FINAL REPORT

Engineered Intrinsic Bioremediation of Ammonium Perchlorate in Groundwater

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List of Acronyms

AI	Artificial intelligence
ATCC	American Type Culture Collection
BSA	bovine serum albumin
cld	chlorite dismutase
ClO_3	chlorate
ClO_4^-	perchlorate
dNTP	deoxynucleoside triphosphates
DoD	U.S. Department of Defense
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)
GA	Genetic Algorithms
GA-ANN	Genetic Algorithm Artificial Neural Network
GMO	genetically engineered microorganisms
LC/MS	liquid chromatography/mass spectrometry
NAS	National Academy of Sciences
NRC	National Research Council
PRB	perchlorate reducing bacteria
pcr	perchlorate reductase
RSM	Response Surface Methodology
SERDP	Strategic Environmental Research and Development Program
UPLC	Ultra Performance Liquid Chromatograph

ABSTRACT

A variety of perchlorate remediation technologies are currently commercially available. One of them, biological degradation involves perchlorate reducing bacteria (PRBs), which are widespread in the environment. PRBs have the ability to grow in either the presence or absence of air, provided proper nutrients are available in the environment. Both in situ and ex situ biological treatment systems have already been applied at full scale to treat perchlorate contamination. Implementation of novel techniques such as optimization and creation of microbial consortia using the Genetic Algorithm have great potential to improve the existing technology. State-of-the-art tools of genomics and proteomics were developed that will allow sensitive, real-time monitoring of processes that remove perchlorate from the environment, such as facilitated remediation and monitored natural attenuation. The results presented suggest that genetic algorithms and environmental proteomics could become tools in environmental microbiology for an efficient control of the functioning of natural and undefined microbial ecosystems.

1.0 OBJECTIVES

The primary objective of this project is to demonstrate that transformation of natural subsurface biofilms by free DNA encoding constitutively expressed genes for perchlorate degradation (reduction) can be employed to establish a process of *engineered natural attenuation* for *in situ* treatment of perchlorate in groundwater. This is accomplished without the addition of genetically engineered microorganisms (GMOs) to the environment. Because the approach relies upon indigenous electron donors as a source of electrons for perchlorate reduction, it avoids the need to drive an aquifer anaerobic by biostimulation.

Project tasks are to: 1) isolate and characterize novel perchlorate-reducing bacteria in addition to those available from culture collections and other investigators; 2) identify members of the strain collection that are most effective at perchlorate reduction by use of genetic algorithm-based optimization; 3) clone and sequence perchlorate reductase (*pcr*) and chlorite dismutase (*cld*) genes from the most efficient strains in our collection; and 4) transform laboratory-based natural subsurface biofilms with free DNA encoding *pcrABCD* and *cld* genes to foster engineered natural attenuation of perchlorate.

2.0 BACKGROUND

2.1 Perchlorate

The reason for an increasing interest in perchlorate pollution includes recent advances in both analytical chemistry and better understanding of perchlorate's health impacts. The advances and developments of chemical methods have allowed detection of low parts-per-billion (microgram per liter [μg/L] (Urbansky, 2000) concentrations, and the toxicological research has suggested that such concentrations may impose a potential risk for developing fetuses and infants (USEPA, 2002; Kucharzyk et al., 2009). Perchlorate inhibits iodide uptake by the thyroid causing disruption in normal thyroid function, which can lead to a number of serious health problems, especially pertaining to early neurological development (Blount et al., 2006).

There have been several high-profile cases of perchlorate contamination of surface waters and drinking water supplies in major metropolitan areas (Gullick et al., 2001) and the parties responsible for the events, such as U.S. Department of Defense (DoD) had to quickly respond to the regulatory and public demand to prevent further exposures and clean up contaminated sites (Stroo et al., 2009). In January 2009, the EPA issued a health advisory to assist state and local officials in addressing local contamination of perchlorate in drinking water. The interim health advisory level of 15 micrograms per liter, or ppb, is based on the reference dose recommended by the National Research Council (NRC) of the National Academy of Sciences (NAS) (Kucharzyk et al., 2009).

Perchlorate is a highly water-soluble anion that has been used by the US DOD for more than 50 years as a component in munitions, pyrotechnics, propellants, explosives, and rocket and missile fuels. Perchlorate is toxic to humans and is persistent in groundwater where it has been frequently observed, especially in the western United States. Perchlorate has been detected in public drinking water supplies that serve millions of people. New technologies, particularly *in situ* approaches, are needed to remove perchlorate from groundwater.

It has been known that microorganisms can reduce oxyanions of chlorine such as chlorate (ClO_3) and perchlorate (ClO_4^-) under anaerobic conditions. The high reduction potential of (per)chlorate and chlorate (ClO_4^-/Cl^- E_o = 1.287 V; ClO_3^-/Cl^- E_o = 1.03 V) makes them ideal electron acceptors for microbial metabolism (Coates et al., 2000). Early studies indicated that unknown soil microorganisms rapidly reduced chlorate that was applied as herbicide for thistle control and the application of this reductive metabolism was later proposed for the measurement of sewage and wastewater biological oxygen demand

(Bryan, 1966). Initially it was thought that chlorate reduction was mediated by nitrate-respiring microorganisms in the environment with chlorate uptake and reduction simply being a competitive reaction for the nitrate reductase system of these bacteria (de Groot and Stouthamer, 1969). This was supported by the fact that many nitrate-reducing microorganisms in pure culture were also capable of reducing (per)chlorate (Roland et al., 1994). Furthermore, early studies demonstrated that membrane-bound respiratory nitrate reductases and assimilatory nitrate reductases could alternatively reduce chlorate (Steward, 1988) and presumably perchlorate.

In the past decade, understanding of the perchlorate reduction progressed dramatically due to the development of the genetic analysis tools for detecting and monitoring dissimilatory perchlorate-reducing bacteria for bioremediative purposes (Achenbach et al., 2006). The perchlorate reduction pathway consists of two central enzymes: perchlorate reductase and chlorite dismutase. The first enzymatic step of the pathway, the reduction of perchlorate to chlorate and chlorate to chlorite, is performed by (per)chlorate reductase (Fig.1).The chlorite formed from this reduction is cytotoxic and requires immediate removal from a cell which is catalyzed by chlorite dismutase converting chlorite to chloride and oxygen (Wolternik, 2005).The generation of oxygen makes anaerobic (per)chlorate reduction unique when compared to other anaerobic respiratory processes. This aspect of (per)chlorate reduction has been of special interest because of its potential to introduce oxygen to anoxic sites to aide subsequent bioremediation strategies (Achenbach et al., 2006).

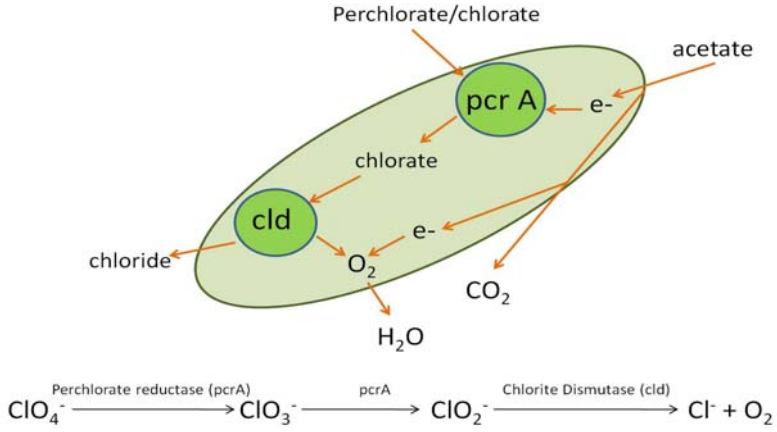

Figure 1. Perchlorate reduction pathway. The reactions are catalyzed by perchlorate reductase (Pcr) and chlorite dismutase (Cld). Note that Pcr is a heterotrimer consisting of PcrA/B complex that is associated with PcrC and Cld is most likely a homohexamer (deGeuss et. al., 2009); each monomer of Cld containing a heme b.

Review papers publish as result of this research:

- Bansal, R., Crawford, R. L., Hess, T. F., and Paszczynski, A. J. 2011. Perchlorate: production, uses, and effects on humans and the environment. Nova Science Publishers, New York (In press)
- Kucharzyk, K. H., Crawford, R. L., Cosens, B., Hess, T. F. 2009. Development of drinking water standard for perchlorate in the United States. J. Environ. Manage. 91:303-310
- Ederer, M., Kucharzyk, K. H., Crawford, R. L., Hess, T. F. 2011. Perchlorate microbial reduction of chlorate and perchlorate: phylogenetic analysis of the genes encoding the degradation pathways. NOVA Science Publishers, New York (In press).

- Kucharzyk, K. H., Hess, T. F., Soule, T., Pszczyński, A. J. 2011. Perchlorate: overview of status and development of remedial options. INTECH Publishers. Book title: "Waste water". (In press)

2.2 Genetic Algorithm outline

Artificial intelligence (AI), such as Genetic Algorithms (GA), covers a wide range of techniques and tools that facilitate decision making and have often been found to be as powerful and effective as gradient search methods in many engineering applications (Schugerl, 2001). Genetic algorithms (GAs) (Holland, 1975) are search and optimization methods based upon the biological principal of evolution through natural selection and mimics biological evolution as a problem-solving strategy. GA tends to thrive in an environment in which there is a very large set of candidate solutions. Inspired by the Darwinian principle of evolution through natural selection, GA borrows part of the vocabulary from biology. Potential solutions to a problem (optimization trials) are conceptually considered to be individuals containing a chromosome encoding the details of the proposed solutions (Reeves, 1993). Such a chromosome consists of genes representing the system variables that are alleles of those genes. GA simultaneously operates on a collection of such solutions, called a population. It evaluates each candidate according to the fitness function that allows each candidate to be quantitatively evaluated (Goldberg, 1989).

