

Biphenyl Degradation Genes in *Rhodococcus* Sp. I24

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Polychlorinated biphenyls (PCBs) have been produced on an industrial scale as dielectric fluids, plasticizers, flame retardants, and hydraulic fluids. The proliferation of such products in routine household and commercial use, without any regulated waste procedures, introduced vast amounts of PCBs into the environment. PCBs were manufactured to be extremely stable; this stability of PCBs and related compounds, such as biphenyl, makes breakdown of the compounds difficult and, in turn, hinders their elimination from the environment. The pollution caused by these xenobiotic compounds can potentially be removed by biphenyl-degrading bacteria used in bioremediation (Asturias & Timmis, 1993).

Biphenyl-degrading bacteria, including the genus *Pseudomonas* and *Rhodococcus* sp. strain RHA1 (RHA1), grow on biphenyl by oxidizing PCBs via a biphenyl catabolic pathway (Masai et al., 1995). Until recently, research on biphenyl degradation has focused on Gram-negative bacteria, in particular members of the genus *Pseudomonas* (Masai et al., 1995). The initial work on the degradation of biphenyl by *Rhodococcus* was done on the dioxygenase genes found in *Rhodococcus globerulus* P6 (Asturias & Timmis, 1993). Studies of *Pseudomonas* showed less desirable characteristics for use in bioremediation, which shifted research to *Rhodococcus*, placing more emphasis on studying whether or not *Rhodococcus* could be used for biphenyl and PCB bioremediation (Kitigawa et al., 2001). As shown in Figure 1, the pathway for biphenyl degradation in RHA1 (elucidated by the Fukuda lab in Japan) includes an upper pathway that breaks down biphenyl to benzoate and two lower pathways that lead to the synthesis of succinate. The four enzymes of the upper pathway are coded by seven genes (*bphA1A2A3A4*, *bphB*, *bphC*, *bphD*) and they jointly convert biphenyl to benzoate and 2-hydroxypenta-2, 4-dienoic acid (Masai et al., 1995). This pathway appears to be regulated by a two-component regulatory system including a biphenyl sensor kinase (*bphS*), which phosphorylates a substrate, and its response regulator (*bphT*) (Masai et al., 1995; Kitigawa et al., 2001; Takeda et al., 2004).

Like RHA1, *Rhodococcus* sp. I24 (I24) can grow with biphenyl as its sole carbon source; however, no research has been published on the role of I24 in biphenyl or PCB degradation, leaving this observation uncharacterized. While the phenotype of RHA1 grown on biphenyl is similar to that of RHA1 grown on sucrose, I24 growth on biphenyl does not match I24 growth on sucrose. The observation that only a fraction of I24 can metabolize biphenyl suggests that a change is occurring in the bacteria that exhibit the ability to grow on biphenyl (Bph+). It is unknown whether the source of the Bph+ phenotype in I24 is genetic or caused by an indirect modification (epigenetic). Additionally, I24 grown with benzoate as their sole carbon source match the growth of I24 on sucrose. Thus, if a genetic event enables I24 to catabolize biphenyl, it must be occurring prior to reaching the benzoate intermediate in the pathway.

A Basic Local Alignment Search Tool (BLAST) search, which enables researchers to compare genetic sequences against published sequences for similarities, found that some genes in I24 have high homology with the biphenyl degradation genes of RHA1 that were discovered by the Fukuda lab. The focus of this work was on I24 homologs to *bphS* and *bphT*, the genes believed to regulate the biphenyl degradation pathway. Using Southern analysis, this study sought to find a large-scale genetic event occurring in the I24 genome that affects a region homologous to *bphS* or *bphT* of RHA1 which allows some I24 to metabolize biphenyl.

