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Phylogeny of *Sphingomonas* species that degrade pentachlorophenol

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Four pentachlorophenol (PCP)-degrading bacteria isolated from geographically diverse areas have been examined in detail as regards their physiology and phylogeny. According to traditional biochemical methods, these strains had been classified as members of the genera *Arthrobacter*, *Flavobacterium*, *Pseudomonas*, and *Sphingomonas*. The PCP degradation pathway has been studied extensively in *Sphingomonas* (*Flavobacterium*) sp strain ATCC 39723 and the first three degradation steps catalyzed by a PCP-4-monooxygenase (PcpB) and a reductive dehalogenase (PcpC) that functions twice are well established. A fourth step appears to involve ring-fission of the aromatic nucleus (PcpA). Molecular analyses revealed that the PCP degradation pathway in these four strains was rather conserved, leading to a phylogenetic analysis using 16S rDNA. The results revealed a much closer phylogenetic relationship between these organisms than traditional classification indicated, placing them into the more recently established genus *Sphingomonas* where they may even represent a single species. With 16S rDNA analysis, many bacterial isolates involved in degradation of xenobiotic compounds that were previously classified into diverse genera have been reclassified into the genus *Sphingomonas*.

Keywords: *Sphingomonas*; pentachlorophenol; systematics; phylogeny; *Flavobacterium*

Introduction

Only within the last 15 years have microbiologists finally been able to establish a satisfactory prokaryotic phylogeny. So difficult has this challenge been that it was once suggested as impossible [37]. Traditional methods for determining eukaryotic phylogenies according to cell morphology and physiological characteristics worked reasonably well, but were not readily applicable to the classification of prokaryotes. To complicate things further, microbiologists have only been able to study those microorganisms that they could cultivate. Since cultivable microorganisms are thought to represent at most about 1% of the microorganisms in nature, this shortcoming strongly biased our perception of microbial diversity. Olsen *et al* [24] also pointed out another barrier to developing a phylogenetic system for prokaryotes; namely, the dilemma posed by a primarily 'negative' definition of a prokaryote. Prokaryotes in the past were defined as organisms lacking the features characteristic of eukaryotes. In other words, if it is not a eukaryote, it must be a prokaryote. The characterization of rRNA nucleotide sequences in the early 1980s changed everything in a dramatic fashion.

Macromolecules had been used to some degree to examine phylogenetic relationships of organisms since the 1950s. Interest in this approach peaked with the publication of 'Molecules as Documents of Evolutionary History' by Zuckerkandl and Pauling [51]. However, the real paradigm shift in the study of prokaryotic phylogeny [8] actually began in the 1970s, when microbiologists started to examine rRNA sequences as determinants of phylogenetic relationships. This change came about after it was realized that ribosomal sequences are well conserved even over geological time scales, and are present in all genomes irrespective of organism type [44].

The prokaryotic ribosome contains a 23S rRNA (~2900 nucleotides) and a 5S rRNA (~120 nucleotides) which are associated with about 30 ribosomal proteins to form the large ribosomal subunit. A 16S rRNA is associated with 20 ribosomal proteins to form the small ribosomal subunit. The actual size of the '16S molecule' may vary between 15S and 18S, or 1500–1900 nucleotides [27]. It is the highly conserved region of this 16S sequence that has become the standard for phylogenetic comparisons of living organisms. As of July 31 1998, more than 30 000 organisms have been placed in the Ribosomal Database Project (RDP) SSU rRNA data files, with approximately 10 000 of these sequences available in aligned form and placed on a phylogenetic tree [20]. From this information, a whole new prokaryotic domain, the Archaea, has been defined to go along with the domains of Eucarya and Eubacteria. Woese and Fox [45] described Archaea as more closely related to the eukaryotic domain than to the Eubacteria. Forterre [7], however, warned that more work is needed before the universal tree can be described indisputably. Discussion also continues on whether the Archaea represent a monophyletic group [7]. Despite the need for filling in the details, 16S rRNA based phylogeny is the new paradigm. For now, this is the way we look at individual microbes and their relationship to one another and the rest of the microbial world [4].

Regardless of progress in establishing phylogenetic relationships, even the definition of a bacterial species has not been established unequivocally. Stackebrandt and Goebel [34] suggested that a 2.5% difference in 16S rRNA sequences should be sufficient to distinguish two sequences as belonging to two species. Others suggest that a 70%

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identity in DNA/DNA hybridization experiments is needed for classification as one species [41]. In addition, other relevant data can be obtained from fatty acid methyl ester analysis (FAME). White [42] suggested that such supplementary data should always accompany a 16S rRNA analysis.