Initially, the first generation of potential solutions is typically generated randomly. A new generation of solutions is created by selecting solutions from the old generation with a probability that is proportional to their fitness value (Vandecastelle, 2006). The selected individuals are called parents. After crossover and mutation are applied, these parents result in children that will make up the next generation of solutions (Fig.2). Crossover is a process that typically occurs with a high probability and in which pieces of chromosome are exchanged between pairs of parents.

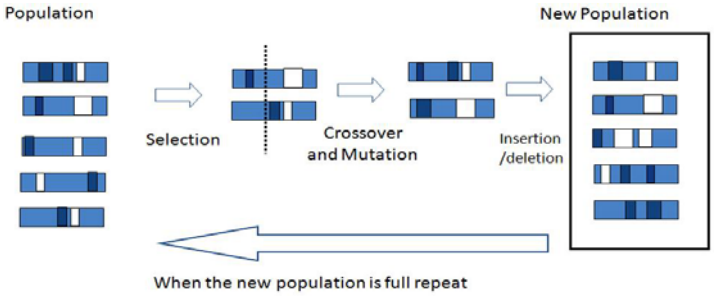

Figure 2. Schematic outline of the operation of a Genetic Algorithm. (T. Soule, University of Idaho, personal communication).

During the process of mutation, each gene has a typically low probability of changing in allele value. The new created generation is then evaluated for fitness, after which the process of selection, crossover, and mutation is repeated. The whole cycle is repeated until an acceptable solution is obtained or until experimental resources run out (Vandecastelle, 2006). This is best summarized with pseudocode, as shown below:

```
begin
        create initial population
        evaluate initial population
        gen = 0
        max _ gen = N
        while (gen < max_gen) do
                gen+ = 1
                select sub-population from initial population
                recombine 'genes' of selected sub-population
```

mutate recombined offspring
evaluate offspring
reinsert best offspring replacing worst parents
end while

Genetic algorithms can cope with multiple interacting variables, operate under considerable levels of noise, and do not require an intricate understanding of the internal dynamics of a system that is to be optimized.

2.3 GA Biological systems manipulation

Stochastical approaches, as GAs, have proven to be extremely suitable for optimization problems regarding many variables, such as fermentation media development (Weuster-Botz and Wandrey, 1996; Weuster-Botz et al., 1995) or in the progress of growth optimization considering the process parameters (Muffler and Ulber, 2004). GAs has been successfully employed to search for the best subset from a large set of microbial isolates that will perform a variety of processes (Vandecasteele, 2004). The processes optimized include biomass production, biomass minimization, and xenobiotic compound degradation. The most recent studies are experimental multi-objective medium optimizations using GA supported by hybrid Genetic Algorithm Artificial Neural Network (GA-ANN) (Franco Lara et al., 2006), optimization of exo-polysaccharide production by hybrid methodology comprising Plackett-Burman design, ANN and GA (Desai et al., 2006), optimization of δ-endotoxin production by Response Surface Methodology (RSM) and GA-ANN (Moreira et al., 2007), modeling and optimization of fermentation factors for alkaline protease production using a feed-forward neural network and GA (Rao et al., 2007), optimization of fermentation media using neural network and genetic algorithm (Nagata and Chu, 2003), optimization of biodegradation of naphthalene by an isolated microorganism by response surface methodology (Martin et al., 2003), and tryptophan-5-halogenase activity assay formulation for enzyme activity optimization (Muffler et al., 2007).

A GA can be used to manipulate microbial ecosystem factors to obtain a desirable functional behavior. There are two ways being used to date. In the first approach efficient mixed cultures can be designed by determining which isolated strains to combine for optimal functional performance (Jarvis and Goodacre, 2005; Vandecasteele, 2004). When designed and constructed appropriately, artificial microbial ecosystems exhibit complex behaviors that are observed in a variety of large-scale ecological systems (Kambam et al., 2008), and perform functions that are difficult or even impossible for individual strains or species (Brenner et al., 2008). These attractive traits rely on two organizing features: communicating with one another and the division of labor. By trading metabolites or by exchanging dedicated molecular signals, each population or individual responds to the presence of others in the consortium (Keller and Surette, 2006). This improves the overall output of the consortium that relies on a combination of tasks performed by a constituent individual or sub-populations (Brenner et al., 2008). If the components of an artificial microbial ecosystem are manipulated, the consequence of altering system complexity can be further explored.

It is also possible to use a genetic algorithm to manipulate environmental conditions and drive an already existing ecosystem in a desired direction, e.g. maximized degradation rate (Kucharzyk et al., 2010, 2011). Certain environmental conditions can influence and cause shifts in ecosystem dynamics (Vandecastelle et al., 2004). Most applications using microbial consortia are in the field of industrial fermentation, where medium compositions are manipulated to maximize production of various chemicals (Bapat and Wangikar, 2004; Etschmann et al., 2004; Fang et al., 2003; Patil et al., 2002; Weuster-Botz et al., 1995; Weuster-Botz et al., 1996). Similar attempts have been made to optimize medium conditions for oil degradation by a pure culture (Li et al., 2004) and for the growth of insect cells (Marteijn et al., 2003). An approach based on changing environmental conditions would start with identifying a set of conditions that influence ecosystem dynamics and that can be manipulated experimentally. Such conditions taken

under consideration may include chemical and physical factors such as temperature, pH, salinity, light treatment, and mixing. They could also include concentrations of electron donors, electron acceptors, and other chemicals (Vandecastelle et al., 2004).

2.4 GA application to optimization of perchlorate biodegradation

Today, a wide variety of *in situ* biological treatment approaches are available to remediate perchlorate from ground and surface waters and soil, and remediation tools and techniques are available from a collection of technology vendors and environmental consultants. Biological *ex situ* treatment systems for perchlorate, as well as the isolation and characterization of numerous pure cultures of perchlorate-degrading bacteria from natural environments, has prompted significant research concerning the potential for *in situ* perchlorate treatment through electron donor amendment to soils and groundwater (Aziz and Hatzinger, 2009). Because of its unique chemical stability under environmental conditions and its high solubility (Urbansky, 2002), microbial reduction of perchlorate was identified as the most feasible method of remediation of contaminated environments. The presented technology avoids the production of hazardous waste streams that require further treatment or disposal and addresses the need to develop *in situ* approaches for the remediation of perchlorate contamination in soil and groundwater.

The overall goal of the *in situ* perchlorate bioremediation with the GA application is to engineer natural subsurface microbial communities (aquifer biofilms) to give them the ability to degrade (reduce) perchlorate, even in the presence of oxygen and without the addition of genetically engineered microorganisms (GMOs) to the environment. This approach is called "*engineered intrinsic bioremediation*." In the search for efficiently degrading mixed microbial cultures two approaches can be implemented. The first uses a genetic algorithm to manipulate environmental conditions and drive already existing ecosystem in a desired direction, and the second approach that is used is to design efficient mixed microbial consortia by determining which isolated strains to combine for optimal functional performance. For that purpose several members of the (per)chlorate strain collection identified and selected as the most efficient in the perchlorate degradation process can be candidates for optimization (Table 1).

Table 1. Examples of known perchlorate- and chlorate-degrading bacteria, used in the GA optimization experiment. *Perchlorate reducers are indicated as PR, chlorate reducers as CR.

NAME	ATTCC / DSMZ	CR/PR*
Pseudomonas chloritidismutans	ATCC # BAA-775	CR
Ideonella dechloratans	ATCC # 51718	CR
Dechlorosoma sp. KJ	ATCC # BAA-592	PR
Dechloromonas agitata	ATCC # 700666	PR
Dechlorosoma suillum	ATCC # BAA-33 / DSMZ 13638	PR
Azosypyra oryzae	DSMZ 1199	PR
Dechloromonas hortensis	MA-1 DSM 15637	PR
Dechloromonas sp. Miss R	Courtesy of J. Coates lab	PR
Dechloromonas denitrificans	ATCC BAA-841, CIP 109443	CR,PR
Rhodobacter capsulatus	DSMZ 155	CR,PR

In the first part of the project a GA was used as an alternative method for directing and artificially defining a set of environmental conditions for naturally occurring microbial consortia and pure cultures to achieve maximum rates of perchlorate degradation. Samples collected from several areas contaminated with perchlorate were used along with pure cultures of perchlorate reducing microorganisms. The initial population (the algorithm's equivalent of a chromosome) was generated at random; a subunit of the bit string (the algorithm's equivalent of gene) gives the value of one parameter. Each experiment was performed in four replicates and a complete chromosome was composed of 36 bits, consisting of 9 medium components of 4 bit each (Table 2).

Table 2. Parameter settings for the genetic algorithm

	GA configuration
Variables	9
Population size	11 (single strains); 12 (consortia)
Generation gap	1
Selection probability	0.5
Mutation probability	0.5
Total bits in chromosome	36

The GA used here followed the generational model and had a population size of 11 (single strains) or 12 (consortia). Each solution was represented as a string of 9 values, encoding values for variables of environmental conditions. In this way, each solution encoded for a specific set of environmental conditions selected in the experiment (Table 3).

Table 3. Ranges of environmental conditions used for the optimization with the GA

INITIAL RANGES OF VARIABLES	
pH	6.8 - 8.0 every 0.1 unit
NH_4Cl	0.125 - 0.375 (g/L) every 0.02 unit
NaH_2PO_4	0.3 – 0.9 (g/L) every 0.1 unit
$NaHCO_3$	1.25 – 3.75 (g/L) every 0.2 unit
KCl	0.05 – 0.015 (g/L) every 0.05 unit
Acetate	1 - 10 mM every 1 unit
Perchlorate	60 - 400 every 10 ppm
Trace minerals	0– 10 (mg/L) every 1 unit
Vitamins	0– 10 (mg/L) every 1 unit

The initial population was generated at random. Fitness values were linearly rescaled, with $\mu' = \mu$ and $f_{max'} = 0$. Roulette Wheel selection was used and no elitism was applied. Single crossover was performed on each pair of selected individuals with probability of 0.5 per bit.

