

Identification of a Putative Chlorocatechol Catabolic Pathway in the Triclosan Degrader *Sphingomonas* sp. RD1

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Abstract

Sphingomonas sp. RD1 is a triclosan-mineralizing isolate from sewage sludge. Previous metabolite analysis has shown that in the catabolic pathway hydroxylated triclosan, 2,4-dichlorophenol and 4-chlorocatechol are produced, suggesting a cleavage of triclosan at the ether linkage connecting the two aromatic rings following hydroxylation. Screening of a fosmid library of total RD1 DNA for metabolic activity against triclosan revealed one clone, pTCSMC-1, which appeared to have particularly high activity. Sequence analysis of pTCSMC-1 did not, however, reveal any obvious genes for enzymes capable of hydroxylating triclosan. Instead, pTCSMC-1 seems to contain at least partial open reading frames (ORFs) corresponding to genes for all the enzymes necessary to catabolize 4-chlorocatechol, one of the identified triclosan metabolites of RD1, to the TCA cycle. Further sequence analysis is necessary to determine whether all the ORFs identified are complete putative genes and a role for this putative pathway in triclosan mineralization remains to be confirmed.

Introduction

- Triclosan is a substituted diphenylether (2,4,4'-trichloro-2'-hydroxydiphenylether) which is commonly used in household and hygiene products as an antimicrobial agent.
- Sphingomonas* sp. RD1 was originally isolated from a triclosan-mineralizing consortium and subsequently shown to be capable of mineralizing triclosan (Hay et al. 2001).
- Metabolite analysis of triclosan mineralization by RD1 indicates that hydroxylated triclosan, 2,4-dichlorophenol, 3,5-dichlorocatechol, and 4-chlorocatechol are intermediates in the catabolic pathway (Kagle, 2004).
- This suggests that RD1 has the metabolic capability to hydroxylate triclosan and to catabolize both 2,4-dichlorophenol (most likely through 3,5-dichlorocatechol (KEGG)) and 4-chlorocatechol.
- The construction and screening of fosmid libraries for metabolic activity against aromatic compounds through accumulation of a brown polymerization product has been successfully used for the identification of genes necessary for their catabolism (Porter and Hay, 2007; Parke, 1992).
- In this study, a fosmid library of total RD1 DNA was constructed and screened for activity against triclosan. The sequence of the clone showing the greatest activity was then obtained and analyzed.

Methods

Strains and Growth Conditions:
E. coli EPI300 (Epicentre, Madison, WI) and *E. coli* EPI300 carrying either pBBRT, pTCSMC-1, or both were grown in LB or LB supplemented with 50 mg/L kanamycin (pBBRT) or 12.5 mg/L chloramphenicol (pTCSMC-1). *Sphingomonas* sp. RD1 was also grown in LB.

Cloning of genes from *Sphingomonas* sp. RD1:
Using a commercially available kit (Epicentre, Madison, WI) ~40 kb fragments of the RD1 genome was cloned into the fosmid pCC1FOS and a library of the resulting fosmids was created in *E. coli* EPI300 (Epicentre, Madison, WI) also carrying the triclosan resistance plasmid pBBRT (Kagle and Hay, 2002). The library was screened for potential triclosan oxygenase activity using a modification of a previously published assay (Parke, 1992). The fosmid conferring the highest level of activity in the screen was named pTCSMC-1 (Figure 1).

Sequencing of pTCSMC-1:
Sequencing and sequence assembly of pTCSMC-1 was performed by Macrogen, Inc. (Seoul, South Korea) using DNA Analyzer 3730XL(Applied Biosystems, Carlsbad, CA).

Annotation:
Initial identification of putative genes was performed using ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Regions of interest were further annotated with Genome Workbench (<http://www.ncbi.nlm.nih.gov/projects/gbench/>).

PCR for Putative Genes in *Sphingomonas* sp. RD1:
Primers for six putative genes possibly involved in a 4-chlorocatechol catabolic pathway in RD1 (Table 1) were designed based on the sequence obtained from Macrogen, Inc. (Seoul, South Korea). PCR was performed using PyroStart Fast PCR Master Mix (Fermentas, Glen Burnie, MD) in a Veriti Fast Thermocycler (Applied Biosystems, Carlsbad, CA) on RD1 genomic DNA purified by standard methods.