MATERIALS AND METHODS

Homology search

A BLAST analysis was done to compare a draft sequence of the I24 genome against the published RHA1 genome to find I24 genes that might be involved in the degradation of biphenyl (Altschul et al., 1990; personal communication with J. Parker and P. Lessard). The sequences of the *bphS*, *bphT*, *benA*, *benB*, and *benC* genes of *Rhodococcus* sp. RHA1 were found in the nucleotide database provided by the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

Strains and plasmids

Growth and culture conditions

Frozen stock of I24 was streaked out onto the standard Luria-Bertani (LB) medium (Miller, 1972) plates containing 2% Bactoagar (Difco Laboratories, Detroit, MI). These plates were identified as the source plates for I24. Wild type bacteria were

in DMSO replacing glucose as the carbon source, as described in Stafford et al. (2001). Defined medium plates were made with 4% Bactoagar. Sucrose and benzoate were provided as carbon sources on some defined medium plates while other defined media plates used biphenyl or naphthalene crystals. These growth conditions allowed for the comparison of I24 growth on different media and ensured the selection of biphenyl utilizing bacteria.

DNA manipulations

Genomic DNA of *Rhodococcus* sp. I24 cultures was isolated following Lessard et al. (2004). The Perfectprep Plasmid Midi and Mini kits from Eppendorf (Hamburg, Germany) were used according to the manufacturer's guidelines to prepare plasmid DNA for restriction enzyme analysis.

Construction of *pbphST*

Polymerase chain reaction (PCR) was performed (Lessard et al. 1999) and pCR-Blunt II-TOPO was used to construct a plasmid which contained portions of *bphS* and *bphT*. This construct was used as a marker during the Southern analysis that was carried out on the genomic DNA of two mutant and four wild type I24 bacteria (Priefert et al. 2004). This probe labeled *pbphST* allowed us to determine if the native forms of *bphS* and *bphT* were present in our samples.

Pulsed Field Gel Electrophoresis

Plugs containing I24 genomic DNA were prepared so that the DNA I24 genomic DNA samples could be resolved by size using the CHEF-DR II PFGE (Bio-Rad) apparatus with a 1% agarose gel (Bio-Rad). Pulsed Field Gel Electrophoresis can resolve much longer bands compared to traditional agarose gels and visualize changes occurring on a scale larger than the 10 kilobases that normal gels resolve.

RESULTS

Gene Homology

The BLAST search yielded several genes that were possible homologs to RHA1 genes in I24 (data not shown). The top match for each gene, its location on the I24 genome, and its sequence were identified (Figure 1).

The rate of mutation

To quantify the frequency of Bph+ colonies, several serial dilution experiments were performed. The number of colonies on the sucrose plates, and in turn, the colony forming units (CFU)/mL of the sucrose plates were always greater than that of the biphenyl plates (Table 1). Colonies of I24 that grew on defined media with biphenyl were collected and designated I24-bph. I24-bph colonies that were plated onto defined media plus biphenyl grew at the same frequency as on the plates with sucrose. That is, after I24-bph was grown in defined media with biphenyl and plated onto defined media plates, the number of colonies on biphenyl plates matched that of colonies on sucrose plates. The biphenyl mutants appeared to level off in their numbers, often times not following the expected 10% increase (Table 1). Variability in the CFU/ml of the biphenyl mutants did not allow for the calculation of a