Clearly, the results of 16S rRNA analyses have already led to reclassification of many species of bacteria, and to establishment of numerous new genera. One such newly established genus is the genus *Sphingomonas* [50]. Many organisms previously classified as members of the Gram-negative genera *Beijerinckia*, *Pseudomonas*, and *Flavobacterium*, and the Gram-positive genus *Arthrobacter* are being reclassified as members of this new genus. Takeuchi *et al* [38] established the phylogeny of the genus *Sphingomonas* as a non-photosynthetic member of the α-4-subclass of the *Proteobacteria*, formerly 'purple bacteria and their relatives' [35]. In traditional terms, the genus *Sphingomonas* groups Gram-negative, non-spore-forming, aerobic, yellow-pigmented, straight rods [50]. The yellow pigmentation in *Sphingomonas* is due to the carotenoid nostoxanthin, rather than to the more commonly occurring brominated arylpolyene (responsible for the yellow color in members of the genus *Xanthomonas*) [11]. Also, members of the new genus are characterized by the presence of sphingoglycolipids. Though common in membranes of eukaryotic organisms, sphingoglycolipids had not previously been found in prokaryotic cells. *Sphingomonas* species also contain octadecanoic acid, 2-hydroxymyristic acid, *cis*-9-hexadecenoic acid, and hexadecanoic acid as major fatty acids [50], the ubiquinone Q10 as the major respiratory quinone, and DNA containing 62–67% GC. Another aspect of the genus *Sphingomonas* is the secretion of gellan-related polysaccharides [2,29,30]. Finally, a comparison of the genus *Sphingomonas* and *Pseudomonas aeruginosa* shows that in the 16S rRNA sequence of the region between 1220 and 1376, nucleotide 1290 is deleted in the 16S rRNA of members of the genus *Sphingomonas*. *Sphingomonas paucimobilis* (NCTC 11030), a pathogenic clinical isolate [10], was proposed as the type species. Other proposed species include *S. adhaesiva*, *S. parapaucimobilis*, and *S. yanoikuyae* [18]. The last named is a strain that lacks the usual yellow pigment. In the same report it was concluded that the non-motile '*Flavobacterium capsulatum*' (type strain ATCC 14666, isolated from distilled water) should be reclassified *Sphingomonas capsulata*. However, the complete organization of the genus has not been established unequivocally [40]. Takeuchi *et al* [38] suggested that the genus *Sphingomonas* be divided into two subgroups; Ederer and Crawford [4] expanded upon these arguments.

The genus *Sphingomonas* groups together an interesting collection of bacteria encoding numerous unusual anabolic and catabolic pathways. Therefore, a better understanding of the phylogeny of this genus, its members, and their respective metabolic pathways will be of continuing interest to both clinical and environmental microbiologists. Many *Sphingomonas* spp can be isolated from oligotrophic environments like the ocean [6], distilled water [18], as well as from soil and/or water [29], and clinical environments [10]. Often they are best grown on dilute media such as 1/10 strength tryptic soy agar (Ederer, unpublished observation). Since the genus *Sphingomonas* was established in 1990, many bacteria capable of degrading anthropogenic compounds have been (re)classified into this genus [4]. Interestingly, there seems to be a relationship between the genus *Sphingomonas* and the capability of bacteria to degrade the xenobiotic compound pentachlorophenol (PCP).

As discussed earlier [4], past research in our laboratory has focused on PCP degradation by *Sphingomonas* (*Flavobacterium*) sp strain ATCC 39723 [33]. The degradative pathway used by this organism is now mostly known (see below). The identification of three other PCP-degrading organisms, *Arthrobacter* sp strain ATCC 33970 [36], *Pseudomonas* sp strain SR3 [32], and *Sphingomonas* (*Pseudomonas*) sp strain RA2 [31] led us to perform a phylogenetic analysis of these organisms and their genes encoding the PCP degradation pathway(s). These four organisms were isolated from geographically diverse areas (Table 1). Their distant phylogenetic relationships as indicated by their classifications led us to suspect horizontal gene transfer between members of these species in the loci involved in PCP degradation [3]. However, our group [5] and others [14,23] found that these four PCP-degrading organisms were not only very closely related, but might even represent a single species.