Over the course of eleven generations of optimization using a GA, a statistically significant 78.9-fold increase in average perchlorate degradation rate by *Dechloromonas spp.* KJ and *Dechloromonas* Miss R was observed, when optimization of consortia (Pl6 and Cw3) resulted in 109 and 143-fold increase in average perchlorate degradation rate (Kucharzyk et al., 2011) (Fig.3). The data obtained in this part of GA optimization provided a composition of an optimal medium for maintaining mixed cultures in further analysis and entailed the use of the GA to artificially construct a consortium from 10 isolates such that the consortium is optimized for the reduction of perchlorate .

In the next experiment, the GA used followed up the generational model and has a population size of 10 (Kucharzyk et al., 2011). A higher population size would most likely increase the efficiency of the optimization; however, we consider 12 experiments in fourfold the maximum number that is logistically feasible. Each solution was represented as a string of 10 bits, encoding the presence or absence of the corresponding microorganism. In this way, each solution was encoded for a specific microbial consortium. The initial population was generated at random. Fitness values were linearly rescaled with $\mu'=\mu$ and $fmax'=2\mu$ (where μ and μ' are the average fitness of the parent population before and after rescaling, respectively, and $fmax'$ is the maximum fitness of the parent population after rescaling). If this yields negative values, the fitnesses is rescaled so that $\mu'=\mu$ and $f'min=0$ (where $f'min$ is the minimum fitness of the parent population after rescaling). Roulette wheel selection is used and elitism is applied. Single crossover is performed on each pair of selected individuals with a probability of 0.90. Mutation is performed by flipping bit values with a probability of 0.01 per bit (Goldberg, 1989). To evaluate the fitness (perchlorate reduction rate/extent) of each individual in a generation, a method for assaying perchlorate concentrations using a fluorescent dye (Kucharzyk et al., 2010) is used.

We expect that the analysis of the various fitness levels associated with particular strain combination show that the effect of single strains on the dynamics of mixed culture will depend on what other strains the organism was combined with. The same strain can have positive, neutral, or negative effect on between-generation variability. Similar to results in several of Vandecasteele (2004) experiments, we expect the GA to be able to optimize efficient mixed microbial cultures in each of the experimental scenarios.

As Vandecasteele et al., (2004) proposed we believe that an ecological mechanism can be proposed to explain formation of highly effective microbial community. It appears as if early on in the optimization, the GA quickly eliminates certain strains from consortia. These could be strains that have a dominating overall negative influence on the productivity of the consortia. On the other hand, some strains quickly seemed to be positively selected. These could be strains that have a high biomass production and an overall positive influence on the consortia. We also propose that the algorithm is seeking out clusters of highly productive organisms (Holland, 1975) that function well together and is then recombining these clusters into larger scale consortia. Such groups of organisms could have a high biomass production because they have a positive influence on each other's growth or because they target different ranges of nutrient sources within the growth medium.

2.5 Advantages and limitations of GA application

The use of stochastic search procedures based on genetic algorithms (GAs) in the experimental optimization of media formulation has been lately applied in an efficacious manner compared to other methods, like statistical design of experiments (Park et al., 1998). The success of this approach is specially associated with the recent advances in the application of miniaturisation and parallelization techniques to bioreactors allowing the implementation of a large number of simple batch experiments which can be carried out simultaneously (Zafar et al., 2010). The careful manipulation of environmental conditions can result in precise shifts in the make-up of a microbial ecosystem, which can in turn translate to desirable changes in overall functionality. The successful execution of the manipulation of microbial systems in either of these two manners will often be a challenging experimental task (Vandecasteele,

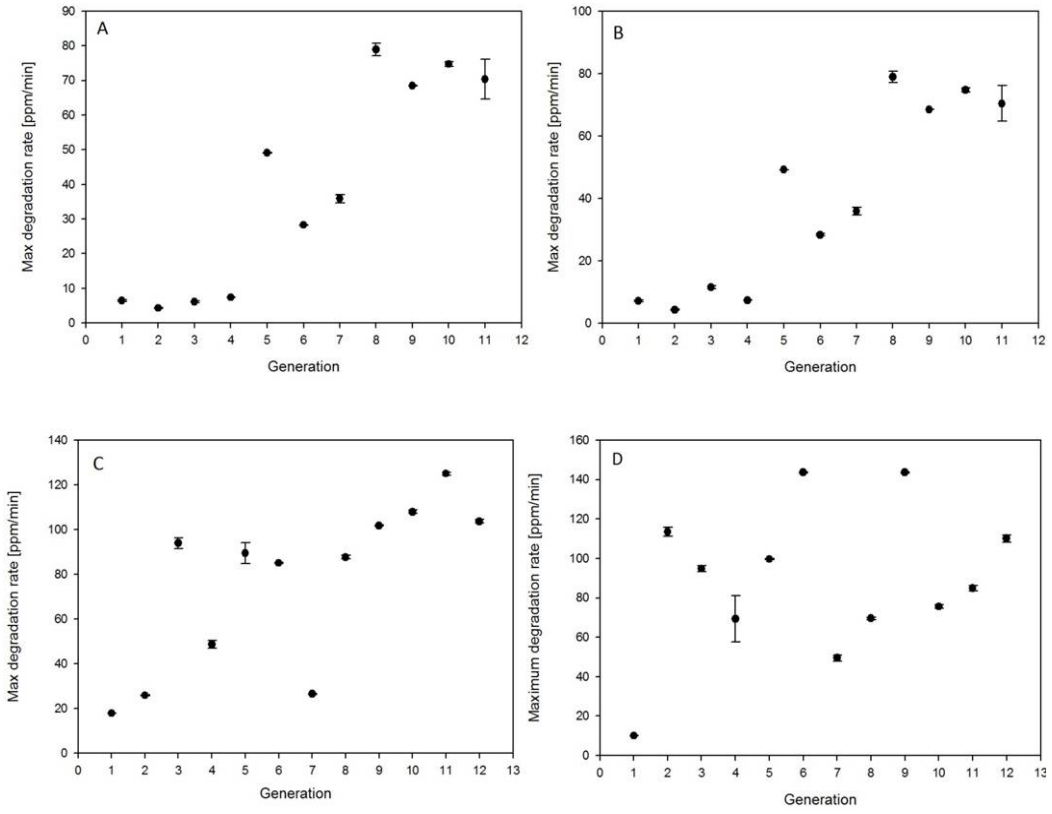

Figure 3: Average degradation rates values of (A) Dechlorosoma sp. KJ, (B) Dechloromonas Miss R, (C) Cw3 consortium, (D) Pl6 consortium.

2006). While statistical methods give better interpretation of an optimized response in terms of variance, GA gives better point of prediction. GA is capable of exploring large variable spaces with the additional advantage of an evolutionary adaptation through selection, information exchange (crossing over) and mutation. The strategy of "survival of the fittest" is applied according to the optimization objectives (Zafar et al., 2010). GA has been successfully utilized for kinetic parameters estimation in biotechnological processes (Park et al., 1997; Saiz et al., 2003) such as alcoholic fermentation.

In the field of bioinformatics there have been a number of reports showing the capability of GA to effect data reduction in order to improve the performance of predictive models. For example, for classification problems using gene expression data (Li *et al*., 2001; Ooi and Tan, 2003), improved classification accuracy was obtained following GA variable reduction. In a similar study, Chuzhanova *et al*., (1998) used GA with the Gamma (near-neighbor) test for feature selection of genetic sequence data, which again leads to improved classification results. GA optimization has also been applied to other bioinformatics-related problems such as sequence alignment (Notredame *et al*., 1998) and phylogenetic tree construction (Lewis, 1998). However, in work related to evolutionary algorithm optimization of laboratory processes, we observe that most noisy fitness functions make use of oversampling to improve the precision of the fitness estimate (Meekof and Soule, 2010). This noise is inherent in both the sensors and in the variability of the processes themselves, particularly in applications to biological processes. GAs cannot effectively solve problems in which the only fitness measure is a single right/wrong measure (like decision problems), as there is no way to converge on the solution. In these cases, a random search may find a solution as quickly as a GA. However, if the situation allows the success/failure trial to be repeated giving (possibly) different results, then the ratio of successes to failures provides a suitable fitness measure.

Detailed information on implementation of genetic algorithms to perchlorate research is included in our recent papers:

- Kucharzyk, K.H., Crawford, R.L., Paszczynski, A.J., Hess, T.F. (2010). A method for assaying perchlorate concentration in microbial cultures using the fluorescent dye resazurin. J Microbiol Methods 81(1):26-32
- Kucharzyk, K.H., Crawford, R.L., Paszczynski, A.J., Soule, T., Hess, T.F., (2011). Use of Genetic Algorithm to increase degradation rates of perchlorate by single strains and consortia. Appl Environ Microbiol (submitted).
- Kucharzyk, K.H., Crawford, R.L., Paszczynski, A.J., Soule, T., Hess, T.F. Use of genetic algorithm to increase degradation rates of perchlorate by artificially designed consortia; *International Symposium on the Bioremediation and Sustainable Environmental Technologies Symposium* (June 27-30, 2010, Reno, NV). Poster.
- Kucharzyk, K.H., Crawford, R.L., Paszczynski, A.J., Soule, T., Hess, T.F. Use of genetic algorithm to increase degradation rates of perchlorate by single strains and consortia; *Seventh International Conference on Remediation of Chlorinated and Recalcitrant Compounds,* Battelle (May 24-27, 2010, Monterey, CA). Poster.
- Kucharzyk, K.H., Ederer, M.M., Green, T., Crawford, R.L., Paszczynski, A.J., Hess, T.F. Using a genetic algorithm to increase degradation rates of perchlorate and expression of genes involved in perchlorate reduction in transformed biofilms; *SERDP-ESTCP Symposium* (Dec 1-3, 2009, Washington DC). Poster.
- Kucharzyk, K.H., Crawford, R.L., Paszczynski, A.J., Hess, T.F. A new method for high-throughput perchlorate analysis. *237th ACS National Meeting* (March 22-26, 2009, Salt Lake City, UT). Poster.
- Bansal, R., Ederer, M.M., Kucharzyk, K.H., Paszczynski, A.J., Crawford, R.L. Cloning and expression of the genes encoding the perchlorate reduction pathway from different bacterial species; *ASM,* (2008, Boston, MA). Poster.

