Characterization of a Novel Chlorobenzoate Degrading bacterium: *Burkholderia phytofirmans* OLGA172, Isolated from a Pristine Environment

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Ecology and Evolutionary Biology department
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Abstract

*Burkholderia phytofirmans* OLGA172 is a chlorobenzoate (CBA) degrading bacterium, known to frequently lose the ability to degrade CBA in the lab. OLGA172 carries the complete set of genes for chlorocatechol degradation (an intermediate in CBA metabolism), *tfdCDEF*, as well as several integrases associated with DNA mobility in proximity to these genes. In this study, putative CBA degradative genes were identified in OLGA172, and an imbalance in regulation between the *tfdCDEF* and CBA degradative genes identified as a cause for incomplete CBA metabolism in this strain. Additionally, expression of the integrase genes was observed to occur constitutively. The role of this expression was not determined but hypothesized to be related to the phenotypic instability seen in OLGA172. Characterization of the strain was carried out with the aim to determine the ecological niche OLGA172 occupies naturally. Results of characterization are discussed in the context of evolution of the chlorocatechol degradative genes.
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List of Frequently Used Abbreviations

2,4-D; 2,4-dichlorophenoxyacetic acid
ABS; activator binding site
Cat-Ben; Catechol-Benzoate
CBA; chlorobenzoic acid
CBD; chlorobenzoate degradative
CC; chlorocatechol
CCD; chlorocatechol degradative
CL-IAA; Chloroindole Acetic Acid
HGT; horizontal gene transfer
IAA; Indole Acetic Acid
INT; Integrase
LTTR; LysR-type transcriptional regulator
ORF; Open reading frame
PCB; Polychlorinated Biphenyls
PGP; Plant growth promoting
RBS; recognition binding site
TNP; Transposase
Chapter 1. Microbial Degradation of Chlorinated Aromatics: A Review

1.1 Introduction

Most chlorinated aromatics are considered to be significant organic pollutants which pose health and environmental concerns due to their toxicity and in some cases, persistence in the environment. Several organochlorines are persistent, lipophillic, bioaccumulative and biomagnified in food chains, characteristics which make them persistent organic pollutants (POPs) and therefore of significant concern. These include pesticides, such as the insecticide dichlorodiphenyltrichloroethane (DDT), which has been banned in most developed countries (Simonich & Hites, 1995); or polychlorinated biphenyls (PCBs). PCBs represent a large group of compounds often found as a mixture, used for a variety of applications from pesticides and plastics to transformer oils, which have been established to have serious health effects on humans and wildlife. In humans, both DDT and PCBs have been found to be globally distributed in human breast milk, especially in areas where fish/marine life are a main source of diet (Polder et al., 2003). In the St. Lawrence River, elevated levels of organochlorines have been found in the blubber of beluga whales, caused as a result of high levels of the contaminant in prey species (Muir et al., 1996).

Large scale production (and emissions) of PCBs started in the 1930’s and peaked in the 1970’s. Production of these chloroaromatics decreased significantly after the 70’s (Breivik et al., 2002), when their toxic effects were established. The EPA banned their use and manufacture in 1977 (Miller et al., 1991). Even though their current use is declining, emissions of PCBs into the atmosphere from old sources is still being seen globally, due to release from soil and sediments, and disposal and incineration of products which contain the chemicals (Breivik et al., 2002). Because of environmental and health concerns, removal of chlorinated POPs such as PCBs are a focus of bioremediation research.

In addition to the major POPs, other chlorinated aromatics are of environmental concern, including chlorophenoxyacetic acids, chlorobenzoates (CBAs), chlorobenzenes, chlorophenols, and chlorotoluenes. CBA’s are mainly present in the environment as byproducts of PCB
degradation, although on their own CBA’s (2,3-6-trichlorobenzoate, 2,5-dichloro-3-aminobenzoate) have been used as herbicides (Martinez et al., 2007). A central intermediate in the degradative pathways of these and many other recalcitrant chlorinated aromatics, is chlorocatechol (Figure 1.1). While partial degradation of some chlorinated aromatics to chlorocatechol (CC) can be carried out by some indigenous microorganisms, CC degradation has been found to be the rate limiting step of the degradation of these compounds and is often a problematic dead end metabolite. If CC is allowed to accumulate, it can oxidize and polymerize to a coloured intermediate which is toxic to microbial cells (Fava et al., 1993). Additionally, in strains that lack the chlorocatechol degradative (CCD) genes, 4CC has been found to be metabolized through the action of a ubiquitous gene from the 3-oxoadipate pathway (muconate cycloisomerase) to form the antibiotic protonanemonin, a normally plant derived compound (Blasco et al., 1995). CC has also been found to directly inhibit the enzymes involved in degradation of PCBs (Sylvestre, 2004).

One strategy for the bioremediation of sites that have been contaminated with chlorinated aromatics is bioaaugmentation, the addition of microorganisms which carry the necessary degradative genes to a contaminated site (Urgan-Demirtase et al., 2006). The microbes added may naturally contain the required degradative genes, or may have been genetically modified to efficiently degrade the pollutant. While genetically engineered microorganisms (GEMs) have been shown to increase degradation in the laboratory, their release in the environment has some limitation (Sayler & Ripp, 2000). Firstly, the addition of GEMs to the environment is perceived negatively, as is the potential for the spread of foreign DNA to the indigenous microbial population. The dissemination of degradative genes from donor strains to indigenous communities has been established to occur in the environment, and shifts in the microbial community structure of soils to which degraders have been introduced has been observed (Dejonghe et al., 2000). In addition to the ethical issues concerning the addition of foreign microorganisms, there are many environmental factors which limit the effectiveness of the introduced bacteria. The instability of introduced genetic material (Urgan-Demirtas et al., 2006), predation by protists, competition with the indigenous population and abiotic environmental factors can all affect bioaugmentation negatively. However, there is evidence that the use of microorganisms from the same ecological niche as the contaminated site may reduce the ecological barriers described (El Fantroussi & Agathos, 2005).
Figure 1.1 Chlorocatechols as a central intermediate (adapted from Schloemann, 1994)
1.2 Horizontal Gene Transfer and Microbial Adaptation

Bacteria have the capacity to adapt to previously persistent compounds, by evolving novel metabolic capabilities and altered enzyme specificities. The genetic mechanisms for evolution of these abilities can be broadly grouped into vertical and horizontal processes. Vertical evolution encompasses the genetic changes which occur over time and can be inherited by daughter cells. Molecular mechanisms for vertical evolution can include point mutations, deletions, duplications, and slipped strand mispairing at the nucleotide level (van der Meer, 1994). These types of mechanisms may facilitate the formation of ‘new’ enzymes with novel metabolic capabilities by changing the substrate specificities of pre-existing enzymes. It is likely that this has occurred for at least some of the genes of the chlorocatechol degradative pathway. For example the chlorocatechol dioxygenase and chloromuconate cycloisomerase of the CCD pathway have similar nucleotide composition (approximately 64%) to the catechol dioxygenase and muconate cycloisomerase of the catechol degradative pathway, and these enzymes exhibit substrate specificity to the substituted compounds over the non-chlorinated analogs (Schlomann, 1994). Substrate specificity of an enzyme can be changed with only a few differences in its amino acid sequence. The chlorocatechol dioxygenases of *Ralstonia eutropha* NH9 and *Pseudomonas* P51 have 95% amino acid similarity, however their substrate specificities are different, having higher activity towards 3,5-dichlorocatehol and 3,4-dichlorocatechol respectively. It was found that this difference in substrate specificity could be attributed to two specific amino acid differences, Val-48 and Ala-52. Site directed mutagenesis at these sites confirmed that these mutations were responsible for the altered specificities (Liu et al., 2005). Thus, small changes can result in altered substrate preferences, which may be selected for if they confer an advantage to the organisms which carry them in a particular environment.

While one mechanism for the acquisition of novel metabolic capabilities is through vertical transfer, horizontal gene transfer (HGT) has been established as an important mechanism for the acquisition of catabolic genes (Top & Springael, 2003). Horizontal gene transfer from one microorganism to another may be one of the dominant forces that shape the bacterial genome, and bypasses the time required to amass beneficial mutations for new degradative abilities. HGT can occur via the transfer of conjugative plasmids (extrachromosomal and independently replicated circular DNA elements) carrying the requisite genes. Several plasmids carrying the
CCD operon have been identified and characterized (for example plasmid pJP4 of JMP134), and the majority of isolates in literature and annotated on Genbank (when location of the gene is specified) carry the CCD genes on a plasmid (Jin et al., 2009) rather than chromosomally encoded.

While transfer of plasmids was historically considered the major mode of DNA transfer, other genetic elements which can cause DNA movement have been identified as being important in shaping the microbial genome (van der Meer & Sentchilo, 2003). These can include transposons, integrons, bacteriophages and genomic islands which are able to move large segments of DNA between plasmids and chromosomes of different individuals or within a single cell. Evidence for past horizontal events can be left behind, often near or flanking catabolic genes are inverted and direct repeats, insertion sequences, as well as genes which code for enzymes capable of DNA excision and integration. The presence of these genes indicate past mobility, and also the potential for future transfer.

1.3 The Problem of Pristine Degraders

While the majority of studies have isolated xenobiotic degraders from contaminated soils and aquifers, there are many examples of microbes with extensive catabolic ability isolated from pristine environments. For example, bacteria indigenous to pristine soils have been found which could degrade 2,4-dichlorophenoxyacetic acid and pentachlorophenol (Kamagata et al., 1997; Shaw & Burns, 2005; Mahmood et al., 2005). Chlorobenzoate degraders in pristine environments have also been found to be widespread around the globe. In a study by Fulthorpe et al., (1996), approximately 600 3CBA degraders were isolated from six geographically disparate regions that had no previous exposure to anthropogenically produced chlorinated compounds or human disturbance (California, Chile, South Africa, Australia, Saskatchewan and Russia). The widespread occurrence of these degraders in pristine soils indicate that not only are CBA degraders more common than previously thought, but questions the hypothesis that the trait has evolved recently due to selective pressure from the presence of anthropogenic sources. The pristine degraders from the Fulthorpe study were also remarkable because they shared the characteristic of being very unstable. Of the 610 isolates that could degrade 3CBA, only 151
maintained this ability after short term storage of 1-2 months in -80 C (Fulthorpe, 1996), even though many of these strains maintained the genes for CBA degradation.

Examples of adaptation through horizontal gene transfer, point mutations and DNA rearrangement in response to the presence of xenobiotic compounds are easily found in literature (Springael & Top, 2004; de Lipthay et al, 2001; van der Meer et al., 1998). The widespread presence of pristine degraders however presents a mystery, namely that there has been presumably no selective pressure and no reason for bacteria to adapt or maintain degradative pathways for a xenobiotic (literally meaning alien to life) that is foreign to the communities enzymatic systems. Without selective pressure the presence of these genes are thought to be a cost to fitness through unnecessary expression and DNA replication. There are at least three possible solutions to this problem. First, the atmospheric spread of anthropogenically produced chlorinated aromatics was so great as to spread to environments far from their use and provide selective pressure for adaptation prior to when these organisms were sampled. The global spread of atmospherically deposited organochlorines in soils, even in remote areas far from their use has been established (Meijer et al, 2003). However, concentrations of PCBs and hexachlorobenzenes combined found in remote soils around the globe at maximum (Meijer et al, 2003) are lower than the 1 uM concentrations of 3CBA considered to be the minimum required amount to act as a selective agent in natural environments (Fulthorpe & Wyndham, 1989). Thus, the widespread presence of 3CBA degradative genes in pristine soils due to selective pressure from remotely produced xenobiotics such as PCBs is considered unlikely.

Secondly, the soils sampled could represent microbial communities who have adapted to these xenobiotics at contaminated sites and then distributed to pristine environments. Alternatively, the strains themselves may not have spread but the genes for CBA degradation distributed via HGT to these pristine sites, where over time without selective pressure these genes would be lost. The CBA degradative operon, and many other catabolic genes are often found to be carried on plasmids, or associated with mobile genetic elements (Top & Springael, 2003). The unstable nature of the 3CBA degrading phenotype of pristine isolates could certainly be indicative the loss of the degradative genes, genetic rearrangement post-isolation or poor regulation of the introduced genes.
Finally, the enzymes used for CBA degradation in these communities could have been used to degrade some natural compound with a similar structure and whose presence preceded the xenobiotic. Chloroaromatic chemicals are not exclusively synthetic, and some do occur commonly in nature. It may be that these xenobiotic structures are not as foreign to bacterial enzymatic systems as the name implies. For example, a high diversity of basidiomycetes have been found to produce natural organohalogenes in ecologically significant quantities. Chlorinated anisyl metabolites are estimated to be produced at a rate of 300 g ha\(^{-1}\) year\(^{-1}\) in European forests, and chloromethane has been found to be produced by white rot fungi, the annual global emissions of which are estimated to be 160,000 t year\(^{-1}\) (Field & Wijnberg, 2003). Natural and widespread production of chlorinated aromatics may have provided selection pressure for organohalogen degraders in pristine environments.

CBA degrading strains from pristine soils are unique systems to examine questions about how the chlorocatechol gene pathway evolved to degrade the manmade compounds that are prevalent today. The presumed lack of selective pressure for these degradative capabilities in pristine systems allows us ask questions about the extent of HGT globally, or alternatively, may give insight in the significance of existing enzymatic diversity to the degradation of man-made pollutants. Because bacteria have only been exposed to many of these compounds since the 30’s, it should be possible to find bacteria in the intermediate stages of evolution of novel catabolic capabilities (Cases & Lorenzo, 2001).

1.4  The Modified Ortho Cleavage Pathway of Chlorocatechol Degradation: Genes and Regulation

The conversion of some chlorinated aromatics to chlorocatechols can be carried out by enzymes with broad specificities which may be nonspecific enough to act on chlorinated compounds in addition to their non-chlorinated substrates (Schlomann, 1994). Ring cleavage of chlorocatechol however, has been found to be the rate limiting step for chloroaromatic degradation and requires specialized enzymes for its complete mineralization (Cavalca et al., 1999). Enzymes for ring cleavage of non-chlorinated analogs of chlorocatechol may not be able to convert this compound because of steric effects or the electron withdrawing effects of the substituent chlorine (Schlomann, 1994). Without the ability to transform chlorocatechol, this substrate accumulates
and is observed as a black pigment after oxidative polymerization (Fava et al., 1993). The modified ortho-cleavage pathway is one metabolic route commonly used for the conversion of the central intermediate chlorocatechol to tricarboxylic acid (TCA) cycle intermediates in microorganisms capable of this transformation.

Complete catabolism of chlorocatechol via the modified ortho pathway requires four genes, typically found as an operon and located on catabolic plasmids (van der Meer et al., 1992). The rate limiting step, conversion of chlorocatechol to chloromuconate is carried out by chlorocatechol 1,2-dioxygenase. The other three genes, chloromuconate cycloisomerase, dienelactone hydrolase, and maleylacetate reductase make up the rest of the chlorocatechol degradative (CCD) operon. The CCD pathways from several bacteria have been characterized, and the most studied genes are clcABDE, tcbCDEF and tfdCDEF (Table 1.1) found in Pseudomonas sp. strain B13, Pseudomonas sp. strain P51, and Cupriavidus necator JMP134 respectively, named differently because the enzymes were found to be most active with different substrates, CBA, 1,2,4-trichlorobenzene, and 2,4-D respectively (Liu et. al, 2001). All of these substrates include a chlorinated catechol as an intermediate. C. necator JMP134, the canonical and most extensively studied chlorocatechol degrader carries two copies of the CCD operon, tfdCIIIdEIIIFII called the module I genes, and tfdDIIICIIIEIIIFII called module II. The module II operon is also associated with the additional genes tfdK, tfdB and tfdA, used for the uptake of 2,4-D, transformation of 2,4-dichloropenol to a chlorinated catechol, and transformation of 2,4-D to 2,4-dichlorophenol respectively. The two modules in JMP134 have been found to have different conversion rates, and slightly different substrate specificities. Though the two modules in JMP134 encode isofunctional enzymes, there is sufficient sequence and amino acid heterogeneity to conclude that the two modules are not the result of duplication and divergence within one host, but are likely to have come from separate origins (Laemmli et al., 2000).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Gene</th>
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<tbody>
<tr>
<td>LysR type transcriptional regulator</td>
<td>tfdR/S, tfdT, clcR, tcbR</td>
</tr>
<tr>
<td>chlorocatechol 1,2-dioxygenase</td>
<td>tfdC, clcA, tcbC</td>
</tr>
<tr>
<td>chloromuconate</td>
<td>tfdD, clcB, tcbD</td>
</tr>
</tbody>
</table>

Table 1.1 Enzymes of the CCD pathway
**cycloisomerase**

**dienelactone hydrolase**

**Maleylacetate reductase**

<table>
<thead>
<tr>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>tfdE, clcC, tcbE</td>
</tr>
<tr>
<td>tfdF, clcD, tcbF</td>
</tr>
</tbody>
</table>

In order for a strain to degrade chlorocatechol, a toxic compound, it needs to possess both the genes for enzymatic degradation and a means for efficiently regulating those genes. The CCD operon is typically regulated by a LysR type transcriptional regulator located upstream and transcribed divergently from the genes it controls (Ogawa & Lang, 2009). In *C. necator JMP134* the regulator of the module II genes is tfdR; an identical but transcribed in the opposite direction from tfdR is another LysR type regulator (tfdS) associated with the operon. Upstream of the module I CCD genes in *C. necator JMP134* is *tfdI*, a LysR regulator which has been truncated by an insertion sequence, and does not code for a functional protein. TfdR has been found to be able to take over the regulatory role of *tfdI* in JMP134, even though the gene is located 8 kb away from the *tfdC1D2E3F1* operon on the same catabolic plasmid (Leveau and Van Der Meer, 1996). In addition to the regulator associated with the CCD operon, the chromosomally encoded regulator of benzoate/catechol metabolism (*CatR*) has been found to be able to cross-activate the *tfdCDEF* operon. The converse is not true, and the regulators of the CCD operon (tfdR, tfdS and *tfdI*) are not able to activate the genes for benzoate/catechol metabolism (Parsek et al., 1994).