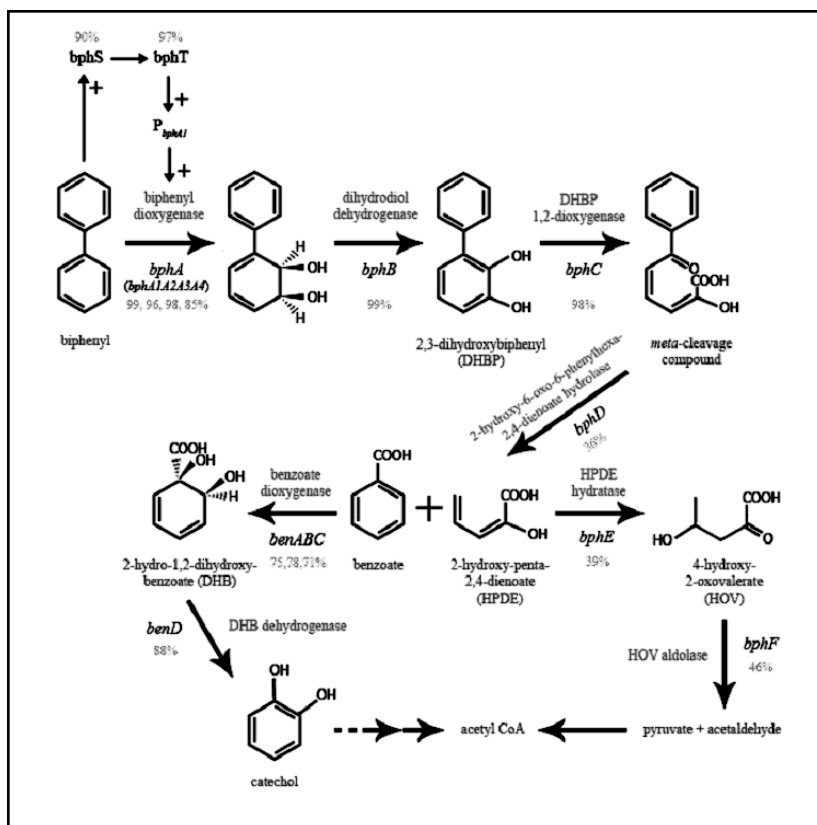


Figure 1 – Proposed biphenyl degradation pathway in *Rhodococcus* sp. RHA1. Intermediates in the pathway are labeled in blue. Enzymes are labeled in orange. Genes encoding enzymes are in dark red. Below each gene name is the percentage of amino acid identity between the RHA1 gene and the best I24 homolog. The two-component system which regulates the degradation reactions is diagramed in purple. Figure adapted from Kitigawa et al., 2001.

Table 1- CFU/mL of I24 grown on defined media with different carbon sources. Plate indicates the sources plate from which the samples were picked.

Plate	Carbon Source	Concentration		
		1x10 ⁻⁵	1x10 ⁻⁶	1x10 ⁻⁷
ACP	Sucrose	507	96	18
	Biphenyl	66	22	5
ACP2	Sucrose	979	82	8
	Biphenyl	57	31	5
TF3	Sucrose	573	60	7
	Biphenyl	51	25	0
TF4	Sucrose	482	68	1
	Biphenyl	58	36	0

specific mutation rate. The range of frequencies was between 0.02% and 62%.

Benzoate on defined media plates

If I24-bph does arise because of a genetic event, then it can affect any of the genes. To test the functionality of the benzoate part of the pathway without performing a Southern analysis, wild-type culture of I24 was grown in LB media and was either plated onto sucrose plates or onto defined media plus sodium benzoate plates. Comparing CFU/mL, the growth on both types of plates was within the same order of magnitude (data not shown).

Southern Analysis

The benzoate data allowed us to use the Southern analysis to test if the location of the regulatory mechanisms coincides with the location of the event causing the Bph+ phenotype. The genomic DNA of a large culture of a biphenyl mutant, of wild type and mutant cultures from the replica plates were digested using restriction enzymes and a Southern analysis was performed. pbphST hybridized to several bands, but no change was detected in the mutants (data not shown). Both sets of Southern analysis showed no obvious change in bphS and bphT at this resolution. The data on the frequency of I24-bph, led to the question of whether or not phenotype changes were being caused by epigenetic versus genetic factors. We approached the question using a second set of enzymes to specifically test methylation patterns. Acc65I and KpnI recognize the same sequence to digest, but Acc65I is sensitive to methylation whereas KpnI is not. The Southern analysis should show subtle differences between Acc65I and KpnI fragments, but due to the low resolution of the gel in the area of interest, nothing definitive could be concluded since the observed difference might be an electrophoresis artifact.

Pulse Field Gel Electrophoresis

Not having found a discernable change using Southern analysis, we tested to see if a change was occurring on the megaplasmid scale. Other species of *Rhodococcus* are known to have mega plasmids which carry functional genes and these megaplasmids can be separated out using pulse field gel electrophoresis (Treadway et al., 1999). We found no discernable differences at this resolution (data not shown). The gel showed similar mobility between the 340 kb bands at this resolution. The 50 kb band was not visible at this resolution.