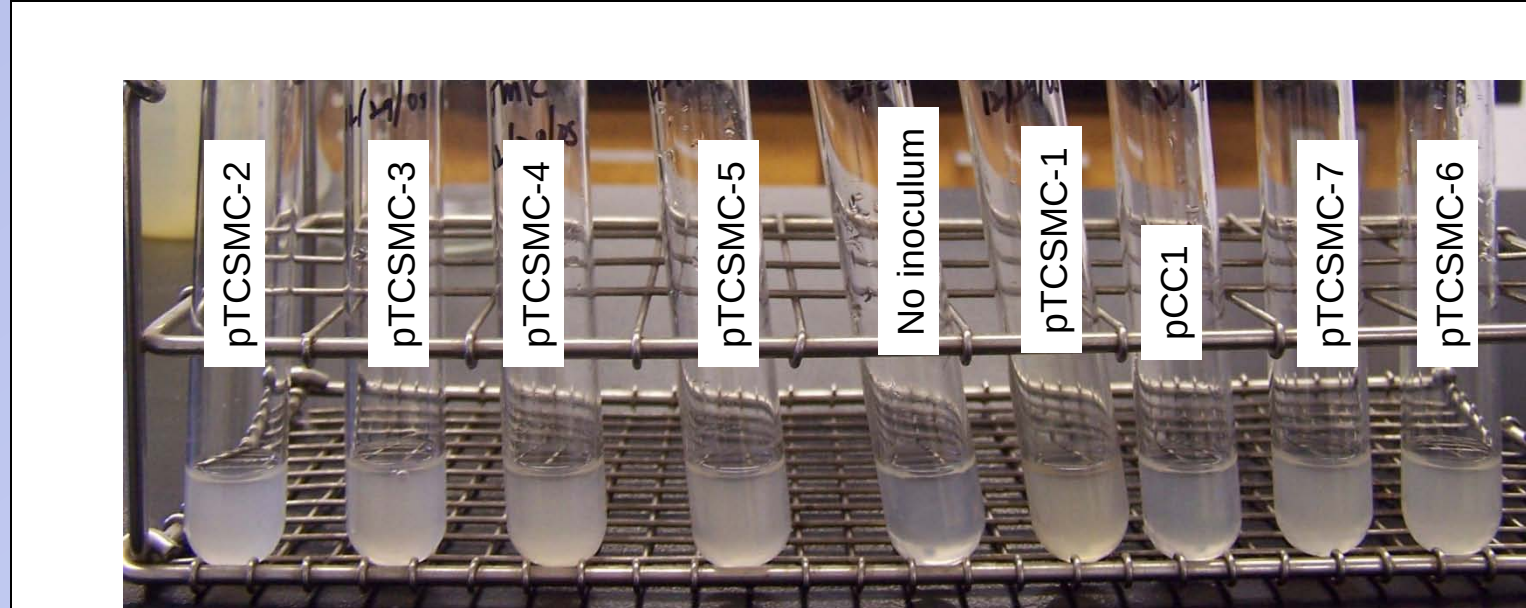


Figure 1. Screening of RD1 fosmid library for activity against triclosan. Brown color indicates a positive reaction. pTCSMC constructs are pCC1 vector carrying ~40kb fragments of RD1 DNA. pTCSMC-1 was chosen for further analysis.

Table 1. Primers used for detection of putative 4-chlorocatechol catabolic genes in *Sphingomonas* sp. RD1.

Target ORF	Putative Enzyme	Primer Name	Primer Sequence (5' → 3')
OrfA	4-Chlorocatechol 1,2-dioxygenase	4-CI Cat F	TTCCCAAGCTATTCAAGGGCCGTA
		4-CI Cat R	TCGACCCATCATGCTCAACAATCC
OrfB	Chloromuconate cycloisomerase	CMCI F	CGAATGTGCCGAGACGATAAAGGT
		CMCI R	CTTCTGGATCGCCTTTGCCGATTTC
OrfC	Carboxymethylenebutenolidase	CMB F	CGGCTACTGGCAGCAATTGAATGA
		CMB R	AGCGCTTCTTTACGCATCGTCTGT
OrfD	Maleylacetate reductase	MAR F	CACATGACAGAGCTTTGTGGTGGG
		MAR R	ATCTACGATGTCGATCTCACGCCTGAC
OrfE	3-Oxoadipate:succinyl CoA transferase subunit A	3QA F	ACTTCCTTCTGCAGCCCAACGAT
		3QA R	TCGAGATCGAGGAACATGCCCCA
OrfG	Beta-ketoadipyl CoA thiolase	BKACT F	ACATTGTGCACAGGCGCTAATCG
		BKACT R	TACCATCGAGCAGTTCGACGTGTCAT