3.0 GENETIC ANALYSIS OF PERCHLORATE DEGRADATION PATHWAY

3.1 Background

Microorganisms capable of perchlorate and chlorate degradation have been isolated from contaminated (Waller et al., 2004) as well as pristine (Coates et al., 1999) environments. All perchlorate and chlorate degrading bacteria identified so far are Gram negative, non-spore forming, facultative anaerobes. Using 16S rRNA gene sequence analyses they were classified into the α-subgroup *(Dechlorospirillum sp*. WD), β-subgroup (*Dechloromonas spp.; Azospira (Dechlorosoma) spp*., *and Ideonella dechloratans* [chlorate only]), γ-subgroup (*Pseudomonas spp*. [chlorate only]), and □-subgroup (*Wolinella succinogenes* HAP-1) of the proteobacteria (Coates and Achenbach, 2004). The dominant groups of perchlorate degrading organisms were found to belong to the β -subgroup of proteobacteria and were classified as *Dechloromonas spp.* and *Azospira spp. (formerly Dechlorosoma spp.).* Most of the perchlorate reducers seem to prefer nitrate as a terminal electron acceptor since perchlorate reduction seems to be induced only after nitrate has been depleted. None of these organisms were found to be able to grow on sulfate (Coates et al., 1999; Logan, 1998) as a terminal electron acceptor.

Only recently with the development of new, more sensitive technology for the detection of low levels of perchlorate and isotope discrimination studies, has it become apparent that perchlorate in the environment not only originates from anthropogenic sources, but is to a large extent generated by naturally-occurring processes. Considering natural, stratospheric generation of perchlorate, a spatially more even distribution

of perchlorate would be expected. However, perchlorate is predominantly found in very arid environments or in areas where man-made contamination exists. One explanation for this localized perchlorate occurrence might be that in environments exposed to significant amounts of moisture, i.e. rain, snow, flooding, etc., the naturally deposited perchlorate does not persist or accumulate because it is regularly leached out by these waters due to its extraordinary solubility of perchlorate. Under anaerobic conditions in these contaminated waters, the perchlorate can be degraded by the ubiquitously present, indigenous, perchlorate-degrading microfloral populations keeping perchlorate levels close to zero.

Perchlorate degradation by microorganisms was first described in the 1960s (Hackenthal et al., 1964). These researchers found that the perchlorate degradation activity was associated with the nitrate reductase enzyme complexes in *Escherichia coli* and *Proteus mirabilis* (Hackenthal et al., 1964; Hackenthal, 1965) and the activity was shown to be inhibited by the presence of nitrate. These organisms however were not able to grow using perchlorate as electron acceptor. Anaerobic enrichment cultures capable of degrading aqueous solutions of 6 g/L or more perchlorate were observed to be associated with a perchlorate-specific activity in the presence of protein based carbon sources (Attaway et al., 1989; Attaway and Smith, 1993). Later Wallace et al., (1996) were able to show that *Wolinella succinogenes*, an organism rather rare and difficult to culture, was able to support perchlorate degradation. This organism was also used in the initial *ex situ* and *in situ* treatment processes of industrial waste streams of rocket manufacturing facilities as well as ground and drinking water treatment applications.

Since then it has been established that specialized enzymatic activities for the reduction of perchlorate and chlorate have evolved. Organisms encoding these enzymes can use perchlorate and chlorate as terminal electron acceptors. Perchlorate can support growth of these organisms when a simple carbon source is supplied. These organisms, however, also encode the gene for the enzyme that catalyzes the subsequent reaction, chlorite dismutation catalyzed by chlorite specific dismutase. This unique enzyme is essential for growth, since it is responsible for the removal of the cytotoxic chlorite from the system by converting it to chloride and molecular oxygen.

Analyses of the reductase enzymes that catalyze the first step in chlorate and perchlorate reduction determined that they belong to the prokaryotic type II DMSO reductase family of proteins and their gene sequences usually were found to be well conserved. The enzyme catalyzing the second step in the perchlorate degradation pathway, the chlorite dismutase, was also found to be conserved.

3.2 Materials and Methods

3.2.1 Growth of bacterial strains and DNA extraction

Bacterial strains were grown and maintained on the media recommended by the culture collections from which they were obtained. *Dechlorosoma* sp. KJ (BAA 592) was grown in ATCC 2361 liquid media under anaerobic condition at 25°C with shaking at 200 rpm. *Dechloromonas hortensis* (DSM-No. 15637) was grown on DSMZ 830a liquid media under microaerophilic conditions in a jar with a GasPak (BD GasPak™ EZ, Campy container system) at room temperature (~25°C). *Pseudomonas chloritidismutans* ASK-1 (BAA 775) was grown on tryptic soy broth under aerobic condition at 25°C with shaking at 200 rpm. *Pseudomonas stutzeri* (DSM -No. 13592) (=*Pseudomonas. chloritidismutans* AW-1) was grown on DSMZ 830-R2A liquid media under aerobic condition at 30°C with shaking at 200 rpm. All of the above media were prepared and sterilized as recommended by either American Type Culture Collection (ATCC) or Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, German Collection of Microorganisms and Cell Cultures (DSMZ). Genomic DNA from bacterial cultures was extracted using the QIAGEN DNeasy Tissue Kit (Qiagen, Valencia, CA).

3.2.2 PCR primers and reaction conditions

The *cld* genes were amplified using several different primer sets. Three primer sets targeting complete and partial chlorite dismutase gene templates were designed based on areas where the different nucleotide sequences were most conserved. Design employed Primer3 software (http://fokker.wi.mit.edu/primer3/input.htm). The primers in set I, cld_1000F and cld_1000R, were designed based on published chlorite dismutase nucleotide sequences from *Dechloromonas aromatica* RCB (CP000089.1) and *Pseudomonas chloritidismutans* (AJ880095) and produce a 1000 bp *cld* amplicon. Primer set II, cld_600F and cld_600R was also designed based on published chlorite dismutase nucleotide sequences from *Dechloromonas aromatica* RCB (NCBI Accession # CP000089.1) and *Pseudomonas chloritidismutans* (NCBI Accession # AJ880095) and amplifies a fragment of 600 bp. In primer set III forward primer, cld_591P_F was designed using the published chlorite dismutase nucleotide sequence of *Pseudomonas chloritidismutans* (AJ880095); cld_591C_F was designed using the published chlorite dismutase nucleotide sequence of *Dechloromonas aromatica* RCB (CP000089.1). Reverse primer, cld_591_R was also designed based on the published chlorite dismutase sequence from *Dechloromonas aromatica* RCB (CP000089.1). Primers cld_591P_F and cld_591_R were used to amplify the partial *cld* gene (591 bp amplicon) from *Pseudomonas chloritidismutans* ASK-1. Primers cld_591C_F and cld_591_R were used to amplify the partial *cld* gene (591 bp amplicon) from all other organisms. Primers were synthesized by Integrated DNA Technologies (Coralville, Iowa).

To optimize PCR conditions, annealing temperatures ranging from 55 to 60°C and different $MgCl_2$ concentrations ranging from 3 to 4 mM were tested. PCR reactions were performed in a Thermo Hybaid MBS 0.2G thermocycler. After optimization, all reaction mixtures consisted of 0.25 mg of bovine serum albumin (BSA), 250 µM deoxynucleoside triphosphates (dNTPs), 10X PCR reaction buffer-$MgCl_2$, 0.4 µM of both forward and reverse primers, 3mM $MgCl_2$, 2 U of *Taq* polymerase (Platinum® *Taq* DNA polymerase, Invitrogen, Carlsbad, CA), 1µl of genomic DNA from the bacterial strains and nuclease-free double distilled water to a final volume of 50 µl. The following PCR conditions were used with primer sets I, II or III to produce the amplicon of predicted size. Reactions were heated to 94°C for 2 minutes followed by 35 cycles of 94°C (30 seconds), annealing temperature ranging from 55 to 60°C (1 minute), 72°C (1 minute); the final extension period was 10 minutes at 72°C. A negative control (without DNA template) was also included. PCR products were observed using gel electrophoresis on a 1.5% agarose gel run in 1X Tris-acetate-EDTA buffer.

3.2.3 Cloning and sequencing of PCR products

PCR products were directly cloned into either the pCR® 2.1 or pCR® 4 TOPO® vector (TOPO TA cloning kit, Invitrogen, Carlsbad, CA). Amplicons produced from genomic DNA of *Dechlorosoma* sp. KJ, *Pseudomonas chloritidismutans* ASK-1 and *Dechloromonas hortensis* were cloned into pCR 4 TOPO®. The *cld* amplicons produced from *Pseudomonas stutzeri* were cloned into pCR 2.1 TOPO® vector. Recombinant plasmids were extracted using a QIAprep® spin Miniprep Kit (Qiagen, Valencia, CA). Sequencing reactions were prepared using M13 primers on a PTC-100™ Programmable Thermal Controller (MJ Research, Waltham, MA). Samples were sent for sequencing to CRB Molecular Biology Core, Washington State University, Pullman WA. Sequencing results for *cld* genes were analyzed using Lasergene 7 (DNASTAR, Inc., Madison, WI). Chlorite dismutase gene sequences obtained from this work have been deposited in the GenBank database under accession numbers EU571095 (*Dechlorosoma sp.* KJ, BAA 592), EU436749 (*Dechloromonas hortensis*, DSM-No. 15637), EU436747 (*Pseudomonas chloritidismutans* ASK-1, BAA 775) and EU436748 (*Pseudomonas stutzeri*, DSM-No. 13592).