### 1.5 Bacterial-Plant interactions in Degradation

Biodegradation in the environment by bacterial inoculants has been found to be variable and has some limitations (Sayler & Ripp, 2000). Microbial biodegradation in combination with phytoremediation has met with some success, and multiple studies have shown increased xenobiotic degradation in the rhizospheres of plants (Shaw & Burns, 2004). The term rhizosphere defines a narrow zone of soil surrounding the root, which is directly influenced by the activity of living plant roots. As a result of the materials deposited into the rhizosphere by the roots, there are increased microbial population densities in this area compared with the surrounding bulk soil, reaching up to $10^9$ and $10^{12}$ cells per gram of rhizosphere soil (Pinton et al., 2001). The increased population densities of bacteria and fungi in this area is referred to as ‘the rhizosphere effect’, thus the rhizosphere is primarily an environment created by root
exudates and the microbes that use them. Rhizosphere enhanced biodegradation has been shown for chlorinated xenobiotics in the past. Siciliano & Germida (1997) showed that a 2-chlorobenzoate mineralizing strain was not able to significantly degrade 2-CBA in unplanted soil. However, when added as a plant seed innoculant, degradation increased by 20% when compared to uninnoculated plants. The addition of a plant growth promoting (PGP) co-innoculant (which could not degrade 2-CBA) increased degradation of 2-CBA by 110% compared to uninnoculated plants. Interestingly, this effect was variable among the plant species tested. 2,4-dichlorophenoxyacetic acid (2,4-D) a pesticide whose degradative pathway include the tfdCDEF gene cluster, is another xenobiotic for which enhanced rhizosphere biodegradation has been observed, and which was found to be variable depending on plant species and plant age (Shaw & Burns, 2004). The variability of degradation depending on plant species and plant age may be mediated through root exudates. Root exudates vary in composition and amount between plant species, cultivar, age and stress level of the plant (Uren, 2000). The addition of a PGP bacterium in the Siciliano & Germida (1997) study may have resulted in microbial induced changes in root exudates, as plant biomass was not seen to increase. In this context, a bacterium which is capable of colonizing within the plant, has plant growth promoting properties and can induce changes in plant exudates, as well as carries catabolic genes for the mineralization of pollutants would have great value for phytoremediation of contaminated sites.

1.6 Description of Study Organism

*Burkholderia phytofirmans* OLGA172 was chosen for in depth study as a representative strain from the collection of CBA degraders isolated from pristine environments. *B. phytofirmans* OLGA172 (formally *Burkholderia* sp. R172) was originally isolated from soil sampled in North Western Russia (38°09E, 60°09N), 5 to 30 cm below the soil surface (Fulthorpe, 1996). OLGA172 is one of the 151 strains that kept its 3CBA degrading phenotype after short term storage in -80°C. However it is still found to be unstable in the lab, sometimes not degrading 3CBA at all, or not to completion as indicated by the accumulation of the coloured intermediate chlorocatechol.

The 16S rRNA gene of OLGA172 is 98 % identical to *B. phytofirmans* PSJN a plant growth promoting bacterium isolated from potatoes, and 97 % identical to *B. xenovorans* LB400, another CBA degrader (originally isolated as PCB degrader).
OLGA172 carries the complete set of genes for the modified ortho pathway of chlorocatechol degradation (like LB400, but unlike PSJN). The tfdCDEF genes of OLGA172 are associated with recombinases and integrases upstream and downstream of the catabolic genes (Jin, 2010). The genome of OLGA172 has been sequenced using Illumina Solexa and Genome Analyzer and Roche 454 GS-FLX methods and the data is currently being assembled. It is believed that OLGA172 does not possess a plasmid as no genes typically associated with the origin of replication in plasmids have been found in the genome (repA, repB, parA and parB). The genes that carry out the complete mineralization of chlorocatechol in B. phytofirmans OLGA172 are tfdCDEF (Figure 1.2). These genes are similar (86% nucleotide similarity) to the module I genes of the canonical strain of C. necator JMP134, however OLGA172 possesses a tfdT gene that is not interrupted. The only other bacterial strain annotated on NCBI that carries a full tfdT gene is Burkholderia NK8, a chlorobenzoate degrader isolated from 3CBA contaminated soil in Japan (Liu et al., 2001).

With the exception of the positive 3CBA degrading phenotype, not much is known about the role of this strain in the environment it was isolated from, or the extent to which it degrades 3CBA. Since this strain is one of the more stable 3CBA degrading isolates, characterizing the conditions that induce stable 3CBA degradation in OLGA172 could help stimulate a more stable phenotype in the other pristine isolates which show instability in the lab.

Figure 1.2 The chlorocatechol degradative operon of B. phytofirmans OLGA172
Using *B. phytofirmans* OLGA172, the overall aim of this work was to answer basic questions which could help elucidate the natural role of the organism and its chloroaromatic degrading genes. This was approached by first characterizing the physiological preferences of this organism as well as the capacity for this organism to degrade 3CBA. The ability for OLGA172 to degrade compounds similar in structure to 3CBA and other chlorinated aromatics was investigated. Although this organism was isolated in forest soil in Northwest Russia, nothing is known about its role or niche in the environment. Because it was found to be highly similar to a well known plant growth promoting bacterium, the ability for OLGA172 to colonize plants and have a pathogenic or plant growth promoting capacity was examined. Once located in the plant, expression studies were carried out to determine if the CCD operon is transcribed in an environment more similar to where the strain was found than simple media. Transcriptional studies were carried out on the CCD operon under conditions of easily degradable nutrient availability to determine if OLGA172 is capable of regulating these genes. Finally, the genes associated with mobile genetic movement near the CCD operon were examined to determine if these genes are regulated in cells (if expressed at all).

My research questions were: 1) Under what conditions does this organism thrive? 2) Can this organism colonize plants and are the degradative genes functional within the plant? 3) Does this organism have a broad capacity to degrade chlorinated aromatics? 4) Are the CBA degradative genes regulated and if so under what conditions are they induced? 5) Are there any conditions that cause OLGA172 to stop degrading 3CBA efficiently? 6) Are the integrase genes in OLGA172 functional and actively expressed in cells?

These research questions were asked with the aim of gaining some insight into the evolution of the chlorocatechol (and chlorobenzoate) degradative pathways. The two main hypotheses as to why these capabilities and genes are found in environments without selective pressure are that a) the genes (or the strains themselves) were disseminated from contaminated sites to the pristine sites the strains were isolated from, and b) the genes preceeded the xenobiotic and were likely used to degrade some natural analog. The results of the research questions studied in this thesis can give insights into whether either of those two hypotheses are likely to have occurred. The research questions, and the result expected if either hypothesis were true are outlined in Table 1.2.
<table>
<thead>
<tr>
<th>Research Question</th>
<th>Result expected if ‘Dessemination of genes’ hypothesis were true</th>
<th>Result expected if the ‘genes preceeded the xenobiotic’ hypothesis were true</th>
</tr>
</thead>
<tbody>
<tr>
<td>Can this organism colonize plants and are the degradative genes functional within the plant?</td>
<td></td>
<td>If the CCD genes are induced in the presence of plants, it could indicate that the pathway is related to some plant exudates.</td>
</tr>
<tr>
<td>Does this organism have a broad capacity to degrade chlorinated aromatics</td>
<td>The CCD and CBD genes of OLGA172 would likely be highly specific to the compound they were evolved to degrade at contaminated sites (CBA)</td>
<td>The CCD and CBD enzymes would likely have broad specificity. They were evolved to be specific to some natural substrate and only incidentally are effective against the man-made compound CBA, it is likely they may incidentally degrade other closely related chlorinated aromatics.</td>
</tr>
<tr>
<td>Are the CBA degradative genes regulated and if so under what conditions are they induced?</td>
<td>Lack of optimized regulation would be seen. The foreign genes would likely not be optimized in the strain they have been introduced to</td>
<td>Optimal regulation of both the CCD and CBD pathways could be expected. The pathways would likely be finetuned over evolutionary time to be expressed only when needed (ie- upregulated in the presence of its substrate, and down regulated in the presence of other easily metabolized carbon substrates). However, the pathway would likely only be regulated to its natural substrate, and not necessarily to CBA.</td>
</tr>
<tr>
<td>Are there any conditions that cause OLGA172 to stop degrading 3CBA efficiently?</td>
<td>Genetic mobility may cause instability seen</td>
<td></td>
</tr>
<tr>
<td>Are the integrase genes in OLGA172 functional and actively expressed in cells?</td>
<td>If expression of the integrase genes associated with the CCD operon is seen, it may be indicative of both genetic movement in the past, as well as the potential for mobility currently.</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2. Plant Colonization Capabilities and Physiological Optima of OLGA172

2.1 Introduction

OLGA172 was originally isolated in forest soil in Northwest Russia, however not much is known about the ecological niche, or physiological preferences of this organism. Establishing the conditions for optimal growth is important for further studies, and may also help indicate the conditions that characterized the environment it naturally occupies. For this purpose, OLGA172 was grown in a range of media, as well as Kreb cycle intermediates to examine nutritional preferences. The temperature and pH optima of OLGA172 were also examined. The pH of the soil that OLGA172 was isolated from was measured to be pH 4.9 (Fulthorpe, 1996), so it was thought that its pH optima would be for mild acidity.

The 16s rRNA gene (housekeeping gene) of OLGA172 was found to be highly similar (98%) to a well known plant growth promoting bacterium, Burkholderia phytofirmans PSJN, so the ability for OLGA172 to colonize plants and have a pathogenic or plant growth promoting capacity was examined. The ability to colonize plants endophytically (and promote plant growth) like PSJN, could indicate that plants are a natural system that OLGA172 lives in.

A bacterial endophyte is a microorganism that is capable of colonizing within plant tissue and which causes no negative effect on its host. Endophytes have been isolated which have xenobiotic degrading capabilities and their potential for bioremeditation is great (Ryan et al., 2008). Endophytes have been shown to aid plants in overcoming the toxic effects of herbicides and in bioremediation. For example, when Pisum sativum (pea) was inoculated with a 2,4-D degrading strain capable of colonizing the rhizosphere and endosphere of the plant, the plant showed none of the toxic effects of tissue accumulation of the herbicide 2,4-D. In this study, inoculated plants were able to remove more 2,4-D from the soil than uninoculated plants (Germaine et al., 2006). Because the internal tissue of a plant has been found to promote horizontal gene transfer, the application of an endophytic strain with degradative genes may not require that the particular strain establish a large population size in order to be affective in the
long term. Dissemination of degradative genes from an introduced bacterium to the indigenous population has been shown in the past (Taghavi et al., 2005).

The mechanisms for the plant growth promoting effects of some bacteria is the subject of a lot research, and several mechanisms have been identified such as plant auxin synthesis, siderophore production, and lowering of plant ethylene levels. Inoculation with PGP bacteria (which carry no degradative genes), has been found to increase contaminant removal by plants used for phytoremediation (Siciliano & Germida (1997).

Auxins are a class of plant hormones naturally present in all plants involved in processes of growth and root development, including stem elongation and root initiation (Reinecke, 1999). Many plant associated bacteria produce auxins or other related indole products, and auxin production plays a key role in both pathogenicity (Glickmann, E. & Dessaux, Y., 1995) and plant growth promotion (Glick, 2010). Indole acetic acid (IAA) is a key phytohormone, involved in almost all aspects of plant growth and development and is also produced by both pathogenic and beneficial bacteria. In pathogenic bacteria, it has been associated with tumor growth, for example in crown gall formation and quorum sensing in Agrobacterium tumefaciens (Yuan et al., 2008). IAA production by beneficial bacteria has been shown to primarily increase lateral root production, but also increase root and shoot length and increased tolerance to environmental stress via increased levels of antioxidant enzymes (Glick, 2010). 4-chloroindole-3-acetic acid (Cl-IAA) is a relatively rare chlorinated auxin that is has been found to be 1.3 to 50 times more active than IAA. Cl-IAA has been found in 9 species of the Fabaceae family, including pea (Pisum sativum), broad bean (Vicia faba), and lentil (Lens culinaris). It has only been found in one species outside of that family, the Scots Pine (Pinus sylvestris) (Reineke, 1999).

One common way to assay for PGP rhizobacteria is to look for production of amino-cyclopropane carboxylic acid (ACC) deaminase, a gene responsible for consumption of ACC, the precursor to ethylene. Ethylene is produced when plants are stressed, ACC deminase can make plants less susceptible to environmental stresses like flooding, phytotoxicants, extremes in weather and microbial pathogens (Sun et al., 2009).

In addition to the well studied mechanisms of auxin production, ethylene regulation, and siderophore production, bacterial mediated plant growth promotion has been found occur
through nicotinic acid mononucleotide (NAM) synthesis (through a poorly understood mechanism). In a mutational knockout experiment on *B. phytofirmans* PSJN, loss of the quinolinate phosphoribosyl transferase (QAPRTase) complex genes (which catalyze the formation of NAM) resulted in a loss of plant growth promoting properties, and complementation of these genes restored these effects. Because NAM has widespread metabolic functions, a specific mechanism could not be determined. The authors hypothesized that the metabolites of this pathway may regulate growth stimulation (Wang et al., 2006). There are likely many other mechanisms for plant growth promotion that are still poorly understood due to the complex nature of bacteria-plant interactions.

PSJN is a well studied plant growth promoting bacterium, which has multiple plant growth promoting mechanisms (IAA and siderophore production, ACC deaminase, QPARTase) though ACC deaminase is the most well studied of these mechanisms in this strain. It is also found to be capable of endophytically colonizing within the tissues of plants, travelling through the stem xylem to leaf tissues (Sun, 2009). The entire genome of PSJN has been sequenced and several growth promoting genes identified. Because the sequences for PGP genes in PSJN are available, they can be used to probe OLGA172 for homologous genes. If OLGA172 were to be capable of beneficially influencing plant growth, colonizing plants endophytically and maintaining the chlorocatechol degradative genes, it could be of interest for phytoremediation of some chloroaromatics.

### 2.2 Methods

#### 2.2.1 Growth of *B. phytofirmans* OLGA172

*B. phytofirmans* OLGA172 was pre-grown on Pseudomonas F media, and the biomass washed 5 times in minimal media prior to being used for inocula. For growth assays in various medias and pH buffers, cells were re-suspended in minimal media and adjusted to an OD of 0.07 in a Biolog turbidimeter (Hayward, CA, USA) at 590 nm (approximately 5 X 10^{11} cells per ul) then 1 µl of suspended cells was inoculated into microtitre plates in triplicate. The optical density at 490 nm was used to measure bacterial growth over 6 days. For temperature assays, pelleted and washed cells were adjusted to an OD of 0.1 (7 X 10^{11} cells per ul), and 10 µl of suspended cells were inoculated into test tubes in duplicate. Bacterial growth was measured as OD at 655 nm and 530 nm over 6 days.
2.2.2 Media used for nutrient optima assays

Cells were grown in various concentrations of the Krebs cycle intermediates - fumarate, alpha-ketoglutarate, succinic acid, and citric acid, as well as the simple sugars glucose and pyruvate. All stock solutions were dissolved in minimal media which was made from 10 mM, pH7 phosphate buffer (in 1L: 17.1 g K₂HPO₄·3H₂O; 3.4 g NaH₂PO₄·H₂O), 10mM Mg/Ammonium Solution (in 1L: 33 g (NH₄)₂SO₄, MgSO₄·7H₂O) and 10 mM trace element solution (in 1L: 12 g Na₂EDTA·2H₂O, 2 g NaOH, 0.4 g ZnSO₄·7H₂O, 0.4 g MnSO₄·4H₂O, 0.1 g CuSO₄·5H₂O, 3 g FeSO₄·7H₂O, 5.2 g Na₂SO₄, 0.1 g NaMoO₄·2H₂O). B. phytorhizans OLGA172 was also grown in common microbiological media: Luria Bertani broth (LB - 10g tryptone, 5g yeast extract, 10g NaCl per litre), Pseudomonas F (PF -10g bactotryptone, 10g proteose peptone, 1.5g K₂PO₄, 1.5g MgSO₄ per litre), Nutrient Broth Media (NB - 5g yeast extract, 8g nutrient broth, 10g glucose per litre), as well as yeast extract (YE) and nutrient broth (NB) on its own (5g per litre). Complex medias were also diluted in order to determine if OLGA172 had a preference for low carbon concentrations using these substrates.

2.2.3 TOC Analysis

In order to compare complex medias where carbon content was not easily calculated (yeast extract, nutrient broth) total organic carbon (TOC) content was measured. TOC analysis was carried out on a Shimadzu TOC-V Series analyzer. Samples were acidified to pH 2 with HCl followed by sparging to remove dissolved inorganic carbon. The sample aliquots were then subjected to high-temperature catalytic oxidation to form CO₂ and subsequent CO₂ concentrations measured with a nondispersive infrared detector.

2.2.4 pH buffers

To adjust the pH of media, various pH buffers were used. For pH 3 to 6 a phosphate-citrate buffer was used by mixing adjusting with 0.2M Na₂HPO₄ and 0.1 M Citrate. For pH’s 7 to 8, Sorensens phosphate buffer was used by adjusting with 0.2M NaH₂PO₄ and 0.2 M Na₂HPO₄. For pH 9-10, a sodium bicarbonate buffer was used by adjusting with 0.05 M NaHCO₃ and 0.1 M NaOH.
2.2.5 Gnotobiotic Root Assay

Pea seeds (*Pisum sativum* cultivar Alaska) and tomato seeds (*Solanum lycopersicum*) were surface sterilized by soaking seeds in 70% ethanol for 5 minutes, 1% bleach for 5 minutes, sterile distilled water for 5 minutes, then rinsed 10 times in sterile distilled water. Seeds were then either inoculated with OLGA172 (number of seeds = 45) suspended in minimal media (4.5 X 10^{11} cells per ul) or in minimal media alone as a control (number of seeds = 45) by soaking the seeds in 10 mL of liquid for 1 hour in a rotator. Seeds were then placed in sterile growth pouches (10 cm X 10 cm) with 10 mL of sterile water. Growth pouches were made by enclosing paper towel in Seal-a-Meal plastic (Jarden Consumer Solutions), adding 10 mL water and autoclaving to sterilize and heat seal the pouches. 2 mL of sterile water were added to pouches every second day once seeds were added. Seeds were measured for germination time. After 10 days, germinated seeds were measured for root length, shoot length, number of lateral roots formed and for total lateral root length. 12 plants from each treatment were then planted in autoclaved potting soil. After 30 days plants were measured for shoot length and number of leaves formed. Significant difference between the two groups was determined by applying a students t-test (p<0.05).

2.2.6 DNA Extraction from *P. sativum* and *S. lycopersicum* plants

Three plants that were treated with OLGA172 and two control plants were used for DNA extraction. Samples of the rhizosphere, leaf, stem, root, pea pod, and pea seed were separated and surface sterilized in 70% ethanol for 5 minutes, 1% bleach for 5 minutes, and sterile distilled water for 5 minutes. DNA was extracted from plants using the FastDNA kit (Qbio gene). Rhizosphere samples were collected by removing 0.2 g of soil adhering to the root. DNA was extracted from soil using the FastDNA for Soil kit (Qbio gene).