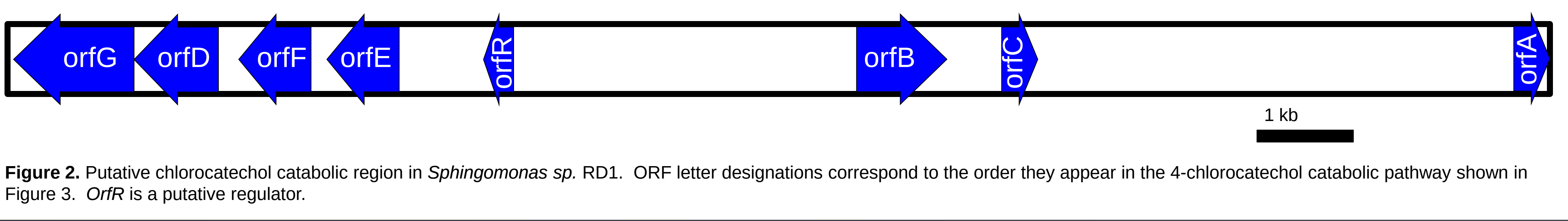


Figure 2. Putative chlorocatechol catabolic region in *Sphingomonas* sp. RD1. ORF letter designations correspond to the order they appear in the 4-chlorocatechol catabolic pathway shown in Figure 3. *orfR* is a putative regulator.

Table 2. Best alignments of open reading frames within the putative chlorocatechol catabolic gene cluster in *Sphingomonas* sp. RD1.

RD1 ORF	Best aligned gene(s)	Enzyme(s) encoded	Source Organism(s)	Pathway(s) and References	BLAST score	E-value	Partial (P) or Full-Length (F)
orfA	clcA	chlorocatechol 1,2-dioxygenase	<i>P. aer. JB2</i>	Chlorobenzoates (Hickey and Focht, 1990)	90	3.45e-11	P
orfB	tfdD	(chloro)muconate cycloisomerase	<i>Sphing. sp. TFD44</i>	2,4-dichlorophenoxyacetate (Thiel et al, 2005)	160	3.45e-30	F
orfC	clcD	Carboxymethylenbutenolidase	<i>Bord. petrii</i>	Chlorobenzenes and chlorobenzoates (Gross et al, 2008)	76	2.18e-7	P
orfD	tfdF	Maleylacetate reductase	<i>Sphing. sp. TFD44</i>	See above	606	3.01e-151	F
orfE	pcaI	3-Oxoadipate:succinyl CoA transferase, subunit A	<i>Pseud. stutzeri</i> A1501	Putative catechol (Li et al, 2010)	444	2.82e-107	F
orfF	pcaJ	3-Oxoadipate:succinyl CoA transferase, subunit B	<i>Pseud. stutzeri</i> A1501	See above	444	2.82e-107	F
orfG	pcaF	Beta-ketoadipyl CoA thiolase	<i>Pseud. knackmussii</i>	Aromatics and chloroaromatics (Gobel et al, 2002)	514	2.82e-126	F
orfR	clcR	Transcriptional regulator	<i>P. aer. JB2</i>	See above	74	7.61e-007	P