Bacterial degradation of PCP

Bacterial PCP degradation is thought to be mediated by two different pathways. The first pathway utilized by two '*Rhodococcus chlorophenolicus*' isolates (PCP-1 and CP-1) was described by Juha *et al* [12] and by Häggblom *et al* [9]. These bacteria produce 2,3,5,6-tetrachloro-*p*-hydroquinone as an intermediate. Uotila *et al* [39] found that the first degradation step, the *para* hydroxylation of PCP, was catalyzed by a cytochrome P450 enzyme. Little is known of the remainder of this pathway.

The second pathway was first studied in *Sphingomonas* (*Flavobacterium*) sp strain ATCC 39723, isolated from a PCP-contaminated soil in Minnesota [33]. The initial catabolic step is catalyzed by a PCP-4-monooxygenase encoded by the *pcpB* gene [25], which has a wide substrate range [48] and significant similarity at both protein and nucleotide sequence levels with other monooxygenases [26]. The second and third catabolic steps are catalyzed by a reductive dehalogenation by PcpC with glutathione as a cofactor [49].

The next pathway enzyme is encoded by the PCP-inducible gene (*pcpA*) [47], since *pcpA* mutants accumulate 2,6-dichlorohydroquinone [1]. Lee and Xun [17] proposed a chlorohydrolase activity for PcpA catalyzing the conversion of 2,6-dichlorohydroquinone. A recent paper by Xu *et al* [46] presents evidence that *pcpA* encodes a 2,6-dichlorohydroquinone dioxygenase, rather than a chlorohydrolase. The evidence for the latter reaction is reasonably convincing, though a few points of confusion remain. There is an apparent difference of about 11.5% in the molecular weight of the PcpA protein as reported by Lee and Xun [17] and Xu *et al* [46]. Also, the product produced by the proposed oxygenase-catalyzed cleavage of 2,6-dichlorohydroquinone has not yet been isolated. Xu *et al* [46] perfor-

Table 1 Phenotypes of the PCP-degrading strains. + or − indicates the presence or absence of a characteristic. The presence of sphingolipids and octadecenoate is indicative of the genus *Sphingomonas* [4]. Δ1290 represents a deletion of nucleotide 1290 in the 16S rRNA gene (numbering with respect to the *E. coli* gene)

Strain	Origin (USA)	PCP[a] mg L^{-1}	Shape	Gram stain	Pigment	Motility	Fimbriae	Sphingolipids	Octadecenoate	Q10[b]	Δ1290	% GC
ATCC 33790	NY	300	rod	neg	+	–	nd	+	62	+	+	66 ± 1
ATCC 39723	MN	100–200	rod	neg	+	–	+	+	57	+	+	66 ± 1
SR3	FL	175	rod	neg	–	+	+	+	59	+	+	64.2
RA2	CO	300	rod	neg	+	+	nd	+	62	+	+	64 ± 1

[a]Highest concentration shown to be degraded.
[b]The presence or absence of ubiquinone Q10 in the respiratory chain.

med sophisticated multiple sequence alignments of peptides produced from their pure protein as compared to a 'PcpA subgroup' of proteins in the NCBInr protein database. These comparisons were suggestive of a relationship of PcpA to extradiol dioxygenases. Thus, additional work is needed to determine conclusively the exact reaction promoted by PcpA. Characterization of the PCP pathway ring-fission product is particularly needed.

Downstream of *pcpB* reading in the same direction, Lange [16] found two further open reading frames possibly involved in PCP degradation, *pcpD* and *pcpR*. The open reading frame (ORF) of *pcpD* showed high similarity to the ORFs of different oxygenase reductases and may transfer electrons from NADPH through a redox center to the flavine adenine dinucleotide group of the PCP-4-monooxygenase. The second ORF, *pcpR*, showed significant similarity with the nucleotide sequences encoding a group of regulatory proteins called the LysR type positive regulators [16]. These similarities have been studied only by nucleotide and amino acid alignments; the actual activities remain to be determined. Figure 1 shows the PCP degradation pathways discussed above. McCarthy *et al* [22] found that *Sphingomonas chlorophenolica* strain RA2 degraded PCP via a degradation pathway identical to that identified in *Flavobacterium* (*Sphingomonas*) sp strain ATCC 39723. A detailed review of microbial PCP degradation has been published [21].