2.2.7 RNA Extraction

*P. sativum* plant samples were collected the same as outlined for DNA extraction. RNA was extracted from all samples using TRIzol reagent (Invitrogen) as outlined by manufacturer. Plant samples were collected and surface sterilized the same as outlined for DNA extraction prior to RNA extraction. RNA was extracted from all samples using TRIzol reagent (Invitrogen) as
outlined by manufacturer. Bacterial and plant cells were lysed in a homogenizer for 40 seconds. Metal beads were added to aid cell lysis.

2.2.8 Polymerase Chain Reaction (PCR)

HotStarTaq Master Mix (Qiagen Inc., Valencia, CA) was used for all PCR reactions. Each reaction contained 1 unit HotStarTaq Plus DNA Polymerase in 1 x PCR Buffer (1.5 mM MgCl2), 200 μM of each dNTP, and 1 μM forward and reverse primer. Annealing temperature was different for each primer set used as outlined in Table X. The thermal cycling program used for amplifying DNA was: 95 ºC for 5 minutes (one cycle), 94 ºC for one minute, one minute at primer specific annealing temperature, extension was at 72 ºC (42 cycles) for one minute followed by a ten minute final extension at 72 ºC (one cycle).

2.2.9 Primer Design

The tfdC gene was used as a probe in plant samples to detect the presence of OLGA172. To control for the possibility of the loss of catabolic genes, primers specific to OLGA172 targeting the 16S rRNA gene and a section of the genome which shows homology to bovine satellite 1.711a DNA (GenBank ascension number; V00115.1) were designed. NCBI does not show any bacterial strains which carry a gene homologous to the section of OLGA172s genome that is similar to bovine satellite DNA. For the OLGA172 specific gene, the 16S rRNA gene of B. phytofirmans PSJN and OLGA172 were aligned, and regions that showed variability were used as sites for primer binding.

Table 2.2.1 Primers used as probes for OLGA172

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ – 3’</th>
<th>Annealing Temperature</th>
<th>Amplicon size (bp)</th>
<th>Region targeted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bov. sat F</td>
<td>TCA CAT GAC TGC CCA TGC</td>
<td>57 ºC</td>
<td>299</td>
<td>Bovine Satellite DNA found in OLGA172 genome</td>
</tr>
<tr>
<td>Bov. sat R</td>
<td>TGA TCA GCG CCA TTA AGT CA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16s OLGA F</td>
<td>CTT AGG ACC TCG CGC TACA</td>
<td>58 ºC</td>
<td>269</td>
<td>16S Fragment from OLGA172</td>
</tr>
<tr>
<td>16s OLGA R</td>
<td>CAG GGT ATT AAC CCA GAG GTT T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TfdC F</td>
<td>TCC GAC CGA TTT CTA TCG AG</td>
<td>58 ºC</td>
<td>205</td>
<td>Chlorocatechol 1,2-dioxygenase (tfdC)</td>
</tr>
<tr>
<td>TfdC R</td>
<td>GTA CTG GGT CGT CAG CGT TT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2.10 First-Strand cDNA Synthesis and Reverse Transcription

RNA was treated with 1 unit of deoxyribonuclease I (Sigma-Aldrich) for each 10 µl volume of RNA and buffer (200 mM Tris-HCl, pH 8.3, 20 mM MgCl2) prior to being used for reverse transcription. Samples were incubated at room temperature for 15 minutes. The reaction was stopped by adding 50 mM EDTA and heating to 70ºC for 10 minutes. PCR amplification targeting the 16S rRNA gene was performed on DNAsed samples to ensure no DNA contamination in RNA samples.

DNase treated RNA was used as template for first-strand cDNA synthesis. RNA, 50 ng/ul random hexamers, 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP, dTTP) and Diethylpyrocarbonate (DEPC) treated water were incubated at 65 ºC for 5 minutes, and 4 ºC for 1 minute. 40 units of RNase inhibitor (RNAseOUT, Invitrogen) and 200 units Superscript III reverse transcriptase (Invitrogen) were used for each sample, incubated at 25ºC for 5 minutes and then heated to 50ºC for 1.5 hours. The reaction was stopped by heating to 70ºC for 15 minutes.

2.2.11 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed with a DGGE-2001 system (C.B.S. Scientific Company., CA, USA) using an 8% (w/v) acrylamide gel (37.5:1 ratio of acrylamide to bisacrylamide) in 0.5X TAE buffer containing a denaturant gradient of urea and formamide. A gradient of 40% to 70% denaturant was used. Gel electrophoresis was performed at 70V at 60 ºC for 16 hours. Gels were stained with ethidium bromide and photographed. A reference sample was loaded onto all gels in multiple lanes to normalize within a gel and allow comparison between gels. Analysis of the DGGE fingerprint profiles was done using GelCompar II Software (Build 4.6, Applied Maths, Kortijk, Belgium). Gels were standardized using a reference ladder included on all gels. The Unweighted Pair group Method using Arithmetic averages (UPGMA) algorithm was used to created a dendrogram clustering data on the basis of their similarity (as determined by the Pearson coefficient) to aid in visual comparison.
2.2.12 Salkowski Colourimetric Assay for Indole Acetic Acid Production and Metabolism

OLGA172 was grown in minimal media supplemented with 5 mM indole acetic acid, 5 mM chloroindole acetic acid, 5 mM indole acetic acid with 0.1% glucose, 5 mM chloroindole acetic acid with 0.1% glucose and 4.5 mM L-Tryptophan (IAA precursor) with 0.1% glucose at 28ºC for 6 days with shaking. Optical density readings at 530 nm were taken daily to measure growth. Bacterial cells were pelleted by centrifugation at 10,000 x g and the supernatant removed and stored at – 20ºC until use. For the Salkowski assay, supernatant was mixed in a 1:2 ratio with Salkowski Reagent (2% 0.5 M FeCl$_3$ in 35% perchloric acid) and incubated at room temperature for 30 minutes. After 30 minutes colour formation was measured at 655 nm.

2.3 Results and Discussion

2.3.1 Nutrient Preferences of OLGA172

*B. phytofirmans* OLGA172 was not able to use the citric acid cycle intermediates succinate, citrate or α-ketoglutarate as sole carbon sources (Figure 2.1). When comparing complex medias, no strict relationship between carbon content and bacterial growth was seen (Figure 2.2). For NB and PF media, higher carbon content always resulted in more biomass. However, for NBM (complex media which contains nutrient broth, glucose and yeast extract), LB and YE, optimal growth occurs at relatively low carbon concentrations (approximately 150-500 ppm carbon). The absence of a direct relationship between carbon content and biomass in complex medias suggests that some other factor in the media is limiting growth such as salinity or nitrogen levels which were not quantified.
Figure 2.1 *B. phytofirmans* OLGA172 growth on Krebs cycle intermediates
Average of maximum triplicate OD measurements (OD 655 nm) over 6 days of growth.

Figure 2.2 *B. phytofirmans* OLGA172 growth on common microbological medias
Average of maximum triplicate OD measurements (OD 655 nm) over 6 days of growth.
2.3.2 pH preferences of OLGA172

*B. phytofirms* *OLGA172* was found to have a preference for mild acidity as expected. Growth occurred in media with a pH of 4-8, with maximal growth at pH 5. Growth in both Pseudomonas F media and Luria Bertani broth with varying pH was found to be similar, although Pseudomonas F media supported better growth at pH 7-8 (Figure 2.3).

![Figure 2.3 pH optima of B.phytofirms in LB broth and Pseudomonas F media](image)

*Figure 2.3 pH optima of B.phytofirms in LB broth and Pseudomonas F media*

Average of triplicate OD measurements (OD<sub>530</sub>) after 3 days of growth in varying pH.

2.3.3 Temperature Preferences of OLGA172

OLGA172 was able to grow well in media at temperatures from 15°C to 35°C. OLGA172 grew slowest at 15°C, and not at all in 4°C over a week (Figure 2.4).

![Figure 2.4 Temperature optima of B.phytofirms grown in Pseudomonas F media](image)

*Figure 2.4 Temperature optima of B.phytofirms grown in Pseudomonas F media*

Average of duplicate OD measurements (OD<sub>530</sub>) after 3 days of growth
2.3.4 *B. phytofirmans* OLGA172 is rhizospheric and endophytic

Using the *tfdC* gene as an indicator for the presence of OLGA172, this strain was found be capable of endophytically colonizing both *P. sativum* (Pea) and *S. lycopersicum* (Tomato). The *tfdC* gene was amplified from DNA extracted from the rhizosphere and leaves of *S. lycopersicum*, and the rhizosphere, roots, stems and leaves of *P. sativum* treated with OLGA172 (Figure 2.5). Uninoculated control plants did not amplify the *tfdC* gene in any sample (data not shown). The presence of amplification products when using the OLGA172 16S rRNA gene specific primers was not found to be in agreement with the presence of *tfdC* (Table 3.1). This is most likely because the primers were not specific enough to target only OLGA172. However, a loss of the catabolic genes in samples that were negative for the presence of *tfdC* but positive for the OLGA specific 16S gene probe could also explain these results. Another explanation could be the transfer of the *tfdC* gene to other endophytic microbial species in the plant. A BLAST primer search for the specificity of the primer pair among Burkholderiales (at least 2 total mismatched bp and 2 total mismatched within the last 5 bp of the 3’ end) resulted in 8 strains that could have amplified with that primer pair. All samples were negative when the BSD primer set was used in plants, though both designed primer pairs were found to amplify a product in OLGA172 positive controls.

**Table 2.2 OLGA12 is an endophyte of *P. sativum* and *S. lycopersicum***

<table>
<thead>
<tr>
<th>Primer set used</th>
<th>Rhizosphere (Soil adhering to the root)</th>
<th>Root</th>
<th>Stem</th>
<th>Leaf</th>
<th>Pea seed</th>
<th>Pea pod</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pea</td>
<td>tfdC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tomato</td>
<td>tfdC</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pea</td>
<td>16S(OLGA)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tomato</td>
<td>16S(OLGA)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

*a. Not positive in the three plants tested*
Microbial community analysis of the stem, leaf, roots and rhizosphere of pea plants by DGGE revealed that although OLGA172 is an endophyte in these samples (interpreted from positive tfdC products in plants) it is not the dominant species (interpreted by band intensity). The detection limit for DGGE in soil samples can be as high as $10^6$ cells per gram of soil, but extraction from plants can inhibit PCR and may raise this detection limit (Solaiman & Marschner, 2007).

When universal 16S primers were used to fingerprint communities, it was not possible to unambiguously identify OLGA172 in samples. There was a multiple banding pattern by the positive control of OLGA172 (amplified from DNA extracted from a pure culture of OLGA172), none which corresponded to bands in samples from inoculated plants (Figure 2.6). The 16S rRNA gene is the most commonly used molecular marker in microbial ecology, however multiple copies of this gene are often present in one strain, and these copies can differ in sequence resulting in multiple bands per strain (Case et al., 2007). OLGA172 has 7 distinct bands in its fingerprint, which suggests 7 copies of the 16S rRNA gene, each which may differ in sequence. DGGE is able to resolve bands that differ in as little as one base pair. B. phytofirmans PSJN carries 5 copies of the 16S rRNA gene, 3 copies on both chromosome 1 and 2. Alignment of the 5 copies of PSJN shows that one copy on chromosome 2 differs by 7 bp, whereas another
copy on chromosome 1 differs from the rest by only 1 bp. In the unassembled genome data from OLGA172 confirmation of 7 different copies of the 16S gene is difficult, because no single section of sequence data from OLGA172 covers the entire 16S gene, and no more than 4 sections of sequence showing homology to the 16S gene were found.

An alternative housekeeping gene to the 16S rRNA gene, is the RNA polymerase B subunit gene (rpoB). RpoB is a single copy gene, and has successfully been used to look at microbial community structure in soil (Peixioto et al., 2002). DGGE using primers targeting the rpoB gene still resulted in multiple bands in OLGA172 (Figure 2.7), likely the result of amplification of non-target DNA. Overall the DGGE profiles using these genes had less bands, and there were bands that had similar GC content to that of OLGA172, and are assumed to be the strain. The community picture that emerges with the rpoB primers is one of low diversity in both inoculated and uninoculated samples, which is not entirely surprising as the seeds were surface sterilized and soil autoclaved prior to planting. When looking at the community profiles of inoculated plants in comparison to control plants, there are more bacterial species in the inoculated plants. There seems to be either one or no strains colonizing the control plants. However, because the rpoB primers also had multiple banding with the OLGA172 isolate, multiple bands in community profiles cannot be said to be the result of higher diversity, it may also be mispriming as well (in strains other than OLGA172). The effects of OLGA172 on community structure does not seem to be dramatic in P. sativum, although its presence may increase diversity in the plant.
Figure 2.6 16S DGGE community profiles of pea plants treated with OLGA172
Plants are labeled with the replicate plant samples are from (a, b, c). Dendrogram is to aid in visualizing similarities between community profiles.
Figure 2.7 rpoB DGGE community profiles of pea plants treated with OLGA172
Plants are labeled with the replicate plant samples are from (a, b, c). Dendrogram is to aid in visualizing similarities between community profiles.
2.3.5 *B. phytofirmans* OLGA172 is plant growth promoting

When *P. sativum* seeds were inoculated with OLGA172, a larger proportion of inoculated seeds were found to germinate after one day when compared to control uninoculated seeds (66% compared to 32% respectively). While the percentage of seeds germinated from the inoculated seeds was consistently higher than the control treatment over 6 days, these results were not found to be significant (Figure 2.8). Final percentage of seeds germinating was 88% for treated seeds, and 86% for the control after 6 days (t-test, p-value: 0.25)

![Germination Time](image)

**Figure 2.8 Germination time after treatment with OLGA172**

In both *P. sativum* and *S. lycopersicum* seeds, treatment of surface sterilized seeds with OLGA172 resulted in longer root length, shoot length and total lateral roots formed after 10 days of growth (Figure 2.9, and 2.10). When *P. sativum* plants reached 30 days of growth, measured shoot lengths and number of leaves formed were not found to be significantly different (Table 2.3). Thus the plant growth promoting effects of OLGA172 seem to be limited to early plant growth.

**Table 2.3 Shoot Length and Leaves formed after 30 days growth**

<table>
<thead>
<tr>
<th></th>
<th>Average Control</th>
<th>Standard deviation</th>
<th>Average. OLGA</th>
<th>Standard deviation</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot length, cm (30 days)</td>
<td>490</td>
<td>201</td>
<td>532</td>
<td>88</td>
<td>0.27</td>
</tr>
<tr>
<td>Number of leaves (30 days)</td>
<td>24.7</td>
<td>4.6</td>
<td>25.5</td>
<td>2.3</td>
<td>0.30</td>
</tr>
</tbody>
</table>
Figure 2.9 Tomato root and shoot growth 10 days after inoculation
Total lateral roots formed were found to be statistically significant when tomato seeds were treated with OLGA172 (P=0.03)

Figure 2.10 Pea root and shoot growth 10 days after inoculation
Inoculation with OLGA172 was found to promote root length, shoot length and total lateral roots formed significantly. Differences in total lateral root length was not found to be significant (P values; 0.03, 0.03, 0.00005 and 0.3 respectively)
2.3.6 OLGA172 possesses genes associated with promoting plant growth

OLGA172 was not found to carry the gene encoding 1-amonicyclopropane-1-carboxylate (ACC) deaminase, a gene that has been correlated with *B. phytofirmans* PSJNs plant growth promoting properties. In order to determine what may be the mechanism for the growth promoting properties of OLGA172, genes associated with increased root growth in *B. phytofirmans* PSJN were used as probes for homologous genes in the genome of OLGA172 using Sequencher. Genes which are correlated with increased root growth are outlined in Table 2.4. All of these genes are also found in *B. phytofirmans* PSJN, including (ACC deaminase).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Present in OLGA172</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indole Acetimide Hydrolase (Iaah)</td>
<td>Gene in the tryptophan pathway for IAA production.</td>
<td>Yes</td>
</tr>
<tr>
<td>Tryptophan-2-monoxygenase (IaaM)</td>
<td>Gene in the tryptophan pathway for IAA synthesis</td>
<td>No</td>
</tr>
<tr>
<td>1-amonicyclopropane-1-carboxylate (ACC) deaminase</td>
<td>Regulates ethylene levels in plants by metabolizing its precursor.</td>
<td>No</td>
</tr>
<tr>
<td>Indolepyruvate decarboxylase (IpdC)</td>
<td>Catalyzes a key step in the indolepyruvic acid pathway for IAA synthesis.</td>
<td>No</td>
</tr>
<tr>
<td>Quinolinate phosphoribosyl transferase (QAPRTase)</td>
<td>NAM biosynthesis</td>
<td>Yes</td>
</tr>
</tbody>
</table>

2.3.7 OLGA172 produces indole products from tryptophan precursors

The Salkowski reagent assay is a commonly used colourimetric reaction to detect IAA, colour is produced in proportion to reagent present. This method was used to measure any possible IAA produced by OLGA172 grown on the IAA precursor tryptophan, and a positive assay result was found. Production of indole product was found in the supernatant after 4 days of growth (Figure 2.11). Analysis of the supernatant from the samples by HPLC showed that the compound being produced was not IAA. A product with a retention time of approximately 2 minutes was found, compared to the retention time of 5.5 minutes for IAA. These retention time differences were consistent in samples over the 6 days. Although the Salkowski reagent assay has been used in multiple studies to quantify the production and consumption of IAA in bacterial strains (Ahmad et al., 2008; Ali & Hasnain, 2007), a study by Glickmann & Dessaux (1994) found that the
Salkowski assay was not specific to only IAA, but showed colourimetric results for the indole products indolepyruvic acid (IPyA), and indoleacetamide (IAM). Both compounds are involved in auxin biosynthesis (Glickmann, E. & Dessaux, Y., 1995) and as result, their production may be the cause of the plant growth promoting effects of OLGA172. Genes homologous to the synthesis genes for both IPyA and IAM were not found in the unassembled genome sequence of OLGA172, however a gene homologous to indole acetimide hydrolase (IaaH) was found (Table 2.4). IaaH is responsible for the conversion of IAM to IAA.

![Graph showing Salkowski reagent colour accumulation in tryptophan grown OLGA172](image)

**Figure 2.11 Salkowski reagent colour accumulation in tryptophan grown OLGA172**
Values are the average of 3 replicates. Samples were collected for 7 days and subjected to Salkowski reagent analysis on the same day.