3.2.4 Comparison of chlorite dismutases

The chlorite dismutase nucleotide and amino acid sequences now available in GenBank and obtained from this work were compared to each other using ClustalX 2.0. The sequences were compared without the signal peptide sequence. The percentages of nucleotide and amino acid similarity were also calculated. Un-rooted trees for chlorite dismutase genes and 16s rRNA genes were also constructed using ClustalX

2.0. The 16s rRNA gene sequences were obtained from Ribosomal Database Project (www.rdp.cme.msu.edu).

3.3 Results and Discussion

We aligned the 16S rRNA gene sequences of the chlorate and perchlorate degraders we studied in our lab. As expected, the organisms split into two groups. *P. chloritidismutans* and *P. stutzeri* clustered together belonging to the γ-subgroup of the proteobacteria and the other organisms belonging to the β-subgroup of proteobacteria (Fig. 4).

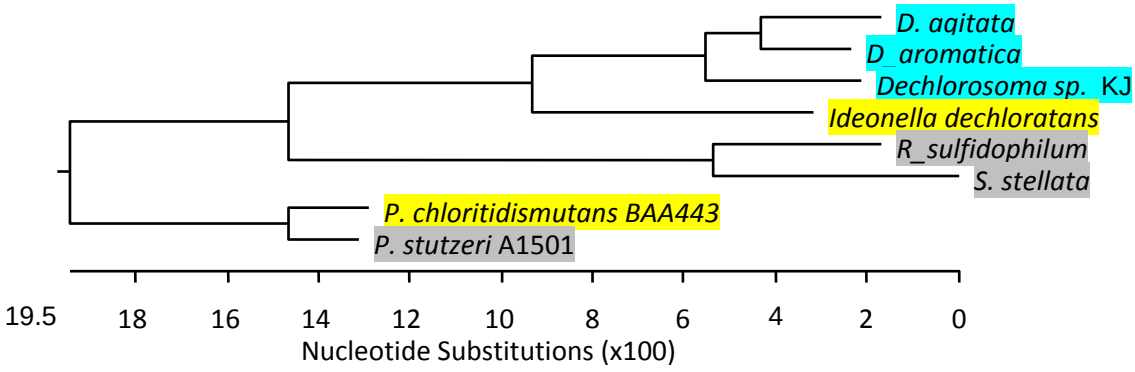

Figure 4. 16S rDNA sequence alignment of known chlorate and perchlorate degraders as well as some close non-degrading relatives. The organisms studied here fall into two groups; *P. chloritidismutans* and *P. stutzeri* belong to the γ -proteobacteria, the other five strains belong to the ß-proteobacteria.

The second group contained the perchlorate degraders we studied as well as *I. dechloratans*, which thus far is the only known member of the β-proteobacteria capable of solely degrading chlorate and not perchlorate.

3.3.1 Arrangement of the genes encoding chlorate and perchlorate degradation activity

Achenbach et al. (2006) outlined the genomic organization of the genes encoding the enzymes involved in perchlorate (*D. agitata* and *D. aromatica*) and chlorate (*Pseudomonas* strain PK and *I. dechloratans*) degradation. We have cloned and sequenced the operons encoding the perchlorate- and chlorate-degrading activities of two additional organisms, *Dechlorosoma sp.* KJ (EU571095) and *Pseudomonas chloritidismutans* (GQ919187), respectively (Fig. 5).

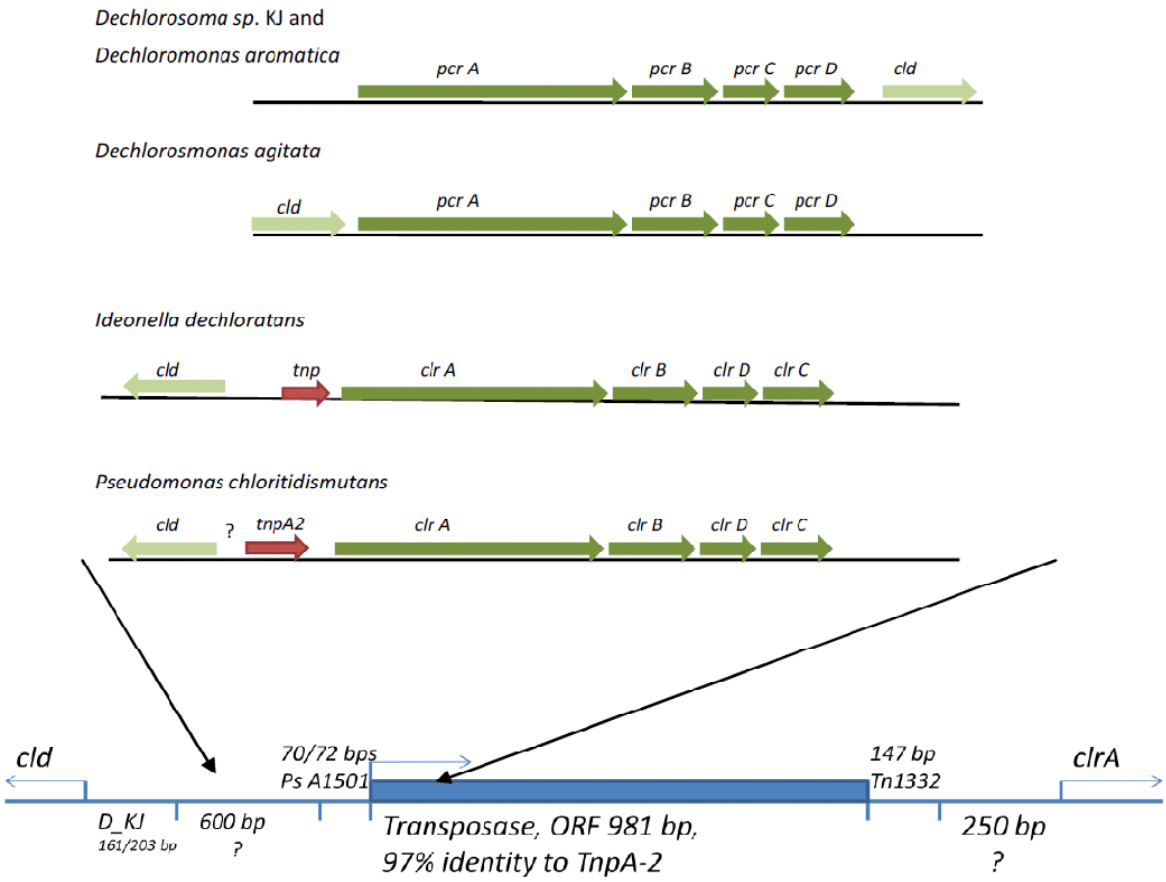

Figure 5. Chromosomal arrangement of the genes/operons involved in chlorate and perchlorate degradation. The operon organization for the perchlorate degraders *Dechlorosoma sp.* KJ and D. *aromatica* is identical, in *D. agitata* the *cld* gene is located upstream of the *pcr* operon. For the chlorate degraders the cld gene is also found to be situated upstream of the reductase encoding clr operon, but transcribed in the opposite orientation. In the *clr* operon the *clrD* gene is located 3' of the *clrC* in both organisms. The intergenic region between *cld* and *clrA* is depicted in more detail to address regional similarities.

