylA–XylM) to catechol by the sequential actions of benzyl alcohol dehydrogenase (XylB), benzaldehyde dehydrogenase (XylC), benzoate dioxygenase (XylX-XylY-BphA3-BphA4), and benzoate *cis*-diol dehydrogenase (XylL) as described for *P. putida* mt-2 [23]. Alternatively, it could be degraded by a toluene dioxygenase (BphA1*-BphA2*-BphA3-BphA4) to toluene-*cis*-dihydrodiol, which can be further degraded to 3-methylcatechol by dihydrodiol dehydrogenase (BphB) as described for *P. putida* F1 [58].

Cluster I, which encodes the entire catechol *meta* cleavage pathway and *bphB* (diol dehydrogenase), is induced by toluene. Toluene *cis*-dihydrodiol was shown to be a substrate for toluene-induced cells by a reaction likely catalyzed by BphB. Cluster V is also induced by toluene, suggesting that the BphA1f-BphA2f-BphA3-BphA4 dioxygenase is present in toluene-induced cells and may catalyze the oxidation of toluene to initiate an F1 type catabolic pathway. There is also evidence that a portion of the mt-2 pathway degrades toluene. Benzyl alcohol dehydrogenase activity (XylB) was evident in toluene-induced cells. Toluene induces oxidation of *m*-xylene, suggesting that transcription of genes encoding xylene monooxygenase, *xylA* and *xylM*, is also elevated in toluene-induced cells. However, these cells did not possess NAD^+ dependent benzaldehyde dehydrogenase activity. Only the ORF1233 aldehyde dehydrogenase is predicted to require a $NADP^+$ cofactor, but the low levels of O_2 consumption from *p*-cresol by toluene-induced cells (Table 1) suggests that genes in cluster M are not induced during growth on toluene. Therefore, if xylene monooxygenase (Xy1A-XylM) can also oxidize toluene, benzaldehyde would be produced by XylB as a dead-end product. Competition for toluene by xylene monooxygenase and subsequent formation of dead-end metabolites may in part explain the poor growth of *S. aromaticivorans* F199 on toluene. Poor substrate specificity by F1 pathway enzymes for toluene and/or its metabolites may also contribute to poor growth on toluene. This possibility is supported by the higher levels of O_2 consumption by toluene-induced cells when catechol was used as the test substrate rather than with toluene (Table 5). These hypotheses are also supported by results from earlier studies on mineralization of ^{14}C-toluene to $^{14}CO_2$. Only 12% of the uniformly labeled ^{14}C-toluene was mineralized to $^{14}CO_2$ by toluene-induced cells [17].

In these same studies, it was demonstrated the toluene-induced cells mineralized more (30% *vs* 12%) ^{14}C-naphthalene to $^{14}CO_2$ than did naphthalene-induced cells [17]. Naphthalene-induced cells did not mineralize ^{14}C-toluene to $^{14}CO_2$. The higher levels of mineralization in toluene-induced cells is likely due to induction of *bphB* and catechol *meta* cleavage pathway genes found in cluster I. Naphthalene-induced cells had very little diol dehydrogenase activity, a function most likely encoded by *bphB*. The ability of naphthalene to support growth in the absence of toluene is, in part, due to low levels of expression of cluster I that occur constitutively. This is supported by the observation that *S. aromaticivorans* F199 grown on one-half strength Luria-Bertani medium expresses catechol *meta* cleavage activity (encoded by either *xylE* or *bphC*), as evidenced by production of yellow metabolites after exposure to catechol. In addition, clones of *bphC* and *xylE* are expressed constitutively in *Escherichia coli* [44].

Genes *bphA1f* and *bphA2f* found in pNL1 cluster V are predicted to encode the substrate binding components of the pNL1-encoded naphthalene dioxygenase since they cluster with other naphthalene dioxygenase binding components [44]. This enzyme is also predicted to catalyze the conversion of indole to indigo. This hypothesis is further supported by the fact that *S. yanoikuyae* B1 mutants in the five gene sets encoding oxygenase binding components, homologous to pNL1 genes *bphA1* (a–b) and *bphA2* (a–b), were not defective in their ability to oxidize naphthalene or biphenyl or to convert indole to indigo (Zylstra, this issue) [57]. The high level of indigo extracted from naphthalene-induced cells suggests that clusters C (*bphA4*), K (*bphA3*), and V (*bphA1f* and *bphA2f*) are induced in these cells. These clusters are also induced in *m*-xylene and *p*-xylene-

induced cells. The lower levels of indigo produced in toluene-, *o*-xylene-, *p*-cresol-, and salicylate-induced cells suggest that these compounds or their catabolic intermediates are poor inducers of naphthalene dioxygenase. The absence of indigo in benzoate-induced cells may be due to repressed expression of genes encoding naphthalene dioxygenase components.