**2.3.8 OLGA172 cannot use CLIAA or IAA as sole carbon sources**

OLGA172 was able to grow well on IAA supplemented with 0.1% glucose, and showed mild growth on IAA. OLGA172 was not able to grow on chlorinated IAA with or without 0.1% glucose. Using the Salkowski reagent assay, the consumption of IAA and CLIAA was not detected. HPLC analysis of the supernatant collected from these cultures confirmed no degradation of CLIAA, or IAA (data not shown).
2.3.9 Expression of the CCD gene within the plant

The *tfdC* gene was found to be expressed in RNA extracted from the rhizosphere, but not within the roots of surface sterilized pea plants. The gene was also found to be transcribed in the stem and leaves of plants inoculated with OLGA172 (Figure 2.12). Amplification of the OLGA172 housekeeping gene *rpoD* using RT qPCR resulted in multiple peaks with differing melting temperatures seen in the product dissociation curves, therefore attempts to quantify transcription levels of the *tfdC* gene in the plant were unsuccessful without a gene to normalize against. When the amplification products for the *rpoD* gene were run on an agarose gel, only one band was visualized. This suggests that the multiple peaks (differing melting temperatures) of the qPCR amplification products are the result of amplification of the *rpoD* gene from at least one other strain in the plant with different nucleic acid sequence.

![Figure 2.12 Expression of the *tfdC* gene within the plant](image)

Figure 2.12 Expression of the *tfdC* gene within the plant
S, Stem; L, Leaf; R, root; Rh, Rhizosphere; O+, OLGA172, (-), negative control.

2.4 Summary

OLGA172 was found have a pH optima for mild acidity (pH 4-6), temperatures in the range of 20-30 ºC, and to grow best on the common microbiological ‘nutrient broth media’. When grown in simple medias consisting of a single carbon source, OLGA172 was found to have a preference for carbon in the range of 100-150 mM, growing best on glucose. This range of carbon is more similar to what would be found near root exudates, or within plants (Darab, 1991), rather than the oligotrophic conditions typically found in bulk soil (Hu et al., 1999).
OLGA172 was found to be capable of endophytically colonizing the plant systems of *P. sativum* and *S. lycopersicum* which may indicate that the strain is plant associated naturally. However, it should be noted that neither *P. sativum* nor *S. lycopersicum* would naturally be found in a boreal forest ecosystem in northwestern Russia (where OLGA172 was isolated), and are not expected to be a natural plant host for OLGA172. Both *P. sativum* and *S. lycopersicum* were chosen as model plants to look at the endophytic capabilities of OLGA172, and its potential for plant growth promotion. Additionally, both *P. sativum* and *S. lycopersicum* can be used to assay for the effects of IAA and ACC deaminase.

OLGA172 was found to have a plant growth promoting effect on shoot and root length, as well as increasing lateral root growth in young plants, though a single mechanism for this effect could not be identified. Because an increase in root length may be related to the increase in shoot length (or the increase in lateral root growth and lateral roots formed) observed, the primary effect of OLGA172 is not known. It may be that OLGA172 only significantly promotes growth of one of these variables, and as a result, other parameters of plant growth are increased as well. Genes in OLGA172 were identified which are associated with plant growth promotion in the closely related strain PSJN (Table 2.4) and may be further avenues for investigation of the plant growth promoting effects of this strain.

Using the *tfdC* gene (the first gene involved in chlorocatechol degradation) OLGA172 was found to be capable of colonizing the rhizosphere and endophytically colonizing within parts of *P. sativum* and *S. lycopersicum* (Table 2.2). However, OLGA172 was not unambiguously identified in plant samples using the designed OLGA specific primers in PCR probing attempts, or in DGGE based methods targeting two different housekeeping genes (Figures 2.5-2.7). In addition, samples which were positive for *tfdC* amplification were not all positive for the other OLGA172 probes. Design of OLGA172 specific primers which do not unintentionally amplify plant DNA or alternatively tagging OLGA172 with an easily identifiable probe such as GFP will aid in observing colonization patterns of OLGA172 in the future. It will also help determine whether the *tfdC* gene has been disseminated to other bacterial strains within the plant.

Interestingly, in RNA extracted samples, the *tfdC* gene was found to be expressed at levels detectable with regular PCR. Attempts to quantification levels of *tfdC* in differet parts of the
plant were unsuccessful because the designed housekeeping genes for normalization did not yield a single product when amplified from plant samples. Design of better OLGA specific primers in this case would help examine whether the tfidC gene is inducible or expressed at higher levels in different parts of the plant in proportion to the population of OLGA1172 found in a specific area of the plant.

Overall, these results do indicate that OLGA172 is likely to be plant associated in the environment was found in.
Chapter 3. Characterization of chlorocatechol degradation in OLGA172

3.1 Introduction

With the exception of a 3CBA degrading phenotype, not much is known about the growth habits of OLGA172. An upper limit of toxicity of 3CBA/CC for OLGA172 has not been established. Based on past observation in the laboratory, OLGA172 can show phenotypic variation in its ability to completely degrade 3CBA. On occasion, partial degradation of 3CBA is observed as a brown colour accumulates in liquid cultures or in the center of colonies grown on 3CBA agar. This is presumed to be due to the accumulation of polymerization products of oxidized chlorocatechol that are toxic to microbial cells (Fava et al., 1993). This colour accumulation is indicative of low levels of chlorocatechol dioxygenase so it is of interest to identify the conditions that cause it. Causes for this phenotypic instability can include the loss or movement of the catabolic genes (genetic instability), or non-optimal transcriptional control of the genes.

Plasmid encoded CCD genes may easily be lost if plasmids are not selected for, however in OLGA172 there is no evidence that the CCD operon is located on a plasmid, or even that OLGA172 carries a plasmid (Jin, 2010). Because the presence of genes associated with mobility are located in proximity to the CCD operon, movement of the CCD genes in OLGA172 is a potential cause for the loss of the 3CBA degrading phenotype in the lab. Insertion of a mobile genetic element in or near the CCD operon may result in disruption of a critical enzyme in the CCD pathway, or disrupt normal regulation of this operon. Insertional inactivation can be seen in JMP134, where the tfdT gene is disrupted by insertion sequence ISJP4, resulting in an early stop codon and a non-functional protein which can no longer properly regulate the CCD operon (Leveau and van der Meer, 1996).

In addition, poor regulation of the CCD operon could also be an explanation for the phenotypic instability seen frequently in the lab. If the enzymes required for CC degradation are not produced in sufficient quantities, CC may accumulate and become toxic to the cells upon oxidation. Data so far indicates that the CCD operon in OLGA172 is present as a single copy and
located chromosomally (Jin, 2010). The advantage of plasmid encoded genes is that they may be expressed in multiple copy numbers in order to produce sufficient levels of enzyme when required. A gene dosage effect has been observed in JMP134, which naturally has been found to contain several copies of the CCD operon in its genome. Derivatives of JMP134 which have been engineered to carry only a single chromosomal copy of one of the CCD operons are unable to grow on 3CBA, conversely when multiple copies of the CCD operon were introduced to this engineered strain, growth on 3CBA was restored (Pérez-Pantoja et al., 2002).

The effects of inoculation volume, and innoulum source were examined to determine if these factors played a role in the phenotypic instability seen in OLGA172. It may be that OLGA172 needs to be present in sufficient population numbers in order to degrade 3CBA at a rate that consumes CC before accumulation and oxidization occurs. If cells are pre-grown on media containing 3CBA, the genes may be induced and the enzyme present in the cell in sufficient amounts to degrade 3CBA efficiently when used as inocula for downstream experiments.

Growth capabilities of OLGA172 in the presence of easily degradable carbon sources as well as 3CBA were carried out to determine if catabolite repression occurs in this strain. If OLGA172 metabolizes an easily degradable carbon source in media with preference to 3CBA (but still partially converts 3CBA to CC), the result may be inefficient turnover of chlorocatechol, resulting in toxicity. Catabolite repression has been found in other chlorocatechol degraders, and has been found to occur at the transcriptional level (McFall et al., 1997).

Another explanation for the phenotypic instability seen in OLGA172 could be that the CCD operon, or at least the peripheral genes that convert compounds to substrates of the CCD operon was not evolved for 3CBA metabolism and therefore does not efficiently convert 3CBA. If that were the case then CC would not accumulate. While it may be unlikely to find the ‘natural’ chlorinated aromatic for the CCD operon in the lab, broad specificity of the CCD genes could indicate that this pathway was not specialized for 3CBA. In order to test this, OLGA172 was grown on chlorinated compounds similar in structure, and some natural chlorinated aromatics to determine if growth and degradation can occur.

To characterize the limits of 3CBA degradation, OLGA172 was grown in various concentrations of 3CBA and growth, growth rate, and rate degradation was measured in various concentrations
of 3CBA. Accumulation of chlorocatechol (seen as colour accumulation) was used as a measure of the CCD pathway functioning improperly. The effects of pre-growing OLGA172 on agar media or liquid media, and on selective and non-selective media prior to inoculation, as well as the effects of varying inoculation volume on colour formation were examined. Catbolite repression was examined by growing OLGA172 on various easily degradable carbon sources in the presence of 3CBA, and finally the capacity for OLGA172 to degrade other chlorinated aromatics was tested.

3.2 Methods

3.2.1 Growth of OLGA172

For growth in 3CBA, cells were pelleted, washed 5 times in minimal media, and then resuspended to an OD of 0.1 in minimal media (7 X 10^{11} cells per ul). 25 mM stock 3CBA was made by dissolving 3CBA in minimal media, with the addition of 1 g NaOH per litre of solution. Stock 4CBA was made in the same way. Growth was measured as OD at 655 nm in a Spectrogenic Genesys 5 spectrophotometer (Milton Roy) for 7 days. When OD readings were taken, bacterial cells were centrifuged and the pellets saved, along with the separated supernatant. Samples were stored at -80 C until used for HPLC analysis. Easily degradable carbon sources used for growth were pyruvate and glucose, and were made as described before (methods 2.2.2) except that 3CBA was added to a final concentration of 2 mM.

To measure the effects of pre-growing cells on different media, OLGA172 was pre-grown on 3CBA agar and liquid media, R2A agar, or Pseudomonas F media, and washed 5 times in minimal media prior to be used as inoculants. Cells were resuspended to an OD of 0.1 (7 X 10^{11} cells per ul), prior to being used for inocula. Cells were inoculated into 2 mM, 4mM, and 6mM 3CBA. Growth was measured as OD at 655 for 7 days. In order to quantify colour formation, bacterial cells were centrifuged and the OD (OD655) of the supernatant measured. The OD of the culture subtracted by the supernatant OD was used to measure growth.

To measure the effects of different inoculation volumes, cells were pre-grown in Pseudomonas F media, pelleted and washed 5 times in minimal media. Cells were resuspended to a OD of 0.1 (7 X 10^{11} cells per ul), volumes of 1 ul, 25 ul, 500 µl and 1000 µl were inoculated into 5 mL of 3 mM 3CBA.
Growth of OLGA172 on other chlorinated compounds was carried out as described for growth on varying concentrations of 3CBA. Compounds examined included, 4-CBA, chloroindole acetic acid, 3,5-dichloroanisaldehyde, 3-chloroanisaldehyde, trichloroguaiacol, 4-chlorocatechol, 6-chlorovanillin, 5-chlorovanillin, trichlorophenol, pentachlorophenol, and chloroacetic acid. With the exception of the chloroanisaldehydes and chloroindole acetic acid, these substrates are not present in the environment naturally.

### 3.2.2 High Performance Liquid Chromatography (HPLC)

HPLC analysis was performed using a Waters 501 HPLC system equipped with a c-18 column, Waters 996 Photodiode Array detector and Waters 717 autosampler. The mobile phase was composed of 40% Acetonitrile, and 60% (1%) Phosphoric Acid. Readings were carried out at 254 nm with a flow rate of 1 mL per minute. Data was analyzed using Waters Millenium software (version 2.15).

### 3.2.3 PCR genomic fingerprinting

Because colour accumulation could also be the result of contamination of cultures with a microbe which has carries broad acting genes which can covert CBA to CC. Genomic fingerprinting of brown samples were carried out to ensure the fingerprints match that of OLGA172. Fingerprinting was done using PCR as described earlier, with the following altered cycle: initial denaturing, 95 C for 2 minutes; 30 cycles of 94 C for 3 seconds, 92 C for 30 seconds, 50 C for 1 minute, 65 C for 8 minutes; final extension 65 C for 8 minutes. A single primer was used for both the forward and reverse primer: Box (5’-3’) CTACGGCAAGGCGACGCTGACG (Rademaker et al., 1998).

### 3.3 Results

#### 3.3.1 Growth of OLGA172 on 3CBA

OLGA172 demonstrated phenotypic instability when grown in varying concentrations of 3CBA. In some instances, OLGA172 was not able to grow in concentrations of 3CBA above 3 mM or 5 mM. In two independent replications of this experiment OLGA172 was found to grow in concentrations of 3CBA up to 8 mM. Despite phenotypic inconsistency in limits of growth in various concentrations of 3CBA, general trends for growth were consistent. Lag time was consistently found to be inversely dependant on 3CBA concentration (Figure 3.1). Increasing
3CBA concentrations also resulted in a slower growth rate of OLGA172 (Figure 3.2), slower mineralization of 3CBA (Figure 3.3), and less 3CBA being degraded overall (Figure 3.4).

**Figure 3.1 Effect of increasing 3CBA concentration and lag time in OLGA172**
Lag time was found to increase with increasing 3CBA concentration. Growth in 9 mM and 10 mM 3CBA never reached an OD$_{655}$ of 0.05.

**Figure 3.2 Growth rate of OLGA172 in increasing 3CBA concentrations**
Growth rate was measured as the maximum growth increase occurring over 24 hours measured for each concentration of 3CBA over 7 days.

Figure 3.3 Rate of 3CBA degradation by OLGA172
Rate of degradation was measured as the maximum 3CBA degraded over 24 hours for each concentration of 3CBA over 7 days.

Figure 3.4 Total 3CBA Degradation by OLGA172
Total 3CBA degradation was measured as the percentage of total 3CBA degraded every 24 hours in varying concentrations of 3CBA.
3.3.2 Effect of inoculation volume of OLGA172 on colour formation in 3CBA

Colour accumulation in samples was visible after 5 days of growth, although colour production was detected by absorbance measurements after 3 days of growth. Higher inoculation volumes resulted in a reduction in lag time, with 1 µl inoculants having the longest lag time (Figure 3.5). Slight colour accumulation (browning) was seen in all samples, however the only samples to turn notably brown were the 1 µl inoculants. Within the triplicate 1 µl inoculant samples phenotypic variation was seen; one replicate appeared very dark, another showed no visible colour accumulation, and the third exhibited intermediate accumulation (Figure 3.6). Samples from all tubes were plated and were fingerprinted and confirmed to have no contamination.

Figure 3.5 Effect of different inoculation volumes on colour formation in 3CBA
A) Growth of OLGA172 in 3CBA. B) Optical density of supernatant only. C) OD<sub>655</sub> readings of growth minus OD<sub>655</sub> readings from supernatant. Curves are the average of three replicates.
Figure 3.6 Phenotypic variation in 3CBA degradation by OLGA172
Replicates of 1 µl inoculant samples, OD$_{655}$ values: 0.124, 0.138, 0.142 (left to right) average of three replicates 0.135. B) Absence of contamination was confirmed by plating and fingerprinting of a representative colony from each tube.

3.3.3 Effect inoculation source of OLGA172 on colour formation in 3CBA

The effects of pre-growing cells on selective and non-selective liquid and agar media was examined. Non-selective media looked at included R2A agar, and Pseudomonas F (liquid). Biomass obtained from R2A agar had a longer lag time than biomass obtained from log phase cells growing in Pseudomonas F media and 3CBA liquid media. Innoculum from 3CBA agar had similar growth to Pseduomonas F and 3CBA liquid media. Slight colouration of samples was visible, however no sample turned brown by eye, regardless of pre-inoculum source when samples were grown in 2mM 3CBA (Figure 3.7). Samples inoculated into 4 mM 3CBA did not start growing until the 8$^{th}$ day, and samples grown in 6 mM 3CBA did not grow at all over 10 days (data not shown).
3.3.4 Growth of OLGA172 on other chlorinated aromatics

Growth of OLGA172 was seen on 4-chlorocatechol (an intermediate of 3CBA degradation), however no growth of OLGA172 was seen on any of the other compounds tested, including 4CBA. For samples where cell death of OLGA172 hadn’t occurred over 7 days (though biomass had not significantly increased), HPLC analysis of the media was carried out to look for any degradation. No significant degradation was seen in any samples other that 4CC (data not shown).
3.3.5 Effect of an Easily Degradable Carbon Source on CC degradation

When OLGA172 was grown in increasing concentrations of pyruvate and glucose, colour accumulation was seen in all samples and found to be dependent upon carbon concentration (Figure 3.8 and 3.9). When the concentration of substrate increased, increased colouration was seen (3CBA concentrations remained the same in all samples). In addition, growth on these carbon sources when combined with 3CBA resulted in less cell growth with increasing substrate concentration when compared to the same media without 3CBA added.

![Figure 3.8 Growth of OLGA172 on pyruvate and 3CBA results in colour formation](image)

Average of triplicates, un; uninoculated sample. Error bars represent standard deviation. Red box is around photo of OLGA172 grown on indicated media, but grown on a different 96 well plate.

![Figure 3.9 Growth of OLGA172 on glucose and 3CBA results in colour formation](image)

Average of triplicates, un; uninoculated sample. Error bars represent standard deviation.
3.4 Discussion

Slower growth rate, degradation and overall growth of OLGA172 was found with increasing concentrations of 3CBA, indicating that at higher concentrations this substrate is toxic to microbial cells. An absolute limit of toxicity was not established for OLGA172 since independent replications of this experiment (with identical inoculation volumes and pre-growth source conditions) resulted in different upper limits for growth. Differences in the condition of cells used for inoculation (age of culture, stage of growth etc) may be potential reasons for the inability to replicate this experiment. Phenotypic variance was most notably seen in replicates when 1 µl inoculant volumes were examined (Figure 3.6A), however since these samples are from the same source and volume of OLGA172 inoculum, differences in the condition of the bacterial population cannot be used to explain this variance.

The lack of significant growth of OLGA172 on the chlorinated aromatics tested (with the exception of growth on 4CC which was expected) indicates that the CCD operon of OLGA172 is not adapted for growth on any of these substrates, nor does OGLA172 carry genes of broad enough specificity to act on these compounds. Lack of conversion or growth on 4CBA indicates regiospecificity and suggests that the pathway for CBA degradation is specialized in OLGA172 to degrade specific isomers. This is in contrast to for example *Burkholderia* NK8, which degrades 3CBA and 4CBA (Liu et al, 2001).