Classification of four PCP-degrading *Sphingomonas* strains

Though the ability to degrade PCP had been considered to be a trait widely distributed in the bacterial kingdom, three independent studies recently questioned this assumption [5,14,23]. The authors of all three studies concluded that four PCP-degrading organisms previously identified as *Arthrobacter, Flavobacterium, Pseudomonas,* and *Sphingomonas* should be considered members of the genus *Sphingomonas,* and may possibly even constitute one species within the genus. The four strains seemed to be more closely related to each other than to the other members of the genus [4]. In support of a close relationship between these strains was the observation that the nucleotide sequences of the *pcpB* gene, encoding PCP-4-monooxygenase, were identical in three of the isolates and only slightly different in the fourth (*Arthrobacter* sp ATCC 33970) [5]. Studies of the *pcpC* gene, which encodes 2,3,5,6-tetrachloro-*p*-hydroquinone (TeCH) dehalogenase, revealed a lower degree of genetic conservation between the four PCP-degrading species (Ederer and Orser, University of Idaho, Moscow, ID, unpublished). Southern analysis using a 0.7-kb *Eco*R1 fragment of the *Sphingomonas* (*Flavobacterium*) sp *pcpC* gene as a probe grouped *Sphingomonas* (*Flavobacterium*) sp strain ATCC 39723 and *Sphingomonas* sp strain RA2 more closely together, as indicated by an identical banding pattern. *Arthrobacter* sp ATCC 33970 and *Pseudomonas* sp SR3 represented a second group (data not shown). Similar analyses for the *pcpA* locus resulted in an identical banding pattern for all four species. These observations are consistent with a more general detoxification role of the *pcpC* gene product. PcpC shares similarities with various gluthathione *S*-transferases (GST). Lloyd-Jones and Lau [19] speculated that GST-encoding genes are widespread among organisms degrading polycyclic aromatic hydrocarbons, particularly sphingomonads. They tested their hypothesis with the help of PCR primers specific for the glutathione-*S*-transferase gene family. However, these GSTs represented a group distinct from the *pcpC* encoded GST of the PCP-degrading species. Contrary to *pcpB* and *pcpA*, the transcription of which is PCP-dependent, the expression of *pcpC* was shown to be constitutive, and thus is not affected by the presence or absence of PCP in the medium [26].

Many pathways for degradation of xenobiotic compounds are encoded on plasmids, but no one has been able to confirm this for the PCP degradation pathway of any of the sphingomonads. The sequence similarities of the *pcpB* genes support the possibility of a plasmid location for the gene, possibly the genes for the entire pathway or at least the inducible genes. Ka *et al* [13] found the 2,4-D pathway gene *tfdB* on a plasmid in a *Pseudomonas* sp, but did not find any hybridization of *tfdB* with isolates identified as *Sphingomonas paucimobilis*. In our hands, a *tfdB* probe from *Alcaligenes eutrophus* plasmid pJMC134 hybridized strongly with the *pcpB* genes of the four PCP degraders. The *tfdB* gene encodes dichlorophenol hydroxylase, a single component monooxygenase similar to the PCP-4-monooxygenase encoded by *pcpB*. However, *Sphingomonas* (*Flavobacterium*) sp ATCC 39723 does not degrade 2,4-D (Hammill and Ederer, University of Idaho, Moscow, ID, unpublished).

Nohynek *et al* [23] studied biochemical and morphological characteristics of four PCP-degrading strains, while Karlson *et al* [14] and Ederer *et al* [5] focused on genetic