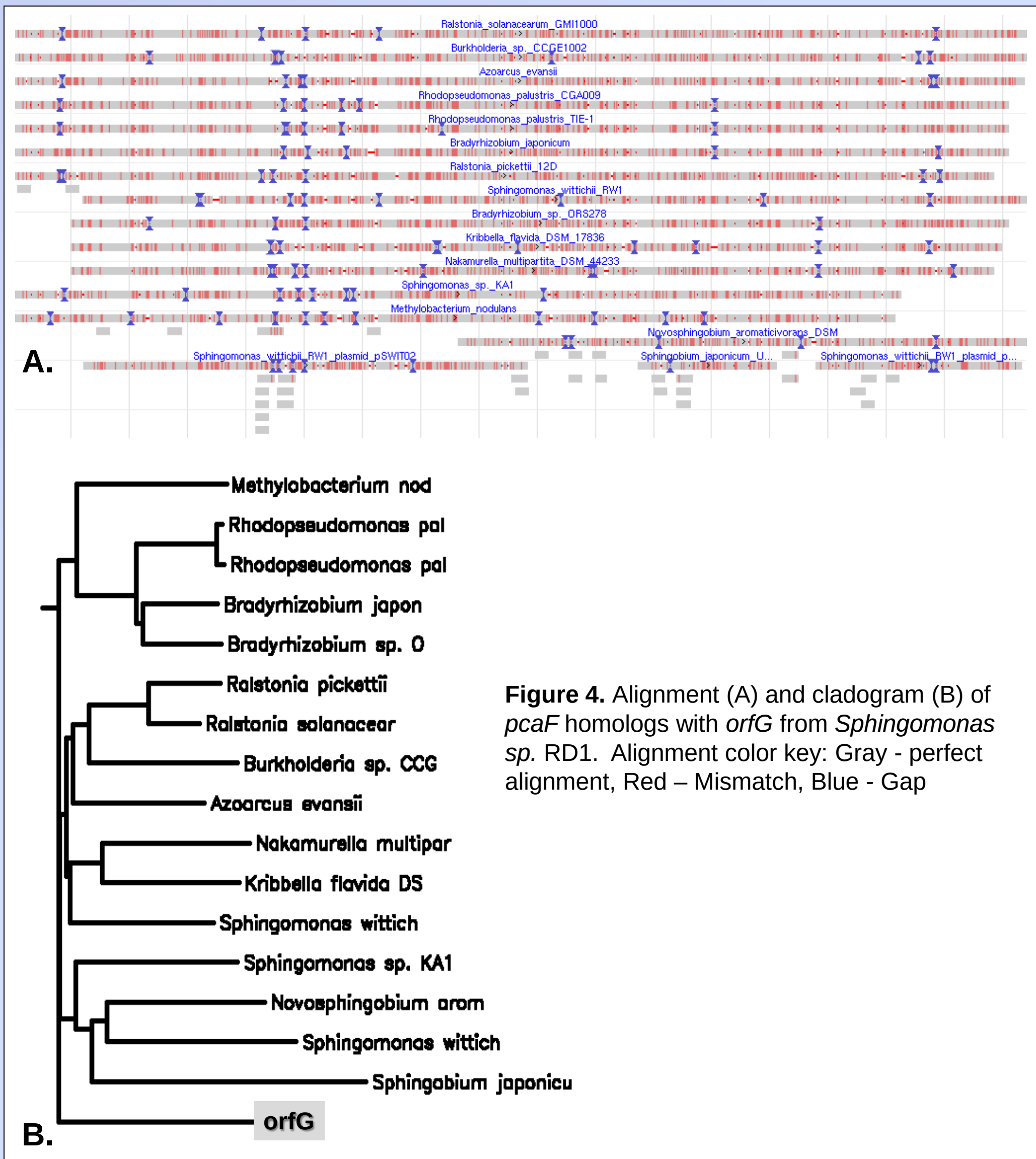


Figure 4. Alignment (A) and cladogram (B) of *pcaF* homologs with *orfG* from *Sphingomonas* sp. RD1. Alignment color key: Gray - perfect alignment, Red - Mismatch, Blue - Gap