Notably, increasing concentrations of easily degradable carbon sources increased colour accumulation. CBA conversion to CC must have occurred, however metabolism of CC did not proceed at a sufficient rate to prevent oxidization of the CC. Note that OLGA172 and all other 3CBA degraders in the collection were selected on 50 ppm 3CBA, which is roughly 0.3 mM, much lower that the concentrations used here. It may be possible that the pathway for CC degradation is catabolite repressed, whereas the pathway for CBA conversion does not have similar regulation at the transcriptional level. This lack of optimization may reflect that the two pathways have not been subject to similar evolutionary pressures, possibly because of HGT of one of the operons, or because CBA is not the substrate that typically gets channeled through the CCD operon naturally. Alternatively, these carbon sources (or metabolites of their degradation)
may interact directly with enzymes present in the cell causing inhibition. In either scenario, the result is cell death and growth restriction in the presence of oxidized CC.
Chapter 4. Imbalance in regulation of the CCD operon and CBA degradative genes in OLGA172

4.1 Introduction

While the genes for chlorocatechol degradation are known in OLGA172, the genes responsible for the conversion of chlorobenzoate to chlorocatechol have not been identified in this strain. The focus of studies on CBA degrading strains thus far has been on the chlorocatechol degradative pathway, since chlorocatechol conversion has been found to be the rate-limiting step, while the work done on chlorobenzoate conversion is less extensive. The conversion of some chlorinated aromatics to chlorocatechols can be carried out by enzymes with broad specificities, which may be nonspecific enough to act on chlorinated compounds in addition to their non-chlorinated substrates (Schlomann, 1994). For example, *C. necator* JMP134, degradation of CBA to chlorocatechol is carried out by a chromosomal, low-specificity benzoate dioxygenase and 1,2-dihydro-1,2-dihydroxybenzoate dehydrogenase which forms 3-chlorocatechol and 4-chlorocatechol (Pérez-Pantoja, 2003). These low specificity enzymes may, over time, be selected for metabolism of compounds with chlorinated substituents, for example the chlorocatechol 1,2-dioxygenase is evolutionarily related to catechol 1,2-dioxygenase but exhibits a preference for chloro- and methyl-substituted catechol over the non-chlorinated analog (van der Meer, 1997).

In a few CBA degrading strains, the genes for metabolism of CBA have been identified. *Burkholderia* TH2 is a 2CBA degrader which metabolizes 2CBA via catechol, and carries the multicomponent 2-halobenzote 1,2-dioxygenase genes encoded by *cbdABC* (Suzuki et al., 2001), as does *Pseudomonas cepacia* 2CBS encoded by *cbdABC* on a plasmid (Haak et al., 1995). The halobenzoate 1,2-dioxygenase has a broad substrate specificity, but benzoate analogs with electron-withdrawing substituents at the ortho position (2-fluoro-, 2-bromo-, 2-chloro) are metabolized preferentially, and converted to kreb cycle intermediates through catechol (Fetzner et al, 1992).

*Burkholderia* NK8, a 3CBA and 4CBA degrader, carries a multicomponent chlorobenzoate 1,2-dioxygenase genes (*cbeABC*) in an operon clustered with its catechol degradative genes. The
chlorobenzoate dioxygenase in this strain, converts CBAs to catechol, 3-chlorocatechol and 4-chlorocatechol (Francisco et al, 2001).

Chlorocatechol oxidizes and polymerizes to compounds which are toxic to bacterial species when allowed to accumulate (Fava et al., 1993). In addition to this toxicity, the activity of the ubiquitous muconate cycloisomerase from the catechol degradative pathway can transform 4-chlorocatechol to form the antibiotic protonanemonin (Blasco et al., 1995). Therefore, efficient transformation of chlorocatechol is essential to bacterial survival. For efficient conversion of CBAs to non-toxic kreb cycle intermediates, synchronized regulation of both the chlorocatechol producing and chlorocatechol metabolizing pathways is critical. An imbalance between the two pathways can result in accumulation of oxidized chlorocatechol and cell death.

In OLGA172, growth on an easily degradable carbon source such as glucose, fructose or pyruvate with 3CBA causes an accumulation of oxidized chlorocatechol seen as a coloured substrate in cultures. Increased colour formation occurs as carbon concentration increases. This suggests that there may be an imbalance between the chlorocatechol producing and chlorocatechol degradative operons, which is influenced by the presence of easily degradable carbon sources.

In order to determine if an imbalance in regulation between these two pathways is the cause for substrate concentration dependant colour formation when grown in CBA, the CBA degradative genes had to be first identified from genome data in order to design primers for transcriptional studies on the two pathways. Quantitative RT-PCR was then used to examine transcript levels of the putative CBA degradative (CBD) operon as well as the CCD operon, measured from the first gene in the CCD operon, tfdC. Additionally, transcription of the muconate cycloisomerase gene in OLGA172 was monitored, to determine if expression of the gene responsible for conversion of chlorocatechol to the antibiotic protonanemonin was differentially expressed in the presence of CBA.
4.2 Methods

4.2.1 Identification of putative chlorobenzoate dioxygenase genes

The genes for the multicomponent chlorobenzoate 1,2-dioxygenase (cbeABC) of Burkholeria NK8 were retrieved on GenBank (ascension number AB024746.1) and used as a probe for homologous genes in OLGA172 using Sequencher software (version 4.1.4, Gene codes corporation). ORFs of putative genes encoding the components of the CBD operon were identified in OLGA172, and amino acid residues were used to search GenBank for homologous genes (BLASTp). Homologous genes in other strains were further analyzed for similar operonic structure and overall amino acid similarity to the putative genes in OLGA172. All alignments were completed using CLUSTALW (ver. 2), available at www.ebi.ac.uk/Tools/clustalw2/index.html.

4.2.2 Primer Design for tfdT, tfdC and the genes of the CBD operon

Primers were designed for the putative CBD operon in OLGA172 with the aid of primer3 software (Rozen and Skaletsky, 2000). Primers used for RT-PCR are outlined in Table 4.1.

**Table 4.1 Primers for genes involved with CBA and CC degradation**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>5’→ 3’</th>
<th>Annealing Temperature</th>
<th>Amplicon Size (bp)</th>
<th>Region targeted</th>
</tr>
</thead>
<tbody>
<tr>
<td>B120 F</td>
<td>GTGAAGACCACCCGAATACC C</td>
<td>58 °C</td>
<td>535</td>
<td>Putative alpha subunit of (chloro)benzoate 1,2-dioxygenase</td>
</tr>
<tr>
<td>B120 R</td>
<td>TGGGATACAGGCAAAAGGTTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB120 F</td>
<td>ACTGGCTTGCCTGCTATGC</td>
<td>58 °C</td>
<td>306</td>
<td>Putative beta subunit of (chloro)benzoate 1,2-dioxygenase</td>
</tr>
<tr>
<td>CB120 R</td>
<td>GTGCCGAAAATCGTGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C120 F</td>
<td>ATGACGCTGACGAAATCTG C</td>
<td>58 °C</td>
<td>554</td>
<td>Catechol 1,2-dioxygenase</td>
</tr>
<tr>
<td>C120 R</td>
<td>TACTTGTCCGTTGAGGACGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TfdC2 F</td>
<td>GTGCCTTACCCAGATCCCA</td>
<td>58 °C</td>
<td>205</td>
<td>Chlorocatechol 1,2-dioxygenase</td>
</tr>
<tr>
<td>TfdC2 R</td>
<td>GACCCAGTCCCCCTCCCTT C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TfdT F</td>
<td>TTGGGACTGCTGCTTGTG C</td>
<td>58 °C</td>
<td>151</td>
<td>LysR-type transcriptional regulator</td>
</tr>
<tr>
<td>TfdT R</td>
<td>CCGAATCCAATATCCACC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMC F</td>
<td>GCTGCCACTTACCTCCGTTCC</td>
<td>58 °C</td>
<td>528</td>
<td>Muconate cycloisomerase associated with the cat-operon</td>
</tr>
<tr>
<td>CMC R</td>
<td>CCTGGTCATCCGCTCCGTTCT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.2.3 Media

OLGA172 was grown in minimal media amended with 2, 25, and 50 mM pyruvate with and without 2 mM 3CBA, as well as soil extract media. Pyruvate was chosen as an easily degradable low molecular weight carbon source because OLGA172 grows very well on pyruvate (see Figure 2.1), and the media is normally clear, so that any accumulation of the coloured intermediate chlorocatechol could be easily observed. Soil Extract media was made by shaking 500 g of soil sampled from mineral soil from under the litter layer of a mixed pine-deciduous forest located near the University of Toronto Scarborough campus in 1 Litre of water for one hour, then removing particulate matter prior to autoclaving.

4.2.4 RNA Extraction

Biomass from log phase OLGA172 cells was centrifuged and washed 6 times in minimal media, prior to being used as innocula. Resuspended cells had an OD of 0.1 (7 X 10^{11} cells per ul), and 10 µl were used to inoculate media. Biomass was then used to inoculate media and incubated for 16 hours at 28°C. Biomass was centrifuged and immediately used for RNA extraction. RNA was extracted from all samples using TRIZol reagent (Invitrogen) as outlined by manufacturer, with the following modifications: Lysis of bacterial cells were aided with beads and a homogenizer for 30 seconds, and an additional chloroform phenol extraction step was added.

4.2.5 DNase treatment of RNA and First-Strand cDNA Synthesis

RNA was treated with 1 unit of deoxyribonuclease I (Invitrogen) for each 10 µl volume of RNA and buffer (200 mM Tris-HCl, pH 8.3, 20 mM MgCl2). Samples were incubated at room temperature for 15 minutes. The reaction was stopped by adding 50 mM EDTA and heating to 65°C for 10 minutes. PCR amplification targeting the sigma factor rpoD gene was performed on DNAseed samples to ensure no DNA contamination in RNA samples. DNase treated RNA was used as template for first-strand cDNA synthesis. RNA, 50 ng/ul random hexamers, 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP, dTTP) and Diethylpyrocarbonate (DEPC) treated water were incubated at 65 °C for 5 minutes, and 4 °C for 1 minute. 40 units of RNase inhibitor (RNAseOUT, Invitrogen) and 200 units Superscript III reverse transcriptase (Invitrogen) were
used for each sample, incubated at 25 °C for 5 minutes and then heated to 50 °C for 1.5 hours. The reaction was stopped by heating to 70 °C for 15 minutes.

4.2.6 Real Time PCR

Real-time RT-PCR using SYBR Green I technology on an ABI 7300 Sequence Detection System (Applied Biosystems) was performed. A master mix for each PCR run was prepared with SYBR Green PCR Master Mix (Applied Biosystems). Final concentrations, in a total volume of 20 µl were: 1x SYBR Green PCR Buffer, 3 mM MgCl₂, 1 mM dNTP, 0.625U Taq polymerase, and 0.25 U Amperase UNG. 1 µl of cDNA was added to each well. 1 µM forward and reverse primer were used. All reactions were carried out on a 96 well plate. The following amplification program was used: 95 °C 2 min, 40 cycles at 95 °C for 15 s followed by 58 °C for 35 seconds. A dissociation step was added (95 °C for 15s, 60°C for 30s, 95 °C for 15s) to analyze the melting curves of products. Primer dimers and other artifacts were evaluated by melting curve analysis and only reactions with a single melting curve peak were considered valid. Representative samples were run on a 1% agarose gel to confirm that products were the expected size. The relative standard curve method was used to quantify transcripts. Dilutions of genomic DNA from OLGA172 ranging from $10^1$ to $10^{-4}$ ng/ul total DNA were included on every reaction plate to create a standard curve for each primer set. The 7300 Sequence Detection System software, version 1.3.1.22 (Applied Biosystems) generated a standard curve by plotting crossing cycle number (Ct) versus logarithms of the given concentrations for each control, and a regression line was drawn between these points. The software calculated the concentrations of the studied genes with the aid of this standard curve. High correlations for standard curves were found for all primer sets used (>99%) Each sample was normalized against the housekeeping gene *rpoD* (using primer set rpoD1). *rpoD* transcript levels were also quantified with the use of a standard curve. Fold difference in target genes relative to the *rpoD* housekeeping gene was calculated by dividing the amount of target gene present by the amount of reference gene present, and taking the average of the normalized values from three biological replicates. PCR efficiency was calculated for each primer set, and for the same primer set if run over multiple plates. PCR efficiency was calculated as \( E = 10^{-\text{slope}} - 1 \), where slope is the slope of the standard curve created for each primer set. PCR efficiencies were all primers used in this study were found to be
in the range of 91-108%, which is within the range considered acceptable for quantification (Taylor, 2010)

4.2.7 Primer Design of housekeeping genes for quantitative RT-PCR

The genes for pyrroline-5-carboxylate reductase (proC) and and sigma factor rpoD (rpoD) were chosen as internal controls for relative quantification in RT-PCR. Both ProC and RpoD have been found to be constantly expressed, rpoD is a critical housekeeping gene and ProC is involved in amino acid biosynthesis (Savli et al., 2003). Both ProC and rpoD have been used to normalize RT-PCR results, and rpoD is an accepted standard for normalizing gene expression in Pseudomonads (Savli et al., 2003; Lesic et al., 2009).

Primers for ProC and rpoD from Savli et al. (2003) were found to have non-specific binding when used with OLGA172 and were not suitable for quantification studies. OLGA172 specific proC and rpoD genes were designed by retrieving the sequences of the genes in B. phytofirmans PSJN from genbank (ascension numbers CP001053.1 and CP001052.1). These sequences were used as probes to locate the genes in OLGA172 genome data using Sequencher (version 4.1.4, Gene codes corporation). OLGA172 was found to carry two rpoD genes, and one ProC gene. Once the appropriate sequences were retrieved, Primer3 software (Rozen and Skaletsky, 2000) was used to design primers for the proC gene and both rpoD genes (rpoD1 and rpoD2). Dissociation curves for each primer set was analyzed using (Light cycler software). Both rpoD2 and proC primer sets were found to have two or more peaks with differing melting temperatures likely due to primer-dimers being formed or non-specific binding. RpoD1 was then used as a housekeeping gene for normalization. Primers tested as housekeeping genes for RT-PCR are outlined in Table 4.2.

Table 4.4.2 Housekeeping Primers Used

<table>
<thead>
<tr>
<th>Primer</th>
<th>5’ → 3’ Annealing Temperature</th>
<th>Region targeted</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoDsav F</td>
<td>GGGCGAAGAAGGAATGGTC 60 °C</td>
<td>rpoD sigma factor (from Savli, et al., 2003)</td>
</tr>
<tr>
<td>rpoDsav R</td>
<td>CAGGTGGCGTAGGTGAGAAGA</td>
<td></td>
</tr>
<tr>
<td>proCsav F</td>
<td>CAGGCCGCGGCAGTTGCTGTC 60 °C</td>
<td>ProC (from Savli, et al., 2003)</td>
</tr>
<tr>
<td>proCsav R</td>
<td>GTCAAGGCCGAGCGTGTGTCT</td>
<td></td>
</tr>
<tr>
<td>rpoD1 F</td>
<td>CCATGGTTCATACGGGGAGA 58 °C</td>
<td>rpoD sigma factor in OLGA172</td>
</tr>
<tr>
<td>rpoD1 R</td>
<td>TAAACATTCCGGGCAATTTG</td>
<td></td>
</tr>
<tr>
<td>rpoD2 F</td>
<td>GGTCCGACTTCGTGGTGTAT 58 °C</td>
<td></td>
</tr>
<tr>
<td>rpoD2 R</td>
<td>GAGTCCGAAGATGCGCAAG</td>
<td></td>
</tr>
</tbody>
</table>
4.3 Results

4.3.1 Identification of chlorobenzoate dioxygenase operon

Genes homologous to the chlorobenzoate dioxygenase genes of *Burkholderia* NK8 were found in a single contig in the unassembled genome sequence data of OLGA172. The identified putative chlorobenzoate degradative (CBD) operon was found to consist of 8 ORFs (Figure 4.1) and operonic structure was found to be highly conserved in the strains with homologous genes. The operon was found to be highly homologous to the catechol-benzoate (cat-ben) degradative operon of *B. phytofirmans* PSJN and *Burkholderia* TH2, as well as the catechol-chlorobenzoate degradative (cat-cben) operon of *Burkholderia* NK8.

ORFs 5-7 were found to code for the alpha subunit, beta subunit, and electron transfer component of (chloro)benzoate 1,2-dioxygenase. ORF8 encodes a short chain dehydrogenase. ORF3 encodes a LysR transcriptional regulator thought to regulate the operon. ORFs 1, 2 and 4 are homologous to muconolactone isomerase (*catC*), muconate cycloisomerase (*catB*) and catechol 1,2-dioxygenase (*catA*) of the catechol degradation pathway respectively. Interestingly, in *B. phytofirmans* PSJN, the *catB* gene is annotated on NCBI as a “muconate and chloromuconate cycloisomerase” (Ascension number CP001052.1). *B. phytofirmans* PSJN is not a CBA or chlorocatechol degrader, and no other genes associated with CBA degradation were found in this strain. No experimental evidence for this gene having activity against chlorinated muconates was found in the literature.

Both TH2 and NK8 posses an additional ORF upstream of ORF1 in the putative CBD operon. The gene is most homologous to *benE* of *Acinetobacter* ADP1, in which the function is unknown. However, the putative *benE* protein is also found to be 69% similar to the benzoate transporter of *Burkholderia ambifaria*, and is likely to play some role in benzoate transport. A gene homologous to *benE* was not found in OLGA172.
**Figure 4.1 Conserved cat-ben operon structure in other burkholderia sp.**

ORFs homologous to those in OLGA172 are in the same colour. Genes that are not found in the cat-ben operon of OLGA172 are (A) a putative benzoate transporter, and (B) transposase, which truncates the alpha subunit of chlorobenzoate dioxygenase in TH2.