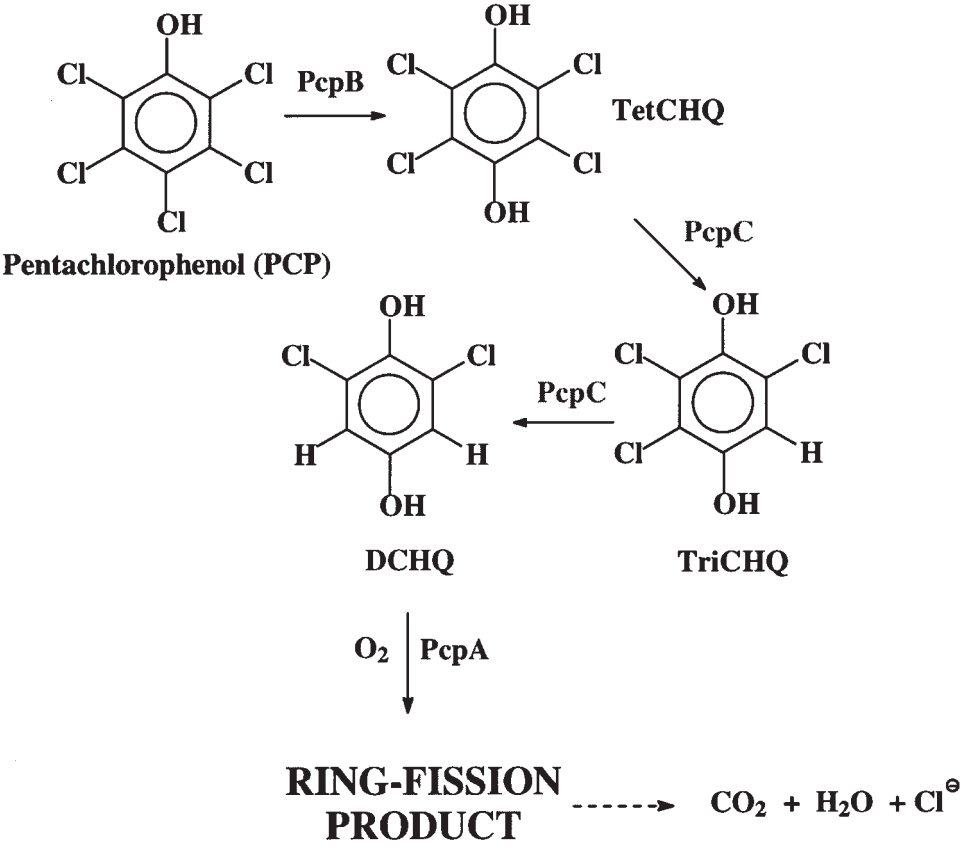

Figure 1 PCP degradation pathway of *Sphingomonas chlorophenolica* ATCC 39723. TetCHQ = 2,3,5,6-tetrachlorohydroquinone; TriCHQ = 2,5,6-trichlorohydroquinone; DCHB = 2,6-dichlorohydroquinone.

characterization of the organisms. All three groups concluded that the four PCP-degrading strains should be reclassified into one genus, *Sphingomonas*. The phenotypes of the four PCP degraders are listed in Table 1.

In comparison with the other members of the genus *Sphingomonas*, the four PCP degraders were characterized by a very similar protein profile and a slow growth rate [23]. 16S rDNA sequence analyses by Karlson *et al* [14] and Ederer *et al* [5] clustered the PCP degraders into a group within the rRNA group IV [28] or the α-subgroup [43] of the class *Proteobacteria* [35]. Whether these organisms can be considered one species [23] is still open to discussion. The 16S rRNA analysis groups *Sphingomonas (Flavobacterium)* sp ATCC 39723 and *Sphingomonas* sp RA2 together, and *Pseudomonas* sp SR3 and *Arthrobacter* sp ATCC 33970 together. Nohynek *et al* [23] suggested that the four PCP degraders be grouped into a single species, *Sphingomonas chlorophenolica* sp nov [14]. For now, this is the accepted classification scheme.

As discussed above and in Ederer and Crawford [4], there are no strict rules about how much divergence in nucleotide sequence is allowable between two organisms regarded as members of a single species. Some researchers base speciation on 70% sequence identity according to DNA/DNA hybridization data [41] and require at least 97% identity between rRNA genes [35]. Both Nohynek's group [23] and ours [5] found very little rRNA sequence variation, supporting the classification of these four strains into one species. However, there are significant differences between the four bacterial strains: First, *Arthrobacter* sp ATCC 33970 shows approximately 10% difference in nucleotide sequence of the *pcpB* gene in comparison to the other three organisms. Also, total DNA *Eco*R1 digests from *Arthrobacter* sp ATCC 33970, probed with the *Sphingomonas (Flavobacterium)* sp *pcpC* gene, resulted in a weaker hybridization signal, also indicating a substantial amount of divergence between the two strains. This is supported by the differences in hybridization pattern between the four species using a Southern analysis and pcpC as a probe (see above). Finally, *Pseudomonas* sp SR3 degrades 2,4 dichlorophenol [32] and seems to exhibit a larger substrate range.

Lake *et al* [15] pointed out that relying on 16S rDNA analysis might not be sufficient to reach a meaningful conclusion about phylogeny. Horizontal gene transfer is an important evolutionary mechanism in prokaryotes as well as eukaryotes. Thus far the four PCP-degrading bacterial isolates can be considered a closely related group of organisms based on the 16S rDNA sequence as well as the nucleotide sequences of *pcpB*. As more sequencing data become available and a better definition of bacterial species is established we will be able to undisputedly classify this group of PCP-degrading bacteria and other organisms.

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