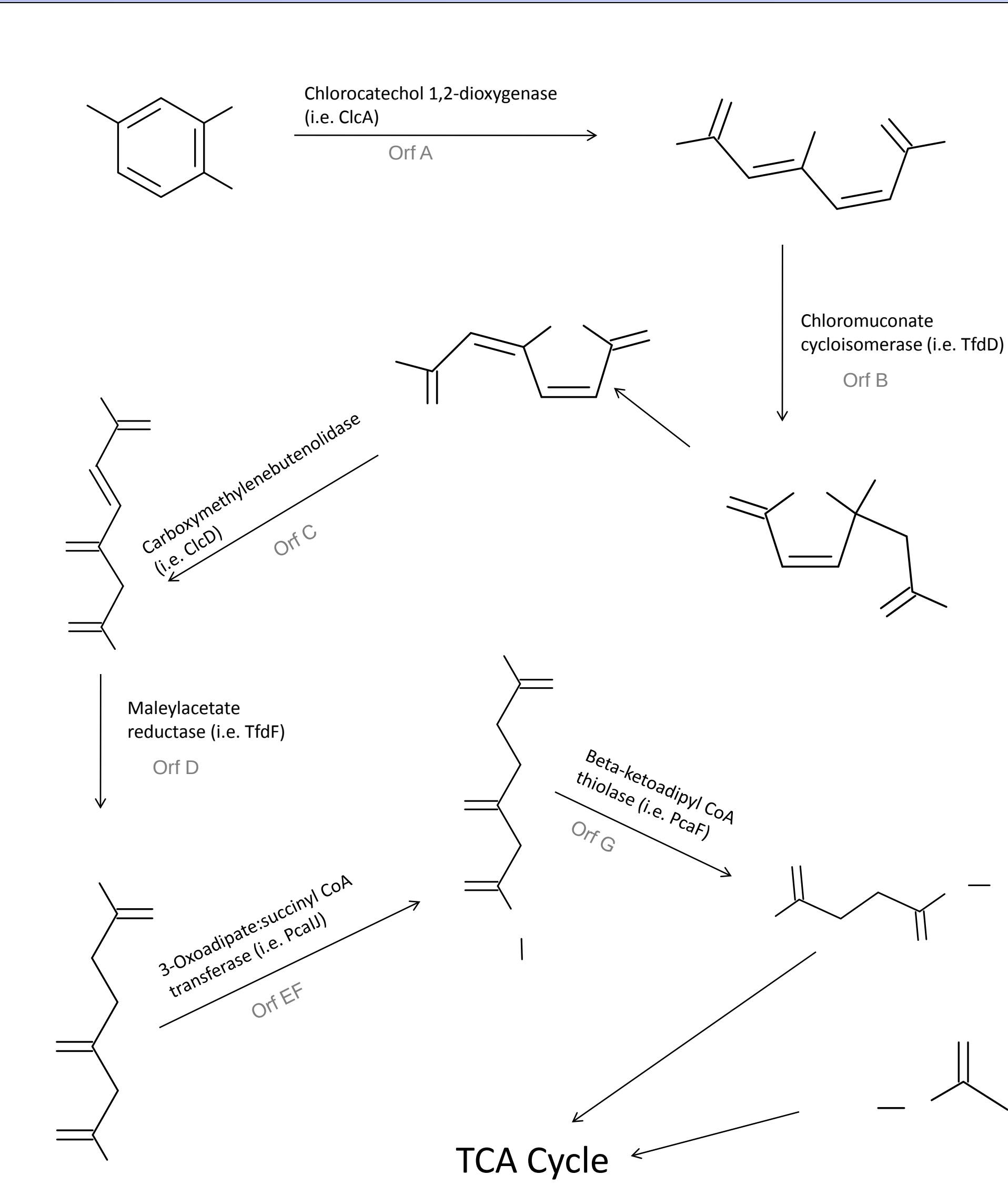


Figure 3. 4-chlorocatechol catabolic pathway. Enzymes required for catalysis of each step are written over the arrows with the corresponding RD1 ORF in grey below.