**Table 4.3 Amino Acid Similarities of the ben-cat operon in OLGA172**

<table>
<thead>
<tr>
<th>ORF</th>
<th>Protein</th>
<th>Acronyms used in this study for RT-PCR</th>
<th>PSJN (cat-ben operon)</th>
<th>NK8 (cat-cben operon)</th>
<th>TH2 (CAT1 operon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>muconolactone isomerase</td>
<td></td>
<td>93%</td>
<td>91%</td>
<td>91%</td>
</tr>
<tr>
<td>2</td>
<td>muconate cycloisomerase</td>
<td>catB</td>
<td>97%</td>
<td>93%</td>
<td>94%</td>
</tr>
<tr>
<td>3</td>
<td>lysR type transcriptional regulator (catR)</td>
<td></td>
<td>93%</td>
<td>90%</td>
<td>90%</td>
</tr>
<tr>
<td>4</td>
<td>catechol 1,2-dioxygenase</td>
<td>catA</td>
<td>90%</td>
<td>86%</td>
<td>92%</td>
</tr>
<tr>
<td>5</td>
<td>alpha subunit of aromatic ring hydroxylase component of chlorobenzoate 1,2-dioxygenase</td>
<td>cbdA</td>
<td>95%</td>
<td>89%</td>
<td>94%a</td>
</tr>
<tr>
<td>6</td>
<td>beta subunit of aromatic ring hydroxylase component of chlorobenzoate 1,2-dioxygenase</td>
<td>cbdB</td>
<td>94%</td>
<td>88%</td>
<td>96%</td>
</tr>
<tr>
<td>7</td>
<td>electron transfer component of chlorobenzoate 1,2-dioxygenase</td>
<td></td>
<td>96%</td>
<td>90%</td>
<td>90%</td>
</tr>
<tr>
<td>8</td>
<td>short-chain dehydrogenase/reductase</td>
<td></td>
<td>92%</td>
<td>86%</td>
<td>93%</td>
</tr>
</tbody>
</table>

Similarity of amino acid residues (%) of putative cbd genes in OLGA172 and homologous genes, a) the large subunit of the (chloro)benzoate dioxygenase in TH2 is truncated by the
presence of a transposase, similarity (%) between TH2 and OLGA172 only represent amino acids coding for the dioxygenase

4.3.2 Regulation of the CCD and CBD operons in the presence of 3CBA and pyruvate

Regular PCR indicated that the genes *tfdC* and CBD operon genes were being transcribed even in the absence of 3CBA. Further analysis using quantitative real-time PCR showed that there were low transcript levels in the absence of 3CBA for all genes looked at (*tfdC*, *catA*, *cbdA*, *cbdB*), but transcript levels for each gene were all significantly induced in the presence of 3CBA (Figure 4.2). Transcription of *tfdC* was found to be repressed by increasing concentrations of pyruvate. Conversely, transcription of the CBD operon (measured by transcription of the alpha subunit of chlorobenzoate dioxygenase, *cbdA*) was not found to be significantly different between samples with varying concentrations of pyruvate and 3CBA, or in varying concentrations of pyruvate only (Figure 4.2).

**Figure 4.2 Transcription of the *tfdC* and CBDO operon**

*tfdC* transcript levels were found to be significantly induced in the presence of 3CBA in all samples tested (one way ANOVA/Tuckey HSD, P<0.05). *** indicates samples where *tfdC* transcripts were found to be significantly different than other samples. * indicates samples that were found to significantly different than expression in cells grown in 3CBA alone. *tfdC*
expression in samples grown in the absence of 3CBA were not found to be significantly different from one another. Error bars reflect standard error.

PCR using primers targeting tfdC to tfdE on reversed transcribed samples indicated that these genes are expressed as a single transcript. The catechol dioxygenase was not part of the same transcript as the chlorobenzoate dioxygenase genes of the CBD operon when using primers targeting the catechol dioxygenase to the beta subunit of chlorobenzoate dioxygenase (data not shown). Transcript levels of the catechol dioxygenase were not significantly different from levels of the chlorobenzoate dioxygenase genes when grown in 3CBA, indicating that though the genes are not transcribed as a unit they are likely under the same regulatory control.

4.3.3 The muconate cycloisomerase of OLGA172 is induced in the presence of OLGA172

The muconate cycloisomerase gene (catB) was significantly induced in the presence of 3CBA, and the gene was not repressed by pyruvate and 3CBA (Figure 4.6). catB is located in proximity, but transcribed divergently from the chlorobenzoate dioxygenase genes identified in OLGA172 (Figure 4.3). The catR regulator of the CBD operon, likely also induces expression of this gene in the presence of 3CBA. Similar to the cbdAB genes, there was no significant difference in catB expression when grown in varying concentrations of pyruvate with CBA, or in varying concentrations of pyruvate only. Expression of the cbdAB genes in the presence of 3CBA were approximately 10 times higher than the catB gene.

Figure 4.3 Expression of muconate cycloisomerase in the presence and absence of 3CBA
All samples tested were found to be significantly induced in the presence of 3CBA (one way ANOVA/Tuckey HSD, P<0.05). Expression was not found to be significantly affected by
altering pyruvate concentration regardless of the presence of 3CBA. Error bars reflect standard error.

4.4 Discussion

The putative chlorobenzoate dioxygenase genes in OLGA172 (cbdAB) are homologous to the chlorobenzoate dioxygenase genes of Burkholderia NK8 (cbeAB), as well as the benzoate dioxygenase genes in B. phytofirmans PSJN and Burkholderia TH2. All three operons are associated with catechol degradative genes, and have similar operonic structure. The cbeABC genes of NK8 have been experimentally identified as containing the genes responsible for degradation of CBAs in this strain. Disruption of the cbeA gene in this strain (alpha subunit of the chlorobenzoate dioxygenase) resulted in the loss of the ability to grow on benzoate as well as monochlorinated benzoates (Francisco et al., 2001). Cell free extracts of the cbeA were found to be most active against benzoate over CBAs. Thus, the cbeA gene of NK8 has broad specificity, which includes the ability to convert monochlorinated CBAs. This enzyme may be the result of adaptation of a benzoate dioxygenase, resulting in the ability to degrade chlorinated analogs. NK8 also carries a CCD operon, however it is not known if these two operons are in proximity to each other in this strain.

There is no experimental evidence in the literature that associate the catechol and benzoate degradative genes of B. phytofirmans PSJN and Burkholderia TH2 as responsible for chlorobenzoate metabolism. PSJN has not been found to be a CBA degrader, although the annotation of a chloromuconate cycloisomerase associated with this operon is interesting. TH2 is a known CBA degrader, however the genes responsible for CBA breakdown have been identified as being a 2-halobenzoate dioxygenase which forms a separate operon from the genes identified in this work to be homologous to the cbdAB genes of OLGA172 (the CAT1 operon of TH2). TH2 possesses two catechol degradative operons CAT1 and CAT2. The CAT1 operon of TH2 is the operon that was found to be homologous to the putative CBD operon of OLGA172. The proximity of the CAT1 operon to the CBD operon in TH2 is not known. Alignment of the large subunit of the 2-halobenzoate 1,2-dioxygenase with the large subunit of benzoate 1,2-dioxygenase in the CAT1 cluster of TH2 showed 59% amino acid similarity between the two genes in the same strain. The large subunit of the 2-halobenzoate 1,2-dioxygenase of TH2 was found to be 60% similar to the large subunit of the chlorobenzoate dioxygenase gene in OLGA172.
While only the *cbd* ABC genes of the halobenzoate dioxygenase have been identified as responsible for CBA metabolism in TH2, both the CAT1 and CAT2 operons have been found to influence CBA degradation experimentally in this strain. Both 3CBA and 4CBA have been found to be inducers of the *catA*_1 gene, however *catA*_1 is not induced in response to 2CBA unless *catA*_2 is disrupted. 2CBA was found to be a *catA*_2 inducer, regardless of the presence of an intact *catA*_1. Additionally, *catA*_1 was found to have enzymatic activity against 3CC and 4CC in this strain converting the substrates to chlorinated muconates (Suzuki et al., 2002). Thus, the catechol dioxygenase genes in TH2, though not identified as being responsible for CBA degradation, appear to respond to different CBA inducers, and are capable of taking part in some steps of their degradation.

The role of catechol dioxygenase (*catA*) in the conversion of CC’s to cis-cis muconates has been found in other strains as well (Kim et al., 1997). Because *catA*_ and the (chloro)benzoate dioxygenase genes are clustered together and are homologous to genes that are associated with CBA degradation in other bacteria, I suggest this operon is responsible for the conversion of CBA to CC in OLGA172. This is supported by evidence that these genes are responsive and significantly induced in the presence of CBA. Whether the chlorobenzoate dioxygenase gene in OLGA172 is a benzoate metabolizing gene with relaxed specificity or a gene which has been specialized for chlorobenzoate degradation cannot be determined though transcriptional studies. Enzymatic activity experiments will have to be carried out in the future to determine which substrates this multicomponent enzyme shows a preference for.

The CCD operon and the CBD operon are not regulated together. The *tfdC* gene was repressed in a concentration dependant manner in the presence of pyruvate. Conversely, the CBD operon doesn’t have similar regulatory control, and wasn’t found to have significantly altered expression in varying concentrations of pyruvate in the presence of CBA. The genes appear to be slightly repressed in the presence of increasing amounts of pyruvate in the absence of 3CBA, however this data was not found to be significant. The imbalanced regulatory control of these two operons may be evidence that these two pathways have not been under similar selective pressure in the past. Differential control could be due to acquisition of one of the operons via HGT, without enough time to fine tune regulation in the presence of CBAs. Alternatively, the natural
role (if any) of the tfdCDEF operon, could involve a pathway(s) that doesn’t include the CBD operon, which is also a plausible reason for why the two operons are not regulated together.
Chapter 5. TfdT is likely a transcriptional activator of the CCD operon

5.1 Introduction

OLGA172 possesses all of the genes necessary to completely degrade 3CBA, including a LysR-type regulator associated with the operon. Because of its amino acid and nucleotide similarity this gene has been identified as tfdT. tfdT is currently only found in two other organisms currently annotated on NCBI, Burkholderia NK8 and C. necator JMP134.

In Burkholderia NK8 the tfdT gene was found to act as a transcriptional activator of the tfdCDEF genes (Liu et al., 2001). In JMP134 the tfdT gene is interrupted by an insertion sequence (ISJP4) resulting in an early stop codon and was for a long time was thought to be nonfunctional. In this strain, an isofunctional tfdR protein associated with the second module CCD operon was found to take over the role of the interrupted TfdT (Leveau & van der Meer, 1997). However in recent a study by Trefault et al. (2009), the partial tfdT gene was found to decrease growth on 3CBA. When mutants of JMP134 which had the partial tfdT gene removed were grown on 3CBA, growth on 3CBA increased by as much as 50%. The interrupted TfdT protein acts as a transcriptional repressor or at least interferes with transcription of the tfdCDEF operon in the presence of 3CBA.

Given how rare the tfdT gene is in comparison to the isofunctional regulatory genes tfdR, tfdS, and cleR, and the fact that there are two conflicting reports on whether this gene is an activator or repressor in the only two strains that carry it, the study of the regulatory function of tfdT in other organisms carrying this gene is needed. There have been no previous studies carried out on OLGA172 to examine how and if the CCD operon is regulated in this organism.

LysR type transcriptional regulators (LTTRs) are the largest family of prokaryotic regulators, with over 100 members in multiple diverse genera (Tropel & van der Meer, 2004). They have been identified as the eighth most abundant protein coding gene in all prokaryotic genomes sequenced thus far (transposases have been found to be the most abundant), and are ubiquitous in prokaryotes (Aziz et al., 2010). LTTRs are typically proteins of approximately 330 aa in
length, and consist of a highly conserved N-terminus (DNA binding) and a poorly conserved C-terminus, where co-inducer binding occurs (Maddocks & Oyston, 2008).

Most LTTRs are found to control a single target operon that they lie upstream of and are transcribed divergently from, however exceptions exist. Examples of co-transcription of a LTTR regulator with the operon it controls (Tropel & van der Meer, 2004), as well as single LTTR control of multiple operons located elsewhere on a bacterial chromosome exist (Maddocks & Oyston, 2008). An example of this is catR, it affects both the regulation of the catBCA and the tfdCDEF operon for catechol and chlorocatechol degradation respectively (Paresek et al., 1994). LTTRs identified with aromatic degradation have all been found to act as transcriptional activators for their target operon in the presence of the appropriate inducer, which in most cases is an intermediate of the pathway (Tropel & van der Meer, 2004). However, the partial TfdT of JMP134, which was recently found to repress 3CBA degradation is a notable exception. It may be that the interruption of tfdT gene with insertion sequence ISJP4 in this strain has resulted in the translation of a non-functional protein which is still able to interact with tfdCDEF regulation, but has lost the ability to induce the operon. The mechanisms of this effect are unclear. DNA binding assays have determined that the interrupted TfdT is not capable of binding to the intergenic region between tfdT-tfdC containing the promoter. Additionally, tfdT was not found to be able to activate a reporter gene in genetic constructs using tfdT as a regulator (Trefault et al., 2009). Regulation of the tfdCDEF genes in JMP134 may be more complex than previously thought.

In the case of chlorocatechol and catechol degradation, the catabolic operons are virtually always found to be associated with a LTTR, the only exception is in Rhodococcus opacus 1CP, a gram positive bacterium whose catechol degradative operon is associated with a Icl-R type regulator (Eulberg & Schlomann, 1998). In addition, in C. necator JMP134, an Icl-R type regulator was found near the module II CCD operon, between the tfdR and tfdS genes. In this strain, disruption of the Icl-R regulator resulted in better growth on 2,4-D, but had little effect on the strains ability grow on 3CBA (Trefault et al., 2009). Both 2,4-D an 3CBA are metabolized through the tfdCDEF genes, though metabolization of 2,4-D requires additional genes associated with the module II operon. The Icl-R regulator was determined to likely be a transcriptional activator of
one of the peripheral genes required for 2,4-D degradation, and is so far the only example of a regulator other than a LTTR involved in transcription of the tfd genes in a gram negative species.

The mechanism through which LTTRs have been found to activate catechol and chlorocatechol degradative genes involve the presence of an inducer to the LTTR which is typically an intermediate of the pathway being regulated. The LTTR protein binds to two sites in the intergenic region between the regulator and the region it controls, a recognition binding site (RBS) and an activator binding site (ABS). The RBS contains a consensus T-N\textsubscript{11}-A sequence at position -70 from the transcriptional start site, where as the ABS is located at the -35 and does not necessarily contain a consensus sequence. Binding of the LTTR to the RBS and ABS sites in the absence of an inducer causes DNA bending in the promoter region. Binding of an inducer to the LTTRs causes a relaxation in DNA bending, allowing a RNA polymerase to bind to the promoter region and initiate transcription. The LTTR also regulates its own expression, though less is known about the mechanisms of its autoregulation (Maddocks & Oyston, 2008). Typically LTTRs are thought to negatively regulate their own expression, possibly to maintain constant levels in the cell. The promoter of the LTTR is usually found to overlap or be close to the promoter of the gene it lies divergently from to allow simultaneous control of transcription bidirectionally. Exceptions to this exist, and in some cases LTTRs may positively regulate their own expression (Schell, 1993).

In addition to the regulators associated with CCD operons, other genes located far from the operon in other organisms have been found to affect the CCD genes. In *P. Putida* RB1 the chromosomally encoded catechol regulator *catR* was found to be able to cross-activate the CCD operon (*clcABD*). However the regulator of *clcABC*, *clcR*, does not activate the catechol/benzoate degradative operon (*catABCD*), although it does interact with the binding sites of the promoter region of the catechol-benzoate degradative operon, (Parsek et al., 1994).

A regulated operon would ideally be transcribed only when needed, and would be able to tie expression with the physiological state of the bacteria. Optimal regulation of the CCD operon in OLGA172 could suggest that this operon has been under selective pressure for some time, and would lend support to the hypothesis that the presence of the CCD genes in OLGA172 (and other pristine degraders) are the result of selective pressure by a compound that preceeded the
xenobiotics that these genes can metabolize. Conversely, poor regulation of the operon could indicate that the genes are in an early stage of optimization of the pathway. There are examples of nutrient availability playing a role in the degradation of CBAs and CCs, and this may be indicative of a metabolic pathway in late stages of optimization (Cases & Lorenzo, 2001). For example, fumarate, a krebs cycle intermediate, was found to bind reversibly to the regulator clcR, competing for the co-inducer binding site resulting in inhibition of chlorocatechol 1,2-dioxygenase expression (McFall et al., 1997). This has the effect of lowering gene expression of a metabolic pathway, when sufficiently high concentrations of an easily degradable carbon source is available, and may imply optimization of this pathway.

Because the tfdT gene is relatively rare, analysis of the amino acid/nucleotide similarity of OLG172 and the only two other tfdT carriers (Burkholderia NK8 and C. necator JMP134) as well as the isofunctional LTTRs of other CBA degrading strains was carried out. Additionally, the tfdT-tfdC intergenic region of OLG172 was compared to other strains carrying tfdC to look for conserved regions involved in regulation. The effect of increasing concentrations of pyruvate on tfdT transcription was examined. In addition, transcription in cells grown in soil extract media was analyzed to determine if the tfdT gene and CCD operon is expressed in media representative of OLG172's natural habitat.

5.2 Methods

5.2.1 Real Time PCR

RT-PCR and quantification was carried out as described in 4.2.7. Primers used to amplify the tfdT gene were (5’ to 3’): TTTGGGACTGCTGTCTTTGA (tfdT F), and CCGAATCCAATATCCACC (tfdT R), an annealing temperature of 58 °C was used. Normalization of transcript levels was made to the housekeeping gene rpoD.

5.2.2 Amino Acid and Nucleotide Alignment

The nucleotides of the tfdT-tfdC intergenic regions of CBA degrading strains were compared by sequence alignment. The tfdT genes of Burkholderia NK8, C. necator JMP134 and OLG172
were also compared, by aligning amino acids. All alignments were completed using CLUSTALW (version 2), available at http://www.ebi.ac.uk/Tools/clustalw2/index.html.

5.3 Results

5.3.1 Analysis of the tfdT gene and regulatory intergenic region in OLGA172

Previous sequencing of the CCD operon in OLGA172 suggested that transcription of the tfdT gene may end early, due to a possible frameshift mutation resulting in a stop codon (GenBank AY168634.1, formally Burkholderia sp. R172). However, sequencing data obtained from two separate next generation sequencing runs failed to detect this mutation. This may either be due to an error in sequencing in the previously annotated sequence, or due to a subsequent mutation in this region. Comparison of the amino acid residues of the tfdT genes of NK8, the truncated JMP134 and the (complete) tfdT gene of OLGA172 showed that the protein is highly conserved (Appendix, A1), except in the C-terminal end of the protein, which was found to be variable in length and in sequence between NK8 and OLGA172. An alignment of the amino acid residues of the three tfdT genes can be found in the appendix. The predicted tfdT product of OLGA172 is shorter in length, being 298 amino acids in length compared with 306 of NK8, and 295 of the tfdR (full gene) of JMP134. The tfdT gene of JMP134 is also short, 211 amino acids in length due to an early stop codon.