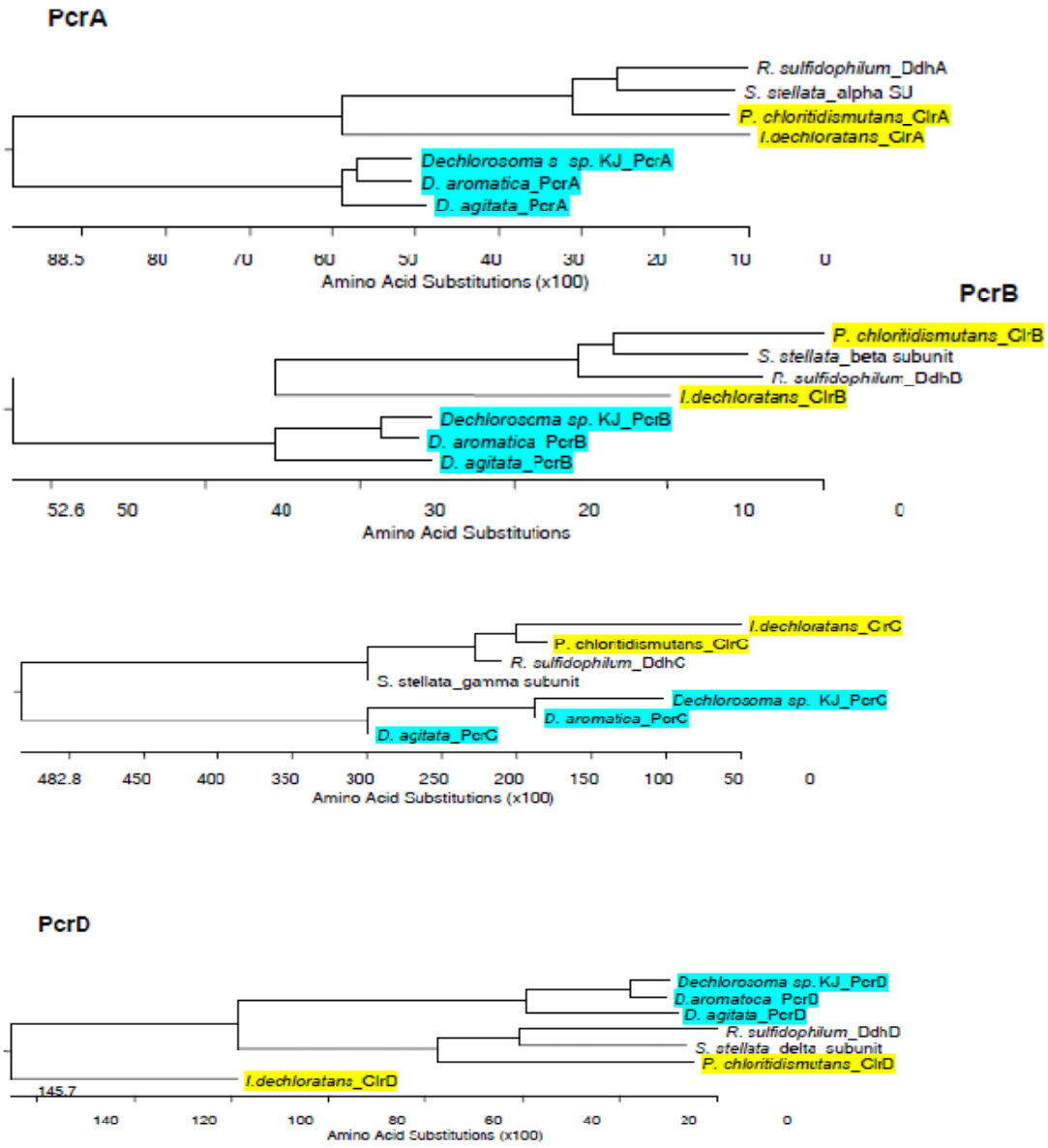

Figure 6. Phylogenetic trees resulting from the alignment of the amino acid sequence of PcrA, PcrB, PcrC and PcrD using the Clustal V option in Megalign (Lasergene 8.0, DNAstar). The alignments for both peptides resulted in the same groupings; the perchlorate degraders *Dechlorosoma sp.* KJ and the two *Dechloromonas spp*. build one, monophyletic group. This is consistent with the 16S phylogeny. The ClrA and ClrB sequences of *P. chloritidismutans* seems to be more closely related to the nitrate reductase of *S. stellata* and the dimethylsulfide dehydrogenase of *R. sulfidophilum*, not following the 16S rDNA phylogeny. The ClrA and ClrB sequences of *Ideonella dechloratans* seems to be rather removed from either group. The perchlorate degraders fall into one monophyletic group. However, the PcrC sequences seem to have much more diverged between these organisms. The peptide sequences of *I. dechloratans* and *P. chloritidismutans* PcrC seem to be more closely related. For the ClrD subunit the *I. dechloratans* sequence seems to have diverged rather distantly from the other organisms. Perchlorate degraders, chlorate degraders.

As expected, the gene arrangement between the two organisms differed. We found the genes in *Dechlorosoma sp*. KJ were arranged in the same way as was reported for *Dechlorosoma aromatica* (Achenbach et al., 2006). The gene arrangement in *Pseudomonas chloritidismutans* matched that of the other chlorate degrader, *Ideonella dechloratans.* The *clr* operon in both organisms was found to be preceded by insertion-type sequences. In *I. dechloratans*, the element encoded a transposase flanked by inverted repeats (ISIde1, Transposase_12, Thorell et al., 2003). We also found an ORF of 981 bps in the corresponding region in the *P. chloritidismutans* nucleotide sequence flanked by inverted repeats that shared 97.6% identity with *Pseudomonas aeroginosa* transposase TnpA-2 (YP_245451) (Haines et al., 2005) (Fig. 6). This particular transposase was previously identified located on pseudomonas plasmid Rsm149, an IncP-6 type plasmid. The 147 bases immediately downstream of the transposase share 98% identify with the repeat region of Tn1332 of *P. putida* (DQ174113). The remaining 250 bps just upstream of the *clr* operon did not reveal any significant similarity to sequences available on NCBI. Eight hundred twenty nine base pairs upstream of the beginning of the transposase gene we found the coding region of the chlorite dismutase. The *cld* gene is transcribed in the opposite orientation as was found to be the case in *Ideonella dechloratans* (Thorell et al., 2003). The *cld* gene of *I. dechloratans* was sequenced by Stenklo et. al. (2001). We found that the roughly 900 bps between the *cld* and *tnpA2* had a region of 161/203 bp identical (79%) to the *Dechlorosoma sp*. KJ *cld* upstream region (see Fig. 5).

The next 600 bps did not reveal any significant similarity with known sequences except for 57/73 bps that are also found in the gene encoding cytc553 of *Pseudomonas alcaliphila.* The remaining 70/72 bps matched a sequence in *Pseudomonas stutzeri* A1501 (CP000304). The region upstream of *cld* in *P. chloritidismutans* was found to be between 97 and 99% identical to genes involved in sugar transport and metabolism of *P. stutzeri* strain A1501. It is noteworthy that this significant identity abruptly ended within a few bases upstream of the 3' end the *cld* gene. In our opinion this abrupt break in sequence identity together with the occurrence of a seemingly functional transposase indicates a rather recent acquisition of the chlorite dismutase gene from a different organism, potentially from *Dechloromonas spp*., since the *cld* sequences obtained for *P. chloritidismutans* and *Dechlorosoma sp.* KJ are 95% identical (Table 4).

Table 4: Distance values between cld amino acid sequences (in % identity) between perchlorate and chlorate degraders. Perchlorate degraders, chlorate degraders.

Cld	*D. agitata*	*D. aromatica*	*Dechlorosoma sp.* KJ	*P. chloritidismutans*	*Ideonella dechloratans*
D. agitata	100	58.7	58	57.9	72.2
D. aromatica		100	94.7	92.1	57.4
Dechlorosoma sp. KJ			100	95.7	57.8
P. chloritidismutans				100	57.1
Ideonella dechloratans					100

These observations, the conservation of upstream regions and the presence of transposable elements further support the idea of horizontal gene transfer as a mechanism for dispersal and acquisition of genes involved in the degradation of (per)chlorate by a wide variety of organisms.

3.3.2 Chlorate reductase and Perchlorate reductase

The chlorate reductases of *Ideonella dechloratans* and *Pseudomonas chloritidismutans* are encoded by the *clrABDC* operon. The *I. dechloratans* operon was initially cloned and sequenced by Thorell *et al.* (2003) and the nucleotide sequence is available at NCBI (AJ566363). The chlorate reductase of this bacterium consists of three different subunits encoded by structural genes *clrA*, *clrB*, and *clrC*. The last gene of the operon, *clrD,* encodes a molybdenum-containing specific chaperon protein. We have cloned and sequenced the homologous region (approx. 10,000 bps) encoding *clrABDC* and *cld* from *Pseudomonas chloritidismutans* (Ederer and Crawford, in preparation).

While the genes encoding the chlorate degradation pathway were found to be arranged in the same way in both chlorate degraders, i.e. *I. dechloratans* and *P. chloritidismutans,* the nucleotide sequence comparison revealed a different picture. The native chlorate reductase of *I. dechloratans* is a heterotrimeric protein with a molecular mass of approximately 160 kDa consisting the subunits ClrA (94 kDa), clrB (35.5 KDa), and ClrC (27 KDa) (Thorell et al., 2003). Further, the chlorate reductase of *I. dechloratans* was most closely related to selenate reductase (*serABDC*) of *Thauera selenatis* (Krafft et al., 2000) and the methyl sulfide hydrogenase *(ddhABDC)* of *Rhodovulum sulfidophilum* (McDevitt et al., 2002). These trimeric periplasimic proteins are molybdopterin containing, iron-sulfur /heme enzymes belong to the DSMO reductase type II family of proteins.

The chlorate reductase of *Pseudomonas chloritidismutans* was purified by Wolterink et al. (2003). These researchers reported that most of the chlorate reduction activity was located in the cytoplasm of the cell and deduced a cytoplasmic location for the enzyme. However, when we aligned the amino acid sequence of *P. chloritidismutans* ClrA with other DMSO type II reductase subunit A peptides (Table 5) and analyzed the sequences using signalP3.0 (Bendtsen et al., 2004), we found strong evidence for a signal peptide indicating that the peptide should be translocated into the periplasmic space.

The N-terminal sequence determined by Wolterink et al. (2003) (LN**M**LEPV…) differs by one amino acid from the one that we deduced by translating the nucleotide sequence (..LN↓**R**LEPV.., ↓indicates suggested cleavage site by SignalP3.0). Table 5 shows the N-terminal amino acid sequences of a number of additional DMSO reductase family II A subunits and their suggested signal peptide cleavage site. The chlorate reductase of *Pseudomonas chloritidismutans* as well as the perchlorate reductase of *Dechlorosoma sp*. KJ were found to encode a potential signal pepide that features a conserved twin-arginine motif indicative of Sec-independent, Tat-mediated protein translocation, thus suggesting a periplasmic target for the mature enzyme. The ClrC subunit of chlorate reductase is thought to be periplamic as well. When we analyzed the amino acid sequences for several C subunits, we found strong evidence for signal peptide cleavage sites (data not shown). SDS-PAGE and gel filtration experiments conducted by Wolterink et al. (2003) revealed potential molecular masses of 97, 38 and 34 kDa for ClrA, B, D, repectively and 167 kDa for the native enzyme. The molecular masses of the *P. chloritidismutans* translation products obtained from the loci cloned in our laboratory resulted in molecular weights of 86.4, 37.3, and 27.1 kDa for the processed peptides.

Table 5. Signal peptides preceding the PcrA subunit of the perchlorate and chlorate reductase. The peptides of all eight organisms are characterized by a conserved twin-arginine motif characteristic of Sec-independent, Tat-mediated protein translocation pathway (Wu et al., 2000). PcrA is assumed to form a cytosolic complex with PcrB (PcrB does not encode a signal peptide). The complex is then thought to be translocated into the periplasm, facilitated by the Signal peptide encoded by PcrA. This process seems to be conserved between Type II DMSO reductase family proteins. Perchlorate degraders, chlorate degraders.

Potential cleavage site

↓

Organism	**Signal peptide**	**Mature protein start**
I. dechloratans	MNSPDEHNG**RR**RFLQF-SAAALASAAASPS-LWA	--SKIQPIEDPLKDYPYRDW
P. chloritidismutans	MGMWKLK--**RR**DFLKGLSVTGAGVMLSGNVWGLN	--RLEPVGETLASEYPYRDW..
D. agitata	--MARLS--**RR**DFLK-ASAATLLGNSLTFKTLAA	-------TMDLSGAFRYSGW..
D. aromatica	--MVQMT--**RR**GFLL-ASGATLLGSSLS-RTLAAA	--------ADLSGAFEYSGW..
Dechlorosoma sp. KJ	--MVQMT--**RR**GFLL-ASGATLLGSSLSLRTLA	------AATDISGAFEYSGW..
R. sufidophilum	--MLRTT--**RR**TLMQGASLVGAGLFAAG-RGWA	LNRLEPIGDTLAEEYPYRDW..
S. stellata	MNMLRMK--**RR**EFLQTSSLAGAGVLAAN-KAWS	LNRLEPIGDTLAEEYPYRDW..