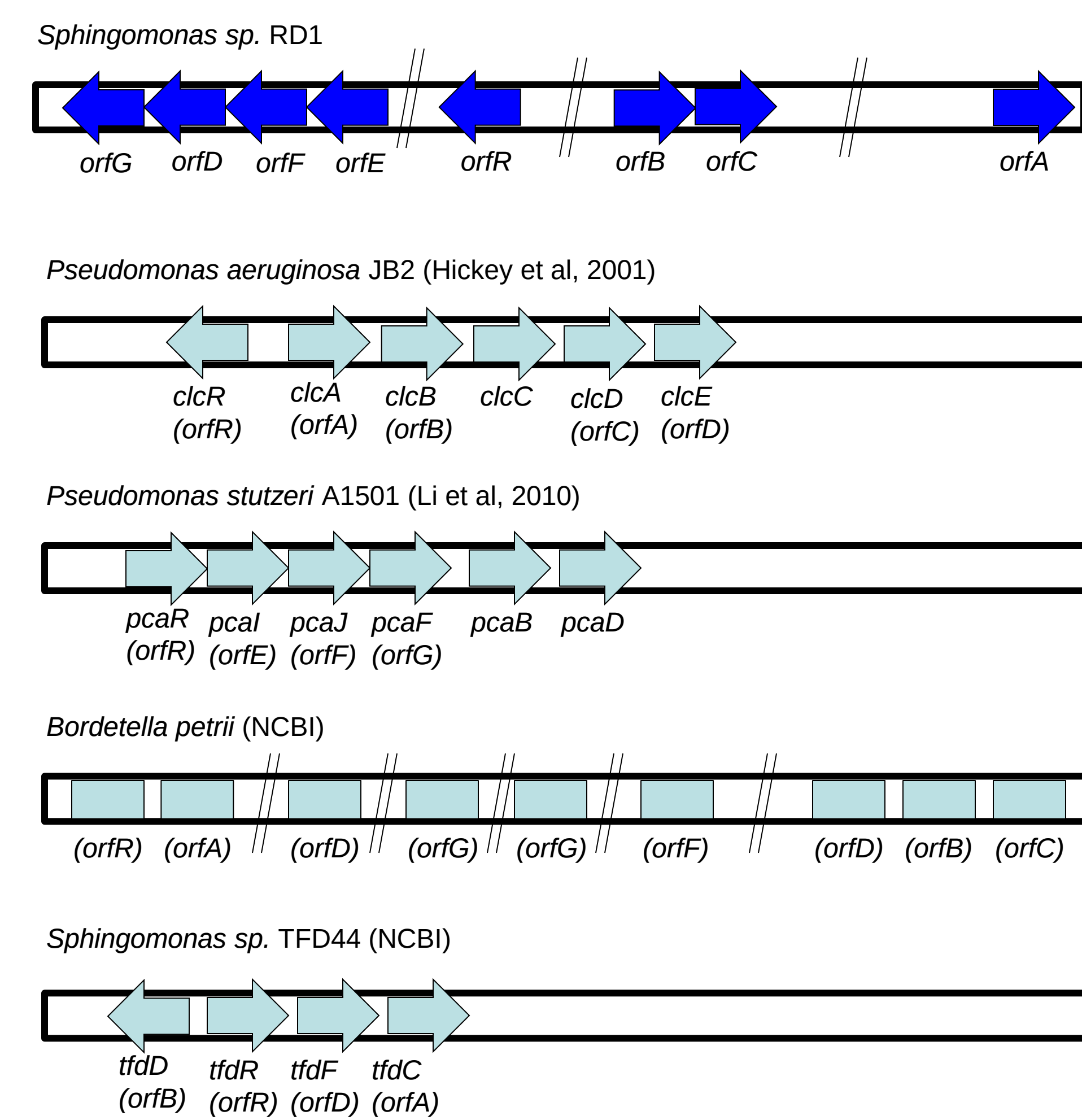


Figure 6. Arrangements of homologous catabolic genes in *Sphingomonas* sp. RD1 and other organisms. Not drawn to scale. Arrows show direction of transcription, if known. Double bar indicates a gap of 1 kb or more.

Results

- Sequencing of pTCSMC-1 by Macrogen, Inc. resulted in two contigs of 19498 bases and 24295 bases, totaling almost exactly the size estimate of 44 kilobases.
- The assembled sequence had multiple points where the exact sequence could not be determined, resulting in "N"s being included in the sequence.
- Initial investigation of the sequence through ORF Finder and BLAST did not reveal any obvious candidates for a triclosan oxygenase, but a 16 kb region of the 24 kb contig appeared to contain genes for all the enzymes necessary for chlorocatechol catabolism (Figures 2 and 3).
- PCR using primers for six of the putative chlorocatechol catabolic ORFs (Table 1) on total DNA from *Sphingomonas* sp. RD1 confirmed the presence of all six ORFs in RD1 (data not shown).
- Further analysis of this region showed homology with genes for experimentally confirmed enzymes from (chloro)catechol-metabolizing organisms for all putative chlorocatechol catabolic ORFs (Table 2).
- The most notable organisms showing homology to genes in this putative chlorocatechol catabolic region were *Sphingomonas* sp. TFD44 (2,4-dichlorophenoxyacetate catabolism) (Thiel et al, 2005), *Burkholderia xenovorans* LB400 (polychlorinated biphenyl catabolism) (Goris et al, 2004), *Pseudomonas knackmussii* B13 (catabolism of aromatics and chloroaromatics) (Gobel et al, 2002), *Pseudomonas aeruginosa* JB2 (catabolism of chlorobenzoates through chlorocatechols) (Hickey and Focht, 1990), and *Bordetella petrii* (catabolism of chlorobenzenes and chlorobenzoates through chlorocatechols) (Gross et al, 2008).
- Analysis of three ORFs resulted in partial alignments: *orfR*, *orfC*, and *orfA* (Table 2).
- An alignment and cladogram of *orfG* with homologs from other organisms does not show a notable clustering with other sphingomonads (Figure 4).
- Examination of the genetic organization of similar catabolic genes in other organisms revealed little to no similarity in organization either to each other or to the organization in RD1 (Figure 5).