The intergenic regions of the LTTR and the chlorocatechol dioxygenase gene upstream of it were compared along with various chlorocatechol degrading type strains. The intergenic regions between tfdT and tfdC of NK8 and in JMP134 (module I operon), as well as the intergenic regions between tfdR and tfdD in JMP134 (module II) were aligned. Also analyzed were Delftia acidovorans, B. xenovorans LB400, P. cepacia p51, Pseudomonas sp CSV90, and P. putida GT241-1. It was found that the intergenic region of OLGA172 does not share high similarity with NK8, although it shares high homology with the tfdT and tfdC genes in this strain. The intergenic region of OLGA172 was relatively similar to the only other tfdT carrier, the module I genes of JMP134 (Table 5.1).

The intergenic region also shared high homology to P. CSV90 and P. putida GT241-1 (Figure 5.1). The genetic information available for these two strains however, only include partial LTTR
sequence along with the full chlorocatechol dioxygenase gene. It may be possible that there are chlorobenzoate metabolizing strains currently isolated that carry genes homologous to the \textit{tfdT} gene. Because the emphasis on characterizing these strains is on the degradative genes rather than the regulatory genes associated with them, the rarity of this gene may be (at least partially) due to this bias in data.

\textbf{Table 5.1 Similarity of the regulatory region of OLGA172}

<table>
<thead>
<tr>
<th>Ascension number</th>
<th>LTTR Protein Length (aa) and similarity (%)</th>
<th>Chlorocatechol dioxygenase (aa similarity)</th>
<th>Intergenic Region (bp similarity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK8</td>
<td>AB050198.1</td>
<td>\textit{tfdT} 306 (77%)</td>
<td>84%</td>
</tr>
<tr>
<td>JMP134 Module I</td>
<td>NC_005912.1</td>
<td>\textit{tfdT} 211 (90%) \textit{a} \textit{tfdR} 295 (46%)</td>
<td>88%</td>
</tr>
<tr>
<td>B. xenovorans LB400</td>
<td>CP000270.1</td>
<td>\textit{clcR} 294 (48%)</td>
<td>59%</td>
</tr>
<tr>
<td>Delftia acidovorans</td>
<td>AY078159.2</td>
<td>\textit{LysR} 294 (50%)</td>
<td>57%</td>
</tr>
<tr>
<td>P. cepacia P51</td>
<td>M57629.1</td>
<td>\textit{tcbR} 276 (52%)</td>
<td>55%</td>
</tr>
<tr>
<td>\textit{Pseudomonas} sp. CSV90</td>
<td>D16356.1</td>
<td>\textit{LysR} 36 (88%) \textit{b}</td>
<td>88%</td>
</tr>
<tr>
<td>P. putida GT241-1</td>
<td>AY493510.1</td>
<td>\textit{LysR} 47 (95%) \textit{b}</td>
<td>88%</td>
</tr>
</tbody>
</table>

(a) Interrupted early due to insertion sequence (b) full sequence not available
Figure 5.1 Alignment of intergenic region between regulator (tfdT/tfdR) and tfdC
Alignment is between various CC degraders. Red box indicates conserved nucleotides among all strains. Underlined nucleotides denote the tfdR recognition binding site of JMP134. The second conserved box is likely the activator binding site of tfdT
5.3.2 Transcription of tfdT

Quantitative rt-PCR indicated that the tfdT gene was induced significantly in the presence of 3CBA and repressed by increasing amounts of pyruvate (Figure 5.2). Like tfdT, tfdC transcripts were found to be repressed in the presence of increasing amounts of pyruvate. When cells were grown on soil extract media, tfdT transcription remained un-induced. When tfdT transcript levels normalized to the housekeeping gene rpoD from all samples were plotted against normalized tfdC values, a sigmoidal plot resulted (Figure 5.3), which would be expected for a regulator which interacted with other factors (such as a co-inducer) during transcription of tfdC. In this instance tfdT does not appear to be co-transcribed as a single unit with the tfdCDEF operon, since transcript levels of tfdT and tfdC differ by two orders of magnitude.

![Figure 5.2 Expression of tfdT in the presence of 3CBA and Pyruvate](image)

**Figure 5.2 Expression of tfdT in the presence of 3CBA and Pyruvate**

Normalized expression of the tfdT gene. Error bars reflect standard error. *** are samples that were found to be significantly different from all other samples. All samples tested were found to be significantly induced in the presence of 3CBA (one way ANOVA/Tuckey HSD, P<0.05). Expression was not found to be significantly affected by altering pyruvate concentration in the absence of 3CBA.
Figure 5.3 Correlation of \( tfdT \) and \( TfdC \) transcription

\( tfdT \) and \( tfdC \) gene expression normalized to the housekeeping gene \( rpoD \). Equation for sigmoidal curve is: \( tfdC = \frac{90.74}{1 + e^{-(tfdT - 0.54)/0.104}} \)

5.4 Discussion

The \( tfdT \) protein of \( \text{OLGA172} \) was found to be highly similar to the \( tfdT \) of \( \text{Burholderia NK8} \) and \( \text{C. necator JMP134} \) and dissimilar to other LTTR regulators of CCD pathways (Table 5.1). Whether this is due to divergence from an ancestral protein is unknown, since to date there are only 3 strains which carry the \( tfdT \) gene for which sequence is available. If the \( tfdT \) isoform were representative of an ancestral regulator, we could expect to see this isoform in more isolates over wide geographic areas. When primers targeting the \( tfdT \) gene were used to probe other CBA degrading strains isolated from pristine environments (from the 1996 Fulthorpe study collection), products were found in 17 of 28 strains tested (Jin, 2010). Though sequence data for these products is not available yet, these results may indicate that this gene is not rare in these environments. If this operon, including the regulator, were representative of an operon which has been under selective pressure for sometime (for example if the operon preceded the man-made chemicals it degrades today), we could expect higher similarity between strains in the nucleotides which code for useful enzymes, and higher variability in the intergenic regions which would be more prone to change over evolutionary time.

There are regions of absolutely conserved nucleotides in the intergenic region of all the strains looked at (Figure 5.1). These regions are used for regulation (LTTR binding sites) of the
tfdCDEF operon regardless of if they possess a tfdT regulator, indicating there is some conserved transcriptional regulatory mechanisms in these operons, and at the very least the tfdT gene likely diverged from a common ancestor to the related isoforms (tfdS, tfdR, clcR).

The highly conserved intergenic regions of Pseudomonas CSV90, P. putida GT241-1 may indicate horizontal transfer of the operon and regulator including the intergenic region. The sequence for these strains only includes a small part of the LTTR, so we can not look at its homology to related genes. P. putida GT241-1 is a 2,4-D degrader isolated from contaminated drainage soil around 2,4-D factories (Yao et al., 2004), and Psuedomonas sp. CSV90 is a 2,4-D degrader isolated from industrial effluent in India. CSV90 carries these genes on a degradative plasmid and readily loses the plasmid when grown in non-selective media (Bhat et al., 1994).

TfdT is thought to act as a transcriptional activator for the CCD operon, as tfdT gene expression increased, expression of the first gene in the CCD operon, tfdC increased. This is similar to the situation with Burholderia NK8 and what has been reported for other LTTRs and CCD operons thus far. It is contrary to the findings of Trefault et al. (2009) which indicated that the interrupted tfdT gene of C. necator JMP134 acts as a transcriptional repressor to the CCD operon, but this changed gene is not expected to behave "normally". In order to unambiguously observe if tfdT is the sole regulator and a transcriptional activator of the CCD operon in OLGA172, it would require that tfdT be deleted or mutated to see if the loss in functionality causes a loss of induction of the CCD genes, which could be done using PCR site directed mutagenesis. Alternatively, the effect of tfdT alone on the CCD genes could be observed by introducing a genetic construct including just the tfdT and CCD genes of OLGA172 to a vector and looking at expression in E.coli, which would not be confounded by the chromosomal genes of OLGA172.

Importantly, tfdT was found to be repressed in the presence of pyruvate in this study (Figure 5.3), indicating that not only is the CCD operon induced in the presence of its substrate, but that transcriptional control of these genes exists which prevents the unneeded expression of the CCD genes in conditions of high nutrient availability. This regulatory control may support the hypothesis that CC degradation has undergone evolutionary fine tuning in this strain.
Chapter 6. Analysis of Integrases near the CCD operon: Homology and Expression

6.1 Introduction

The phenotypic instability displayed by OLGA172 is likely not solely attributable to transcriptional control. An imbalance between the CC producing and CC degrading pathways, explains the accumulation of oxidized chlorocatechol seen in samples degrading 3CBA in the presence of an easily degradable carbon source. However, this is not the only condition that a failure to efficiently metabolize 3CBA has been observed in OLGA172. OLGA172 has repeatedly been found to spontaneously accumulate coloured CC in culture. An example of its inexplicable phenotypic instability can be seen when replicates of OLGA172 were grown in 2 mM 3CBA with no additional carbon source, and were found to differentially accumulate coloured CC (Figure 3.6A). Because inoculation volume, inoculum source, and media were all identical between these replicates these are not likely sources for the variation seen.

Another possibility for the instability seen could be genetic rearrangement near or within the CCD degradative pathway. Two things suggest that DNA rearrangement may be responsible for the observed phenotypic instability. First is the presence of numerous integrase and recombinase genes which are associated with DNA mobility. Secondly, is the example of a mobilizable CCD operon in Pseudomonas putida B13. The clc element of B13 carries both the CCD operon, and an integrase gene which can carry out the excision and self transfer of the CCD operon (Sentchilo et al., 2003). This sets a precedent for the idea that the CCD operon in OLGA172 may be mobilizable through the action of an integrase, and this mobility may be what causes the instable phenotype observed.

Approximately 3500 bps of the region upstream from the CCD operon in OLGA172 harbours three integrases in tandem or a "recombinases in trio" (RIT) element (Figure 5.7), a recently coined term for the discovery that integrases commonly occur in threes (Houbt et al., 2009). The RIT element in OLGA172 has been named RITBphylO1 (Jin, 2010) and was found to share high homology with the RIT elements discovered so far in other strains, where the integrases themselves are not homologous to each other, but the three integrases in tandem share homology...
with the three integrases found in tandem in other distantly related strains (the RIT elements as a whole are homologous) (Jin, 2010). In addition to the complete integrases of the RITBphytO1 element, there is a partial integrase located downstream of the CCD operon, downstream of the tfdF gene. The partial integrase, as well as all the genes in the RITBphytO1 element have been found to contain the conserved catalytic residues required for cleavage and ligation, meaning that the CCD operon in OLGA172 is flanked on either side by integrases which carry the catalytic residues required for genetic movement (Jin, 2010). The functionality of these genes has not been established.

The tfdT and CCD operon of OLGA172 was found to display high homology to Burkholderia NK8 and C. nedator JMP134 (Table 4.3). In JMP134 the intergenic non-protein coding tfdT-tfdC region was found to be highly homologous to OLGA172 (62% similarity), however NK8 showed lower similarity (40%) to OLGA172 in this region. In this chapter I discuss sequence analysis of the homology between OLGA172 and these two strains in the regions outside of the CCD operon. In addition, the sequence of the genes involved in DNA mobility in OLGA172 were analyzed in order to have a better understanding of past or current mobility of this operon. Finally, quantitative transcriptional studies were carried out in order to determine if the integrase genes of the RITBphytO1 element are actively transcribed in this strain.

6.2 Methods

6.2.1 Real Time PCR

Real-time RT-PCR using SYBR Green I technology on an ABI 7300 Sequence Detection System (Applied Biosystems) was performed. A master mix for each PCR run was prepared with SYBR Green PCR Master Mix (Applied Biosystems). Final concentrations, in a total volume of 20 µl were: 1x SYBR Green PCR Buffer, 3 mM MgCl₂, 1 mM dNTP, 0.625U Taq polymerase, and 0.25 U Amperase UNG. 1 µl of cDNA was added to each well. 1 µM forward and reverse primer were used. All reactions were carried out on a 96 well plate. The following amplification program was used: 95 °C 2 min, 40 cycles at 95 °C for 15 s followed by 58 °C for 35 seconds. A dissociation step was added (95 °C for 15s, 60°C for 30s, 95 °C for 15s) to analyze the melting curves of products. Primer dimers and other artifacts were evaluated by melting curve analysis
and only reactions with a single melting curve peak were considered valid. Representative samples were run on a 1% agarose gel to confirm that products were the expected size. The relative standard curve method was used to quantify transcripts. Dilutions of genomic DNA from OLGA172 ranging from $10^1$ to $10^{-4}$ ng/ul total DNA were included on every reaction plate to create a standard curve for each primer set. The 7300 Sequence Detection System software, version 1.3.1.22 (Applied Biosystems) generated a standard curve by plotting crossing cycle number (Ct) versus logarithms of the given concentrations for each control, and a regression line was drawn between these points. The software calculated the concentrations of the studied genes with the aid of this standard curve. High correlations for standard curves were found for all primer sets used (>99%) Each sample was normalized against the housekeeping gene rpoD (using primer set rpoD1). rpoD transcript levels were also quantified with the use of a standard curve. Fold difference in target genes relative to the rpoD housekeeping gene was calculated by dividing the amount of target gene present by the amount of reference gene present, and taking the average of the normalized values from three biological replicates. PCR efficiency was calculated for each primer set, and for the same primer set if run over multiple plates. PCR efficiency was calculated as $(E) = 10^{(-1/slope)} - 1$, where slope is the slope of the standard curve created for each primer set. PCR efficiencies were all primers used in this study were found to be in the range of 91-108%, which is within the range considered acceptable for quantification (Taylor, 2010).

Primers used to amplify the integrases of the RITBphytO1 element are outlined in Table 6.1. Normalization of transcript levels was made to the housekeeping gene rpoD.

**Table 6.1 Primers for integrase genes in the RITBphytO1 element of OLGA172**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>5’→ 3’</th>
<th>Annealing Temperature</th>
<th>Amplicon Size</th>
<th>Region targeted</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS66 F</td>
<td>CTCGGATTGTCGCCATTATC</td>
<td>58 °C</td>
<td>177</td>
<td>Integrase 1</td>
</tr>
<tr>
<td>IS66 R</td>
<td>CTCTGGCTCGGACATGAATC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Int2 F</td>
<td>ATACCATGCAACCAGCCTTC</td>
<td>58 °C</td>
<td>211</td>
<td>Integrase 2</td>
</tr>
<tr>
<td>Int2 R</td>
<td>CGACCAACTCTGCCCCTCTAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Int3 F</td>
<td>CAGATCCTCAAACGCAACCT</td>
<td>58 °C</td>
<td>198</td>
<td>Integrase 3</td>
</tr>
<tr>
<td>Int3 R</td>
<td>ATGGGCCACATTGTCGCTT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.3 Results

6.3.1 Homology of the regions outside of the CCD operon to NK8 and JMP134

Non-protein coding region upstream of the CCD operon

NK8 has two regions upstream of the CCD operon that are homologous to regions of OLGA172 (Figure 6.1). Interestingly, these stretches of DNA (158 bps and 142 bps in length) are separated by a 12 bp region in OLGA172 which shares very little homology to the 441 bps which separate these regions in NK8. These regions were not found to contain any ORFs in either OLGA172 or in NK8. No homology to the same regions were found in JMP134, or in any other strain when a blastn search was carried out for nucleotide similarity of this region to other organisms. JMP134 was not found to have any regions similar to OLGA172 in the region immediately upstream of the CCD operon.

Integrase and partial integrases/transposases

A partial IS66 family transposase (IS66 tnp1) was found upstream of the RIT\textit{BphytO1} element in OLGA172 with homology to an IS66 family transposase found in JMP134 (80\%). The entire transposase in JMP134 is 1635 bps long, but OLGA172 only possess 259 bps in total that are homologous to the C-terminal of the protein. The nucleotides homologous to the last 12 bps of the C-terminal of this gene were found further upstream and inverted 65 bps away from the partial integrase (Figure 6.1). IS66 tnp1 in JMP134 is located on a megaplasmid, and not on the plasmid which carries the CCD operon in this strain (plasmid pJP4).

The partial IS66 tnp1 in OLGA172 was also found to show high homology to several IS66 family transposases located in \textit{Burkholderia xenovorans} LB400 (77\% similarity), which is another CBA degrader, originally isolated as a PCB degrader. In this strain the closest partial IS66 tnp was found to be located over 650,000 bps away from the CCD operon.

The IS66 tnp1 is located 6 bps upstream from a 30 bp inverted repeat (IR) which flanks the RIT\textit{BphytO1} element and is absolutely conserved (Figure 6.1). Downstream of the RIT\textit{BphytO1} element is another IS66 family transposase fragment (IS66 tnp2) 276 bps in length, which is also located 6 bp away from the IR outside the RIT\textit{BphytO1} element. The two fragments are of the C-
terminal of the proteins they code for, and do not represent a single IS66 family transposase protein which was interrupted by insertion of the RITBphytO1 element. When the two partial IS66 transposase fragments were aligned using clustalW, they were found to be 34% identical, and are therefore not likely to be the same protein. IS66 tnp2 was found to be most homologous to transposases found in Ralstonia eutropha H16 (77% similarity), and Variovorax paradoxus S110 (75% similarity). Neither of these strains are CBA degraders. A RIT element homologous to RITBphytO1 in OLGA172 is also found in H16.

There is another partial integrase located downstream of the CCD gene in OLGA172 (Figure 6.1). This protein showed the highest homology to a lambda phage integrase found in Burkholderia sp. 383 which is not a known CBA degrading strain. This partial integrase is missing both the N-terminal and C-terminal ends, though it does carry the catalytic residues (Jin, 2010).