The enzyme perchlorate reductase is encoded by the *pcrABCD* operon and was first purified from strain GR-1 (Kengen et al., 1999). Strain GR-1 was later classified as *Azospira (Dechloromonas) oryzae* DSM11199 (Wolterink et al., 2005). The mature protein was determined to have a molecular mass of 420 kDa and a compostion of α3β3 with PcrA approximately 95 kDa and PcrB approx. 40 KDa. The periplasmic location of the enzyme was indicated by activity assays using whole cells and inhibition by methylviologen (MV) which cannot cross the cytosolic membrane. Perchlorate and chlorate degradation activity was shown to reside in the same enzyme (Kengen et al., 1999). The GR-1 enzyme was also capable of reducing bromate, iodate, and nitrate. Further, perchlorate reductases were identified in the genomes of *Dechloromonas agitata* and *Dechlorosoma aromatica* (Bender et al., 2005). It was well established that the ORFs encoded by *pcr*ABCD operon showed significant identity on the level of amino acid sequence with other microbial reductases, i.e. nitrate reductase, selenate reductase (*ser*ABDC), dimethyl sulfide dehydrogenase (*ddh*ABDC), ethylbenzene dehydrogenase (*ebdABCD*), and chlorate reductase (*clrABDC*), all of which are members of the type II DMSO reductase family (Bender et al.,

2005). The same was found for both, *Dechlorosoma sp. KJ* perchlorate reductase and *Pseudomonas chloritidismutans* chlorate reductase sequence.

The cloned *pcrA* gene from *Dechloromonas sp*. KJ encodes a structural protein of about 101 KDa. The protein encoded by *pcrB* was estimated to be 37kDa. SignalP3.0 analysis of the β-subunit did not reveal the presence of a signal peptide, hence it has been postulated previously that PcrA and PcrB form in the cytoplasm and translocate into periplasm mediated by the Tat-protein translocation mechanism where PcrB 'hitch-hikes' along. Further, the peptide encodes four cysteine-rich clusters for Fe-S center binding which are likely responsible for electron transfer to the molybdopterin-containing α–subunit of perchlorate reductase (Bender et al., 2005; Kengen et al., 1999).

The *pcrC* gene encodes the γ-subunit, a c-type cytochrome responsible for connecting the reductase to the membrane (Bender et al., 2005). PcrC is translated with a signal peptide for translocation, but is only thought to be only loosely bound to the PcrA/B complex. That is also the argument why Kengen et al. (1999) did not detect PcrC in the reductase complex. The mature peptide encoded by our clone is estimated to be 23.2 kDa.

The final 675-bp pcrD gene, based on sequence identity with SerD, DdhD, EbdD and NarJ, likely encodes a system-specific molybdenum chaperone protein (Bender et al., 2005). This finding is supported by the absolute requirement for molybdenum for active perchlorate reduction (Chaudhuri et al., 2002). The SerD, DdhD, and EbdD proteins are believed to be involved in assembly of the mature molybdenum-containing selenate reductase, dimethyl sulfide dehydrogenase, and ethylbenzene dehydrogenase, respectively, prior to periplasmic translocation via the Tat pathway. However, these proteins are not believed to be parts of the active enzymes (Krafft et al., 2000; McDevitt et al., 2002; Rabus et al., 2002). The *Dechlorosomas sp.* KJ peptide has a proposed mass of 25.2 kDa.

Fig. 7 shows the phylogeny resulting from the amino acid alignments for the four peptides. The sequences for PcrA and PcrB cluster together in agreement with the 16S data. We found that the *P. chloritidismutans* ClrA and the ClrB amino acid sequences are more similar to that of the non-chlorate degrading organisms, *R. sulfidophilum* and *S. stellata* than to either the other chlorate reductase or the perchlorate reductases. The sequences for the PcrC and D peptides of the perchlorate degraders cluster together, however the extent of the sequence conservation is much less.

3.3.3 The chlorite dismutases

The *cld* sequence of *P. chloritidismutans* was found to be identical to the *cld* gene of the chlorate degrader *P. stutzeri* DSM 13592. These loci also showed a surprising 99.2% identity to the *cld* genes of perchlorate degraders *Dechloromonas hortensis* DSM15637, and 92.1% identity to the *cld* gene of *Dechlorosoma sp*. KJ (cloned and sequenced by us, see below). Futher, the *cld* sequences of *Dechloromonas aromatica* was found to be 89.6% identical (CP000089.1) and a 68.5% identical to *D. agitata* (AY124796). The nucleotide sequence of *I. dechloratans* was determined to be 67.5% identical and seems to be more closely related to the *cld* sequence of the perchlorate degrader *D. agitata* (Fig. 7). As expected, the recently identified functional chlorite dismutase from *Candidatus Nitrospira defluvii* (Maixner et al., 2008) showed the least amount of sequence identity (Fig. 7).

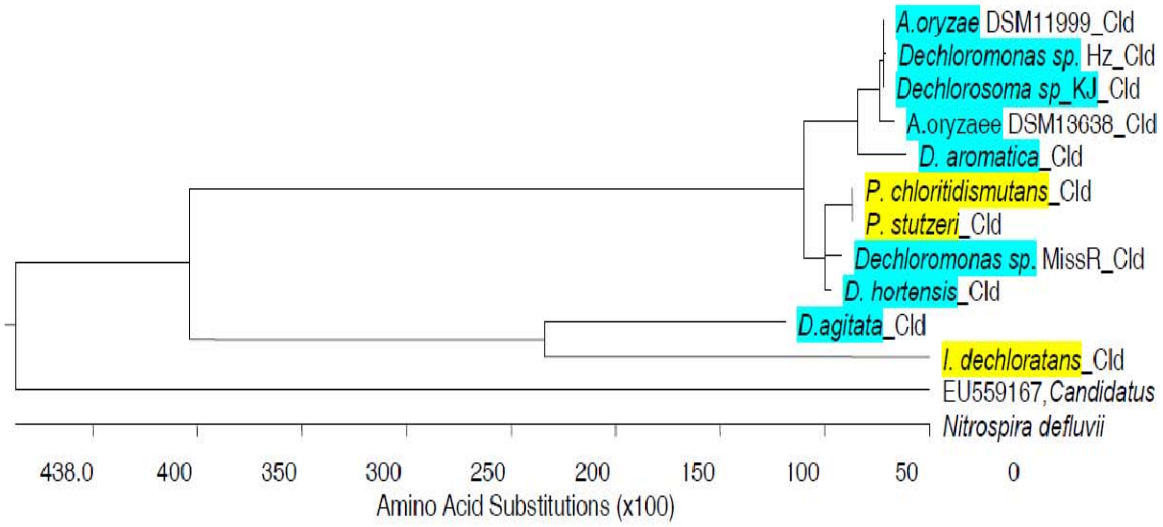

Figure 7: Alignment of the Cld amino acid sequences. The resulting phylogeny does not follow the 16S rRNA gene phylogeny. Further, the chlorite dismutase sequence of the chlorate degrading pseudomonads were found to be much more closely related to the sequences determined for the perchlorate degraders, rather than to the other chlorate degrader, *I. dechloratans*. The Cld amino acid sequence of the perchlorate degrader *D. agitata* was found to be more closely related that of I. dechloratans than to those of the other perchlorate degraders. Since not all genes of the chlorate and perchlorate degradation pathways show the same distribution of relatedness, they may have been acquired from different sources, even though they are located adjacent to each other on the chromosome in all bacteria studied thus far. As expected the Cld amino acid sequence derived from *Candidatus Nitrospira defluvii*, a nitrite-oxidizing bacterium, was determined to be the most diverse. This organism was recently found to have a functional chlorite dismutase without having the ability to degrade perchlorate or chlorate (Maixner et. al., 2008). Perchlorate degraders, chlorate degraders.

Chlorite dismutase is an essential enzyme in the degradation pathways of perchlorate and chlorate since chlorite is extremely cytotoxic. The enzyme is one of only two currently known enzymes that generate molecular oxygen (the other one being of course the water splitting complex in photosynthesis). Chlorite dismutase catalyzes the dismutation of chlorite to molecular oxygen and chloride. The enzyme was first purified from the Gram negative bacterium strain GR-1 by van Ginkel et al. (1996). These researchers determined that the enzyme was a homotetramer with a molecular mass of 140 g/mol. The vmax was measured as 2,200 U/mg protein and the Km = 170 µM. More detailed biochemical analyses for chlorite dismutase from *D. aromatica* strain RCB was conducted by Streit and Dubois (2008). One of the more notable characteristic of the heme containing chlorite dismutase was the irreversible inactivation of the enzyme after ~1.7 x 10E4 turn-overs per heme prosthetic group.

Mehboob et al. (2009) purified the chlorite dismutase from *Pseudomonas chloritidismutans* and determined that the enzyme had similar characteristics except that it was not inhibited by hydroxylamine whereas the enzyme isolated from *Azospira oryzae* GR-1 was. Further the *P. chloritidismutans,* enzyme was inhibited by azide whereas the GR-1 enzyme was not.

De Geus et al. (2009) solved the crystal structure of heme-based chlorite dismutase isolated from *Azospira oryzae* strain GR-1. These researchers found that the enzyme actually consists of six rather than four subunits. This discrepancy can be explained by earlier measurements were determined by gel filtration assays which apply the Stokes radius of globular proteins as a determinant for the molecular weight of proteins which lack precision.

The chlorite dismutase activity is an enzymatic activity shared by perchlorate and chlorate degraders. Surprisingly, when we aligned the nucleotide sequence of the *cld* genes from these organisms, we found that the *cld* sequences of the perchlorate degrader *D. agitata* and chlorate degrader *I. dechloratans* were more similar to each other than to the other organism in this study. In fact, the nucleotide sequence of the *cld* gene from the chlorate degrader *P. chloritidismutans* and *P. stutzeri* were nearly identical to the sequences determined for *Dechlorosoma sp.* KJ and other perchlorate degraders (Table 4, Figure 7).