Consumption of oxygen with toluene, xylene, salicylate, and naphthalene was highest in naphthalene-induced cells. This suggests that naphthalene, or a catabolic metabolite, induces expression of genes encoding enzymes that catalyze the initial oxidation of these substrates. The induced clusters necessary for naphthalene dioxygenase activity are V, K, and C, which encode *bphA1f*, *bphA2f*, *bphA3*, and *bphA4*, respectively, and those required for xylene monooxygenase activity are clusters H and G, encoding *xylA* and *xylM*, respectively. High levels of oxygen consumption with benzoate and *p*-cresol substrates by naphthalene-induced cells suggest that cluster J, which encodes the benzoate dioxygenase XylX and XylY substrate binding components, and cluster M, which encodes *p*-cresol methylhydroxylase components PchF and PchC are also induced during growth on naphthalene. Clusters encoding toluene dioxygenase and salicylate oxygenase are also induced, but the identities of the genes encoding these functions is not known.

S. aromaticivorans F199 grows well on *p*-cresol, producing large mucoid cells on agar surfaces and high cell densities in liquid media with this compound as the sole source of carbon and energy. Induction of the *p*-cresol degradative enzymes is not coupled to expression of other aromatic 'upper pathway' catabolic genes encoded on pNL1. The surprising finding that O_2 consumption by *p*-cresol-induced cells provided with 4-methylcatechol are significantly higher than with catechol or 3-methylcatechol suggests that *p*-cresol may be oxidized to 4-methyl catechol rather than, or in addition to, 4-hydroxy benzyl alcohol. The 4-methyl catechol could then be further degraded by pathways described in fungi where either the ring is cleaved by a 1,2-methylcatechol dioxygenase [43] or where the ring undergoes a second oxidation at the methyl group to form 3,4-dihydroxybenzyl alcohol [30].

Homologs to genes that encode the enzymes *p*-cresol methylhydroxylase (*pchFa*, *pchFb*, and *pchC*) and *p*-hydroxybenzaldehyde dehydrogenase (*nahF*) responsible for degradation of *p*-cresol to *p*-hydroxybenzoate are found in cluster M. Interestingly, these enzymes show strong homology to *Penicillium simplicissimum* enzymes, vanillyl-alcohol oxidase and vanillin oxidoreductase, which degrade vanillyl alcohol to vanillic acid via vanillin. The latter compounds are the products of ferulic acid degradation, a common monomer in plant lignins. Although we have not tested ferulic acid as a growth substrate, we have demonstrated that strain F199 can grow on vanillic acid and another lignin degradation product, syringic acid (Fredrickson *et al* this issue). *S. yanoikuyae* strains B1 and Q1 were not able to grow on either compound. Recently, the sequences of three genes responsible for non-oxidative decarboxylation of vanillic acid in *Streptomyces* sp D7 were deposited in Genbank (Accession number AF134589). PNL1 ORFs 1244, 1272, and 1280, also found in cluster M, have extensive homology to the strain D7 vanillic acid catabolic genes, *vdcB*, *vdcC*, and *vdcD*, respectively. Since the products of the D7 genes catalyze the conversion of vanillic acid to guaiacol [59] we can predict that a similar metabolic pathway is utilized by strain F199. Gene homologs responsible for degradation of *p*-hydroxybenzoate or guaiacol were not found on pNL1. The two remaining genes, *orf1242* and *orf1251*, predicted in cluster M have no homologs and are likely candidates for producing an enzyme that catalyzes this activity.