The integrase genes of the RITBphytO1 element were the only full genes associated with mobility found near the CCD operon. The RITBphytO1 element contains 3 integrases which overlap by the nucleotides ATGA, suggesting translational coupling. The RITBphytO1 element genes of OLGA172 are not found in either NK8 or JMP134.
Figure 6.1. Regions of similarity between OLGA172, NK8 and JMP134
Shaded boxes show homologous DNA regions between a given strain and OLGA172. DNA coding for protein is labeled with the protein name, empty boxes and black lines indicate non-protein coding regions. Black lines and triangles denote areas that are not highly homologous to OLGA172 (<60% similarity). Percentages reflect similarity in nucleotide composition of the homologous region. Vertical black line in tfdT of JMP134 is an insertion sequence which disrupts the protein. (*) Similarity to NK8’s TfdT was calculated using 908 aligned nucleotides, similarity between the entire gene and OLGA172 (926 bp) was 70%. Length of regions of interest given in bp. IS66 triangle upstream of IS66 tnp1 of OLGA172 is homologous to the last 12 bp of the C-terminal of IS66 tnp1, however the bps are inverted.
6.3.2 Expression of the integrases of the RIT element

Quantitative RT-PCR was used to measure transcript levels in the integrase genes of the RIT element. The three integrases examined were int1, int2, and int3 (int3 is closest to the CCD operon). All integrases were found to be expressed in low amounts in samples grown in varying concentrations of pyruvate with and without 3CBA (Figures 6.2–6.4), as well as in soil extract. Although all samples prior to reverse transcription were tested with rpoD primers to ensure that no DNA was present, due to the low expression of these genes, DNased samples were re-checked using int1 primers from representative samples to ensure that low expression was not an artifact from DNA contamination in the original samples. Lack of contamination was confirmed in samples tested.

The nucleotide sequences of the integrases in the RITBphytO1 element overlap by the nucleotides ATGA, so the possibility that these genes are coupled in translation was investigated. PCR using primers targeting the first and third integrase did not yield a PCR product in reversed transcribed DNA, indicating that the integrases in the RITBphytO1 element do not form a single transcript. Because the expression pattern of int1 and int2 look similar, whereas int3 looks differentially expressed (Figures 6.2-6.4), PCR targeting the first and second integrases was performed, and int1 and int2 were found to be expressed as single transcript. No product was found when PCR targeting int2 and int3 was carried out, confirming that the three genes are not expressed together.

There were no ORFs immediately near the RITBphytO1 element with homology to a regulator. The closest identified regulatory gene to the RITBphytO1 element is the LysR-type regulator of the CCD pathway, tfdT. When transcript levels of tfdT normalized to the housekeeping gene were plotted against normalized expression of the integrase genes, no significant correlation was found to exist (Figure 6.5).
Figure 6.2 Expression of the int1 gene in OLGA172

Figure 6.3 Expression of the int2 gene in OLGA172

Figure 6.4 Expression of the int3 gene in OLGA172
No correlation in expression if int genes and tfdT

No relationship was found between expression of \textit{tfdT} and any of the integrase genes. $R^2$ values were 0.18, 0.02, and 0.48 for \textit{int1}, \textit{int2}, and \textit{int3} respectively.

### 6.4 Discussion

The integrase genes in the RIT\textit{BphytO1} element all overlap by the nucleotides ATGA. This is also true of all other RIT elements identified to date and this nucleotide overlap is thought to indicate translational coupling (Janssen et al., 2009). In OLGA172, PCR using primers targeting the first to third integrase (\textit{int3}) did not yield a PCR product in reversed transcribed DNA, indicating that the integrases in the RIT\textit{BphytO1} element are not a single transcript. Similarly, PCR targeting the second to third integrase did not yield a product. The first and second integrases were found to be part of a single transcript using this method.

This is the first expression study of the integrase genes comprising a RIT, and these results could indicate that the three genes of the RIT element are differential regulated, though a regulator has not been identified in this study. The closest identified regulatory gene to the RIT\textit{BphytO1} element is \textit{tfdT}. However no significant correlation between expression of the \textit{tfdT} gene and any of the individual integrase genes could be found (Figure 6.5). It has been suggested that the three integrases in the RIT element may have evolved to play a specific role in RIT mobility (Houdt et al., 2009). The differential expression of these integrase genes may support the idea that each gene plays a different role, and may need to be expressed at different levels. It is not currently known if all three integrases are needed for mobility, if they work in concert, or if mobility can be carried out by the action of one or two members of the element.
Low (expression levels of 2-18% of housekeeping gene rpoD expression), but constitutive expression of the RITBphytO1 element genes (though differentially expressed) was seen in all samples tested. With the exception of the int1 and int2 genes in cells grown in 50 mM pyruvate, expression of the RITBphytO1 element genes were observed in higher amounts in the presence of 3CBA than in its absence (Figure 6.2-6.4), however this observed increase in expression was not found to be statistically significant. These results are similar to the findings in Psuedomonas B13, a CBA degrader which carries its CCD operon on a mobile genetic element called the clc genomic island. The clc element is 105 kb in length and is present in two copies on the chromosome of this strain. The clc element is self mobilizable, and transfer of the genomic island is facilitated through expression of an integrase gene (intB13) located at the end of the genomic island. In a gfp fusion study which allowed individual cells to be monitored for intB13 expression, it was found that while overall expression of the intB13 gene was always very low, expression in the presence of 3CBA, as well as in stationary phase cells was higher (this increase was not found to be statistically significant). Interestingly, they also found that no more than 13% of the population expressed intB13, and its expression was extremely random (Sentchilo et al., 2003). Because this is contrary to what would be expected for inducible expression, the authors concluded that expression of intB13 could be the result of random microscopic events and transcriptional factors present at the individual cell level. Whether the expression of a single integrase associated with mobilization of a genomic island can be inferred to be similar to OLGA172 is not known, since thus far the RIT element and the CCD operon in OLGA172 has not been found to act as a genomic island or be mobilizable at all. While it is now known that the integrase genes are transcribed, it is not known if they are responsible for mobility in this region of the genome.

The RITBphytO1 element of OLGA172 shows highest homology to two RIT elements in Cupriavidus metallidurans CH34, and one from Ralstonia eutropha H16. Interestingly, in both identified RIT elements found in CH34, the RIT element is flanked by partial fragments of an IS66 tnp gene (Houdt et al., 2009). In H16, the RIT element is found duplicated and flanking a region of DNA approximately 3000 bps in length. The duplicated RIT elements in this strain forms a large stem in a stem loop structure, and the ends of the RIT elements at the base of the stem are both flanked by IS66 tnps (Schwartz et al., 2003; Houdt et al., 2009). RIT elements flanked by partial IS66 tnps in these three strains may reflect an affinity for this element at
specific sites. In OLGA172 the distance between the end of the inverted repeat and the partial tnp fragment on both ends of the RIT is 6 bps (Figure 6.1), this also supports the hypothesis that the RIT element integrates into these sites in a highly specific manner.

Further studies need to be carried out to determine if there are any conditions that will induce the integrase genes of the RIT element, and if they are capable of mobility. The possibility of secondary structure formation involving the RIT element, similar to what is found in H16 is also worth further exploration since it may be the cause for the inability to sequence beyond this region in the efforts to date.

Analysis of the homology of the region upstream of the CCD operon in OLGA172 and NK8 also indicates the potential for past mobility in this region. *Burkholderia* NK8 was found to have two regions of high homology to OLGA172 in the non-protein coding regions upstream of the CCD operon (Figure 6.1). In NK8 these regions were separated by 441 bp, whereas in OLGA172 these regions were separated by 12 bp. These regions of high homology have no similarity to any other sequenced strain on GenBank and thus are not likely to be involved in any conserved protein or binding motif. These regions of variable homologies may be indicative of past excision and ligation of the regions upstream of the CCD operon (and possibly including the CCD operon) between these two strains, or similar microbes. These regions, in combination with the presence of multiple full and partial integrases indicate that it is likely that this region was involved in mobility in the past.
Chapter 7. Conclusions and Future Work

Most chlorinated aromatics are xenobiotic compounds that are relatively recalcitrant and frequently toxic in the natural environment, and as such their fate is of concern for the environment and for human health. The degradation of chlorobenzoate has been a focus of chloroaromatic degradation research for a long time. Ironically it informs our research in this area precisely because it is not typical of xenobiotic chlorinated aromatic compounds, it is relatively easily degradable, and yet is an intermediate to the breakdown of one of the most recalcitrant groups of compounds - the polychlorinated biphenyls. As it is not known to be naturally occurring, it represents a compound that bacteria have adapted to degrade within just a few decades. Our understanding of how this has occurred holds promise for the understanding of how bacteria may adapt for the break down of other more problematic compounds. This has implications for the applied field of bioremediation and to the study of microbial evolution. The study of OLGA172 in this work has implications in both areas and will be discussed separately.

Bioremediation

Microorganisms with degradative capabilities have been exploited for bioaugmentation (addition of organisms to a contaminated site), biostimulation (the stimulation of naturally occurring degraders using organic or nutrient amendments) and in phytoremediation. In bioaugmentation trials, genetically engineered microbes often do not compete well in natural systems because they have been selected for unrealistic laboratory conditions. Introduced genetic material can become instable and lost (Urgan-Demirtas et al., 2006), and predation and competition with indigenous microbes may limit the extent to which the introduced bacterium can proliferate and aid in degradation. Using bacteria (or their degradative genes) that were isolated from the same ecological niche as the contaminated site for genetic modification has been found to increase the success of introduced bacteria for bioremediation. For example, researchers were able to enhance bioaugmentation of activated sludge by introducing foreign degradative genes isolated from Comamonas testosteroni R5, into a Comamonas sp. found to be dominant in the activated sludge population (Watanabe et al., 2002). In this instance, using a species already adapted to a particular ecological niche aided degradation.
In this work, OLGA172 a soil isolate from a pristine site, was found to have plant growth promoting effects on seedlings, and be capable of endophytically colonizing both *P. sativum* and *S. lycopersicum*. Additionally, expression of the *tfdC* gene was found to occur within the plant, though these levels were not successfully quantified in this study. This combination makes OLGA172 an attractive strain for exploiting for phytoremediation. The endosphere of the plant is thought to be a protected environment for microbes where there is consistent nutrient, moisture, pH. Microorganisms in the endosphere are thought to be specifically selected for by the plant, and therefore there are limited numbers of competitors which fill this niche (Backman & Sikora, 2008).

One of the major goals of this study was to determine the ecological preferences of OLGA172, which was originally isolated from soil. A carbon optima in the range of what would be found near or within plants was found, in combination with the colonizing capabilities and plant growth promoting effects seen indicate that it is very likely that OLGA172 is typically associated with plants in the environment it was found in. What role plant association may have on shaping evolution of the CCD pathway remains to be determined. One major work that still needs to be accomplished when characterizing OLGA172 is the designing of OLGA172 specific primers. Locating OLGA172 in the various parts of the plant by use of primers other than those which target *tfdC* will aid characterization in several ways. It will allow for unambiguous detection of OLGA172 within various parts of the plant, and will allow us to identify whether the *tfdC* gene has been disseminated to other bacteria within the plant. It will also (perhaps more importantly) allow us to quantify expression levels of the *tfdC* gene in different parts of the plant. Determining if the *tfdC* gene is induced or expressed at higher levels in some parts of the plant can potentially give us insight into whether plant associations have helped shape the evolution of the CCD genes or their regulation.

**Microbial Evolution and Chlorinated Aromatic Adaptation**

Manmade chlorinated aromatics such as PCPs, some chlorinated phenols, chlorinated benzenes and CBAs are generally thought to have never been encountered by microorganisms prior to their large scale manufacture and release into the environment. The presence of these anthropogenically produced compounds is thought to have resulted in selection of bacteria with novel degradative pathways enabling bacteria to take advantage of new carbon sources (van der
Meer, 1997). Evidence for the selection of catabolic bacteria at contaminated sites is abundant (Springael & Top, 2004; de Lipthay et al, 2001; van der Meer et al., 1998). What is less clear however, is why or how degraders found in environments with presumably no selective pressure have come to adapt to these compounds.

The 3CBA degraders isolated from pristine environments by Fulthorpe et al. (1996) were found across the globe, in 6 regions over 5 continents. This clearly indicates that 3CBA metabolism is widespread and common in areas where no known selection pressure exists. Only 150 of the 600 strains originally isolated in that study were found to maintain their 3CBA degrading phenotypes post-storage (Fulthorpe et al., 1996). This collection of isolates is a great sample set to study questions about how microorganisms adapted to degrade the chlorinated aromatics that are prevalent today. The instability of the majority of these strains makes them difficult to study, but also very interesting.

There are two main hypotheses for why 3CBA metabolizing strains can be found so readily in pristine environments. The genes for degradation may have pre-dated the mass release of chlorinated xenobiotics, probably to degrade a natural analog, or the degradative genes from bacteria that did evolve them in a contaminated environment, indiscriminately and ubiquitously spread them across wide geographic areas through horizontal gene transfer. The results of characterization of OLGA172, a representative pristine degrader isolate can be discussed in this evolutionary context. Briefly, the main findings of this study are as follows:

1. OLGA172 does not display a broad capacity to degrade chlorinated aromatics other than 3CBA. Degradation was not seen on any of the other compounds tested, most notably 4CBA, indicating that the degradative pathway is regiospecific and likely specialized to a limited number of compounds. Degradation was also not seen on the natural chlorinated aromatics tested, including chlorinated indole acetic acid, found as a phytohormone in some plants, or on chlorinated anisaldehydes, compounds that are naturally occurring in places where white rot fungi are active.

2. Accumulation of oxidized and toxic chlorocatechol was found when OLGA172 degrades 3CBA in the presence of an easily degradable carbon source. This can be explained by
imbalanced transcriptional control of the CC producing pathway, and the CC degradative pathway.

3. The regulator of the CCD operon in OLGA172 is *tfdT*, which was found to be induced in the presence of 3CBA. OLGA172 also possesses a mechanism to reduce expression of the CCD genes when a carbon source which is easier to degrade is present (carbon catabolite repression), seen when *tfdT* (and as a result *tfdCDEF*) expression is repressed in the presence of easily degradable carbon sources.

4. Analysis of homology of the *tfdT-tfdC* intergenic regions in the two only other strains which carry this gene, show variable homology to OLGA172. This may suggest horizontal transfer of the entire CCD operon in the past when homology to the intergenic region was relatively high (JMP134). Conversely, low homology in the intergenic region, but high homology of the surrounding *tfdT* and *tfdC* genes (seen with NK8) may indicate that the operon has existed for sometime, and accumulated mutations in the intergenic regions which has less selective pressure to maintain specific nucleotides.

5. OLGA172 possesses multiple full and partial genes associated with mobile genetic movement in proximity to the CCD operon. The three integrases which comprise the RIT element upstream of the degradative operon were found to be expressed at low levels in OLGA172 regardless of the presence of 3CBA. Additionally, one of the integrases (*int3*) was found to be differentially expressed.

The inability of OLGA172 to degrade other chlorinated aromatics, including 4CBA suggests that the enzymes are not of low specificity, and may have been fine-tuned over evolutionary time. Similarly, regulation of the CCD operon, which involves being induced in the presence of its substrate, as well as being repressed in the presence of easily degradable carbon source suggests that OLGA172 has evolved the ability to save the energy costs of expressing and translating these genes when an easy to degrade carbon source is available. The imbalance in regulation of the CCD operon and the CBD operon, can be seen as poor regulation of the CBA degradative pathway in OLGA172. This lack of optimization may reflect that the CBD and CCD operons have not been subject to similar evolutionary pressures in the past, possibly because of HGT of
one of the operons, or because CBA is not the substrate that typically gets channeled through the CCD operon naturally.

Evidence of past genetic transfer events are present in OLGA172. The presence of partial integrases and tranposases as well as the intact RITBphytO1 genes indicate that this region likely was involved in past (or current) mobility events. This could involve transfer of the genes from OLGA172 to another organism, transfer into OLGA from a foreign source, or genetic rearrangement with the strain. The observation that the RITBphytO1 genes are expressed indicates that current mobility in this region is a possibility. The RITBphytO1 genes were found to be constitutively and differentially expressed, with no statistically significant induction seen under the conditions tested. Future work on this strain should involve characterizing these genes further. Specifically, experiments to determine if these genes are inducible or if they are under specific regulatory control needs may reveal if these genes are capable of mobility in response to some environmental stimuli.

The regulator of the CCD operon is a (presumably) rare gene, tfdT, which is found in only two other species annotated on GenBank. Analysis of the homology between the operons of OLGA172 and these two strains showed variability in the tfdT-tfdC intergenic regions, and some interesting homologies upstream of the CCD operon. The intergenic non-protein coding nucleotides between the tfdT gene and tfdC gene can be expected to accumulate some changes over evolutionary time if they do not effect the tfdT/tfdC genes negatively or change the fitness of the organism. When OLGA172 was compared to Burkholderia NK8 and C. necator JMP134, the intergenic regions were found to have 40% and 62% similarity (compared with approximately 80% similarity of the tfdT and tfdC genes). This could support the idea that these strains have been under varying selective pressure for some time.

Burkholderia NK8 was found to have two regions of high homology to OLGA172 in the non-protein coding regions upstream of the CCD operon. In NK8 these regions were separated by 441 bp, whereas in OLGA172 these regions were separated by 12 bp. These regions of variable homologies may be indicative of past excision and ligation of regions upstream of the CCD operon (and possibly including the CCD operon) between these two strains, or similar microbes.
Preliminary data on the pristine degrader collection indicates that some of the pristine isolates may also carry the tfdT gene (Jin, 2010). 28 strains were probed for the tfdT gene using PCR and 17 were found to have a product of the expected size. Future work should involve the sequencing of these genes as well as the tfdT-tfdC intergenic regions. Since the tfdT gene seems rare thus far, studying microbes which carry this particular isoform may be a manageable group to examine evolution of the CCD pathway.

Finally, it is important to note that no real conclusions on why the CCD genes are found readily in environments with no selective pressure can be drawn from the study of a single pristine isolate. OLGA172 seems to indicate both the possibility of horizontal gene transfer, and adaptation over long time scales. Congruency between the tfdC gene and a housekeeping gene like rpoD or the 16S rRNA gene in other pristine degraders could indicate if these operons were horizontally transferred. Additional data, such as similarity of the intergenic tfdT-tfdC regions in these strains, and the ability to regulate the CCD operon (induction in the presence of CBA and repression in the presence of easily degraded carbon) could resolve an idea as to whether these genes have a natural role, or whether they have been horizontally transferred. Horizontal transfer and maintenance of the CCD genes in regions which have no selective pressure for them, could indicate that the fitness costs to maintaining ‘useless’ genes are less than previously thought. It would indicate quite a widespread potential for horizontal gene transfer to spread genes over large geographic areas, and could have some interesting ramifications for other microbial adaptations in the absence of selective pressure, like that of antibiotic resistance.
References


Reinecke, D. 1999. 4-Chloroindole-3-acetic acid and plant growth. *Plant Growth Regulation.* 27(1):1573-5087


### Appendices

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**Figure A.1 Alignment of tfdT proteins of OLGA172 with tfdT of other strains**

JMP134 possesses an incomplete tfdT gene which is interrupted by an insertion sequence. There is high homology among the three strains, however the C-terminal of the protein is not conserved in OLGA172 and NK8.