Toxicity of Biphenyl

The growth of I24 on a sucrose plate, a biphenyl plate, and on a plate with sucrose and biphenyl was compared at each dilution to establish if biphenyl is toxic to I24. The growth on the sucrose plate with biphenyl plate yielded results similar to that of I24 on biphenyl plates (Table 2).

Table 2- Determination of biphenyl toxicity on I24. The table shows the CFU/mL of I24 at differing concentrations of growth media.

Carbon Source	Concentration		
	1x10 ⁻⁵	1x10 ⁻⁶	1x10 ⁻⁷
Sucrose	3510	353	77
Biphenyl & Sucrose	84	74	72
Biphenyl	24	27	30

DISCUSSION

The high homology found between the I24 sequence and the published RHA1 sequence indicates that I24 has the basic genetic code to degrade biphenyl (Aloy et al., 2001). Higher frequencies of I24 that can metabolize biphenyl than actually occur might be expected because of such high homology. The question is why, despite the high homology, are these "mutations rates" not higher. That is, despite the fact that we have high homology between RHA1 bphS and bphT and I24 genes, only a fraction of I24 grew, but not all, grew on plates

with biphenyl as a carbon source. This suggests two possible explanations; first, a change could be occurring in the active domain of a protein, such that despite many amino acids being identical, the enzyme is inactivated. It is also possible that the cause could be epigenetic or stochastic, rather than genetic.

The apparent plateau of the growth of the biphenyl mutants, as seen in table 2, also raises several questions. The ability of I24-bph mutants to grow well on defined media plates with biphenyl shows that are no severely limiting factors in the laboratory conditions. Also, the fact that defined plates with biphenyl which were not plated with I24 showed no growth indicates that the biphenyl was not contaminated with an organism that behaves like I24 or that competes with it. There is no explanation on why this phenomenon is only being observed when wild type cultures are plated onto defined media with biphenyl. This observation could point to part of the process that changes I24.

The growth of I24 on benzoate implies that the pathway that breaks down benzoate to succinate is functional. According to the pathway elucidated by Kitigawa et al. (2001), this result implies that the benABC genes must be functional. The functionality of benABC and the part of the biphenyl pathway that follows the genes might indicate that the mutation that allows I24 to grow on biphenyl is occurring in the first four steps of the biphenyl degradation pathway, in bphS, or in bphT.

The results of the pulse field gel suggest that the Bph+ phenotype does not arise because of a change in the megaplasmid or because I24-bph integrates a megaplasmid into the chromosomal genome. Neither Southern analyses nor the pulse field gel was able to detect direct genetic changes for this phenotype. Nonetheless, it is still possible for a genetic change to be occurring on a scale outside of the resolution of both these experiments.

Since the Southern analysis and the pulse field gel electrophoresis did not identify any discernable genetic differences between I24 and I24-bph, we tested the toxicity of the biphenyl. It is possible for a stochastic event to cause phenotypic noise resulting in the cell to cell variation seen in a clonal population (Elowitz et al., 2002). There could be some compound in the cell which, at a certain level, allows I24 to survive their first exposure to biphenyl. The observation that I24 colonies grown on sucrose and biphenyl appear at similar frequencies as I24 grown on biphenyl as opposed to on sucrose alone suggests that biphenyl is toxic to I24, but that this is independent of I24's ability to degrade biphenyl.

In conclusion, no discernable genetic changes were found in I24 that could grow on biphenyl as compared to wild type I24. The variability in the appearance of the phenotype may be due to the toxicity of biphenyl. Once a colony has been exposed to biphenyl, it can grow at the same rate as I24 on sucrose, suggesting it has a working mechanism for biphenyl degradation. Epigenetic factors, which vary from cell to cell, may allow some cells to have higher amounts of yet to be determined compound, which helps the cell survive the initial exposure to biphenyl. Further research on the toxicity of biphenyl to I24 can lead to engineered I24 that can more

efficiently degrade biphenyl, thus helping in the bioremediation of biphenyl in the environment.

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