Another question that remains unresolved is the identity of the catechol intradiol cleavage pathway genes. Intradiol catechol cleavage activity is induced in toluene- and benzoate-induced cells (Table 3). Although this activity was not measured in *p*-cresol-induced cells, the low levels of extradiol activity suggest that intradiol cleavage predominates when *p*-cresol is supplied as the growth substrate. O_2 consumption from catechol or methyl-catechol by *p*-cresol- or toluene-induced cells was higher in whole cells than in cell extracts (Table 5). This suggests that the catechol oxygenase expressed during growth on toluene or *p*-cresol was inactivated as a consequence of disrupting cells or that intact membranes are required for activity. Neither extradiol cleavage enzyme (BphC and XylE) is predicted to reside in the membrane (Figure 1). Since no homologs to intradiol cleavage enzymes were found on pNL1 we can only speculate that novel genes encoding this activity also reside on pNL1. The fact that homologs to several of these pNL1-encoded genes are adjacent to genes that encode 'lower pathway' cleavage enzymes supports our speculation that novel genes on pNL1 carry out similar reactions. For example, homologs of the putative regulator encoded by *orf007* are found adjacent to, and in some cases are known to regulate, homogentisate degradative genes in *Pseudomonas aeruginosa* (contig 90, WIT database at http://wit.mcs.anl.gov/WIT/) and protocatechuate 3,4 dioxygenase (contig 92, WIT database), catechol intradiol degradative genes in *Rhodococcus opacus* [13], and the protocatechuate degradative operon in *Acinetobacter* [18]. Similar cross-genomic comparisons can be made with several of the other ORFs that have no ascribed function in aromatic catabolism.

The results of these induction studies suggest that higher levels of naphthalene and toluene mineralization might be achieved if both substrates are used together to induce gene expression. Analyses of putative promoter regions indicative of regulatory binding sites provide additional evidence that gene clusters required for mineralization of naphthalene or toluene are induced by more than one substrate. Interaction of BphR and Sigma 54 with RNA polymerase is predicted to occur only at promoters that direct transcription of clusters D (*bphR*), E (unknown oxygenases and possible cryptic transport proteins), N (*bphD* and unknown dehydrogenases), B (*xyIL*), P (unknown membrane protein), and clusters found in the conjugation and replication regions of the plasmid. In *S. yanoikuyae* B1, Sigma54-mediated regulation is only predicted to regulate expression of *bphR* and the gene cluster encoding *bphAla*, suggesting that expression of aromatic catabolic enzymes in this strain will also require multiple inducers. The putative regulator, encoded by orf007, is likely involved in regu-

lation of additional aromatic catabolic gene clusters. However, evidence of common repeats or palindromes that may serve as the binding target of this protein was only evident upstream of cluster G (*xylM*) and M (*p*-cresol/vanillic acid catabolic genes and unknowns). The linkage of these clusters is unusual, since we expect *xylM* to be necessary only for xylene and toluene degradation and genes on cluster M only to be necessary for *p*-cresol or vanillic acid degradation. Metabolites of toluene 4-sulfonate monooxygenase [4] and toluene-4-monooxygenase [55] feed into the *p*-cresol catabolic pathway. Perhaps pNL1 encodes a novel oxygenase with a Xy1M component that produces aromatic metabolites that also feed into the *p*-cresol pathway.

It is obvious that additional studies are needed to decipher the complex regulatory network that dictates expression of aromatic catabolic genes encoded on pNL1. Whether or not predicted gene clusters correlate with actual operons needs to be established. Since the inducers tested are also substrates of aromatic catabolic enzymes, it is not known if the induction patterns observed were due to the aromatic compound added or to a catabolic metabolite. Biphenyl, lignin model substrates, and mixtures of substrates need to be tested to determine what combination of substrates leads to complete mineralization of aromatic compounds. Efforts are underway to advance our understanding of the regulation of pNL1-encoded pathways by making measurements using the reporter gene encoding green fluorescent protein cloned downstream of pNL1-encoded promoter regions.

The origin of *S. aromaticivorans* F199 in pristine sediments that were buried millions of years ago [45] suggests that the evolution of aromatic catabolic genes on pNL1 probably did not result from the presence of man-made environmental pollutants. Instead, they may have evolved originally in response to the availability of complex mixtures of aromatic compounds that result from humification of plant materials by other organisms. Over time, some ancestors to strain F199 were buried during natural sedimentary processes, and acquired additional adaptations that enabled them to survive in conditions where nutrient availability was poor or infrequent. Other *Sphingomonas* strains remained at the surface or were carried back to the surface in groundwater and gave rise to strains such as B1 that have more recently adapted to the presence of man-made pollutants. These adaptations in strain B1 are evidenced by its better ability to grow at the expense of various aromatic compounds than strain F199. We hypothesize that strain B1 arose through transfer (ie by transposition or illegitimate recombination) of aromatic catabolic genes, from an ancestor shared by strain F199, onto the chromosome. Some genes (*p*-cresol degradation and hypothetical membrane proteins encoded by pNL1 ORFs 1038, 1042, 1201, and 1217) were discarded while new capabilities were acquired (the hypothetical membrane protein, BphX). The regulatory response pathway was also modified to reflect the differing stimuli and substrates available to these strains.

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