Discussion

Although the screening procedure used was designed to identify fosmids carrying *Sphingomonas* sp. RD1 DNA which conferred triclosan oxygenase activity, it appears that a putative chlorocatechol catabolic region from RD1 has been identified. Putative genes or gene fragments for all enzymes required to take 4-chlorocatechol to the TCA cycle have been identified in this region of DNA from RD1. The best alignments for the eight putative chlorocatechol-related ORFs in this region are with genes from known or presumed (chloro)catechol metabolizers, two of which are capable of catabolizing xenobiotics similar to triclosan (Goris et al, 2004; Thiel et al, 2005).

The gene arrangement of the putative 4-chlorocatechol catabolic region in RD1 differs from the gene arrangement in other organisms with chlorocatechol catabolic regions that can be determined from the available studies and GenBank sequences. This suggests that although the genes themselves are highly conserved, their genetic arrangement is not. The lack of conserved gene arrangement along with *orfG* not clustering most closely with homologous genes from other sphingomonads suggests these genes were acquired via horizontal gene transfer. This would not be particularly surprising, as the genes for similar catabolic pathways are frequently found on plasmids and other mobile genetic elements (i.e. Vallaeys et al, 2006; Hickey et al, 2001; Romine et al, 1999).

The fact that the catabolic genes for triclosan degradation are not clustered in a classical operon is consistent with the complex organization of catabolic genes in other sphingomonads (reviewed in Stolz, 2009). It is interesting to note, however, that in the case of RD1, the ORFs corresponding to the "upper pathway" (4-chlorocatechol to maleylacetate) and "lower pathway" (maleyl acetate to acetyl-CoA and succinyl-CoA) are each clustered together and the two clusters are transcribed in opposite directions.

Several of the ORFs identified appear to be partial, in particular *orfR* (regulatory protein), *orfC* (carboxymethylenebutenolidase), and *orfA* ((chloro)catechol 1,2-dioxygenase). There are three possible reasons for these apparently incomplete genes. First, the genes might in fact be incomplete. Knowing from previous metabolite analysis of RD1 that 4-chlorocatechol is an intermediate metabolite in triclosan catabolism, in this case the gene(s) encoding the related 4-chlorocatechol catabolic activity would have to be located elsewhere in the RD1 genome. Second, errors in the sequence could incorrectly curtail the ORFs detected. A more precise sequencing of the regions surrounding these partial ORFs would address this issue. Finally, specifically for *orfA* ((chloro)catechol 1,2-dioxygenase), the fragment of DNA cloned into pTCSMC-1 appears to end in the middle of the putative gene for (chloro)catechol 1,2-dioxygenase. It is therefore not possible to tell from this cloned fragment whether the sequence in this putative chlorocatechol catabolic region represents a complete (chloro)catechol 1,2-dioxygenase gene in RD1.

Further analysis of this region must be performed to confirm its involvement in 4-chlorocatechol catabolism. Although subsequent sequence analysis can be done using pTCSMC-1, its ability to confer 4-chlorocatechol catabolism would be hindered due to the incomplete *orfA* (chlorocatechol 1,2-dioxygenase). All metabolic experiments must therefore be done on *Sphingomonas* sp. RD1 or new constructs including a complete *orfA*. We hope in the future to show that this region represents the genes required for a portion of the lower pathway of triclosan catabolism in *Sphingomonas* sp. RD1.

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