3.3.4 Transformation of laboratory-based natural biofilms with free DNA encoding pcrABCD and cld

The *cld* gene and *pcrAB* operon fragments were cloned into the *Bam*HI site or into the *Sma*I/*Bam*HI site, respectively, in the same orientation as *gfp* and under the control of the *pnpt2* promoter of broad host range vector pSP1 (Figure 8) and transformed into *E. coli* DH5α.

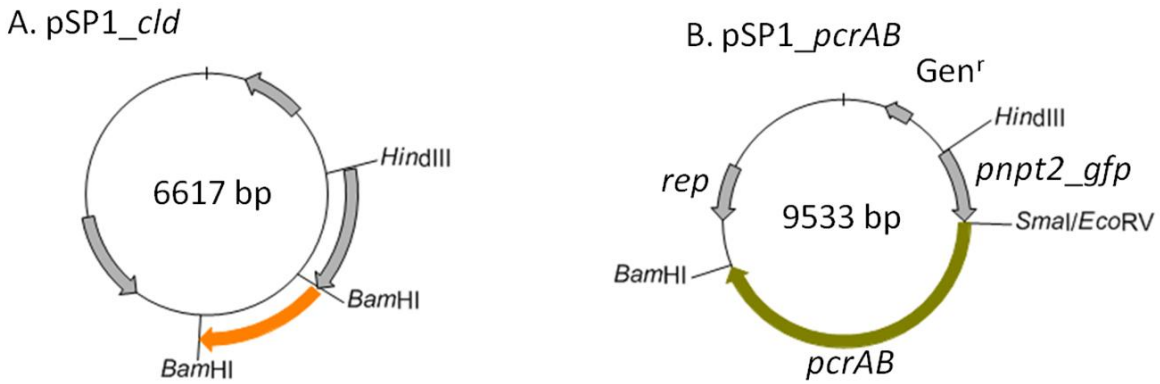

Figure 8: Cloning of the *cld* gene and *pcrAB* operon fragments into broad host range vector pSP1

3.3.4.1 Biofilm formation. Biofilms of *Acinetobacter baylyi*, a readily transformable Gram-negative bacterium, were grown on polystyrene coupons in 200 µl BHI. After incubation for 3-4 days at 25°C with shaking at100 rpm, the biofilms were ready for transformation.

3.3.4.2 Transformations. Biofilms were transformed by adding purified plasmid with cloned DNA (0.4-1.1 µg) (Figure 8) under selective conditions (gentamycin 10 mg/L), and incubated at 25°C and 100 rpm for 24 hrs. After 48 and 72 hrs additional aliquots of plasmid DNA were added: pSP1 vector only, pSP1_*cld* and pSP1_*pcrAB*. Transformation of the biofilm was monitored microscopically by detection of GFP fluorescence (ex599 nm/ em619 nm) with 400x magnification (Figure 9). The transformed biofilm was physically removed from the polystyrene coupons and aliquots were plated on BHI containing 10 mg/l Gentamycin to re-grow transformants and visualized by monitoring GFP fluorescence (Figure 10).

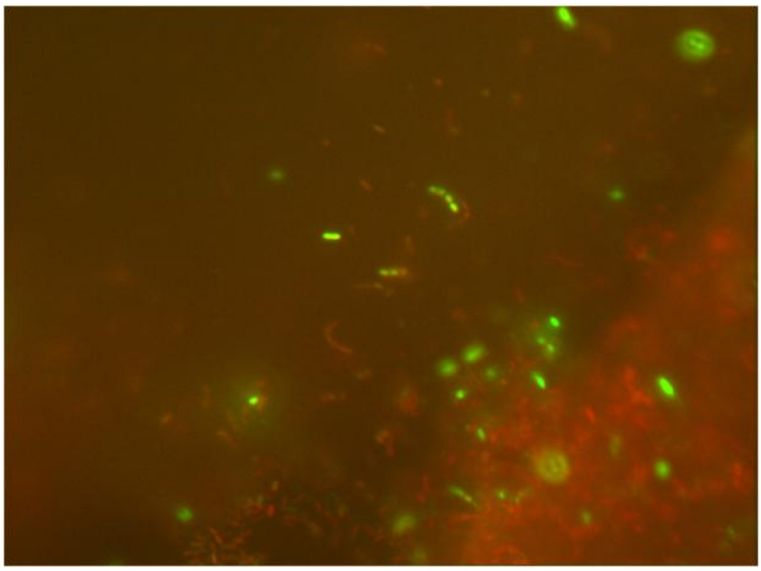

Figure 9: *Acinetobacter baylyi* biofilm transformed with pSP1_*cld* plasmid DNA. All cells (live and dead) in the biofilm were stained with SYTO 64 (red), the picture was then overlayed the same frame viewed through a 515-555 nm filter to visualize GFP (green).

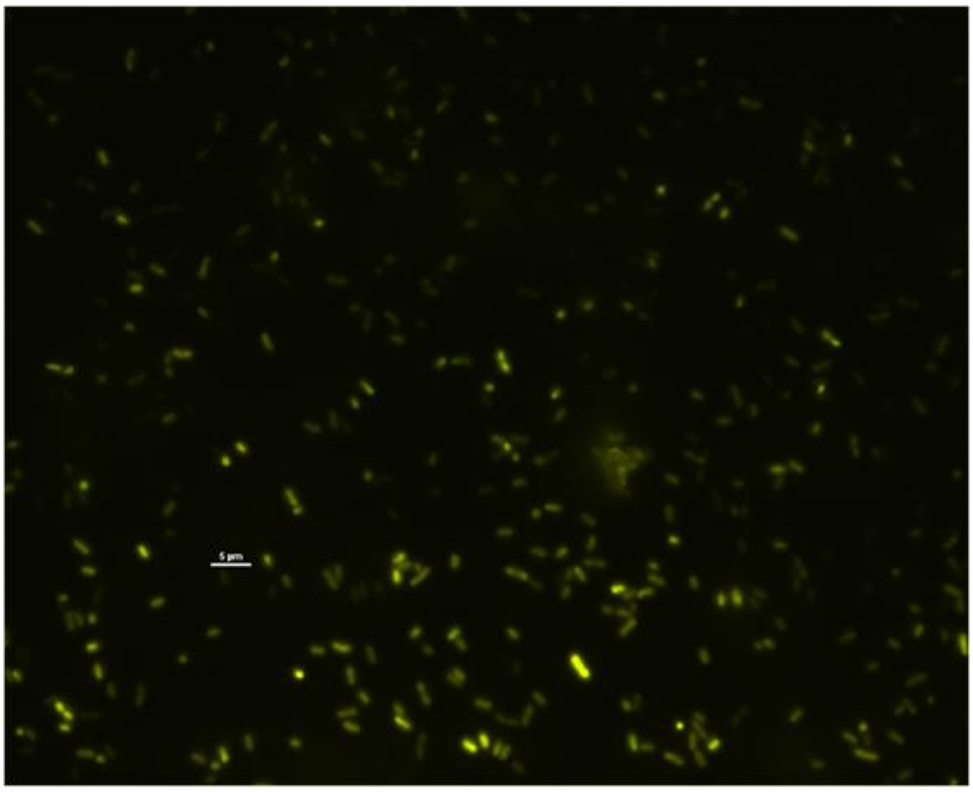

Figure 10. GFP expressing *Acinetobacter baylyi* transformed with pSP1_*cld* and subsequently grown in batch culture

3.3.4.3 Proteomic analysis. Actively growing cells were harvested by centrifugation, subjected to formic acid extraction at 95°C and subsequent trypsin digestion O/N at 37°C. The resulting peptides were separated using reverse phase liquid chromatography and analyzed by ESI-MS/MS. This work is continuing. We are in the process of confirming expression of *gfp*, *pcrAB,* and *cld* in *A. baylyi* biofilms by proteomic analysis

3.4 Conclusions

These results confirm previous reports (Achenbach et al., 2006) that horizontal gene transfer has been the mode of acquisition of chlorate and perchlorate degradation genes. The gene encoding the detoxifying chlorite dismutase activity (*cld*) may have arisen from a second, independent, probably later transfer event. At least for the *P. chloritidismutans* and *I. dechloratans,* this transfer could have been facilitated by a transposable element which was detected in the intervening sequence between the *pcr* and *cld* promoters. The chlorate reductase activity of *P. chloritidismutans* may have evolved from dimethylsulfide dehydrogenases like, ddh in *R. desulfidophilum* or nitrate reductase from *S. stellatum*, whereas the cld activity in this organism may have originated from organisms like *Dechlorosomas spp.*. The difference seen cannot be explained by sequence constraints in the chlorite dismutase, particularly since recently a rather diverse; however still catalytically active chlorite dismutase was discovered (Maixner et al., 2008). If the chlorate and perchlorate degradation pathways have evolved more than once and from different sources, why then are the two operons clustered? One plausible explanation might be that in order for an organism to grow using perchlorate and/or chlorate as electron acceptor the chlorite dismutase activity to detoxify chlorite is essential, hence these organisms may experience strong selective pressure to assure coordinated expression of the two operons.

One paper was published related to this section:

Smith, S. A., Benardini III, J. N., Strap, J. L., Crawford R. L. 2009. Diversity of aerobic and facultative alkalitolerant and halotolerant endospore formers in soil from the Alvord Basin, Oregon. Systematic and Applied Microbiology 32: 233–244

4.0 PROTEOMICS APPROACH TO MONITORING OF PERCHLORATE ENVIRONMENTAL FATE

4.1 Background

It is important to determine the exposure of a given environment to perchlorate contamination and characterize the current perchlorate degradation status in that environment as it relates to the potential effects on the health of humans and exposed ecosystems. There are several approaches to accomplish these objectives. The U. S. DOD and the U. S. EPA have recommended various chemical analytical methods using ion chromatography and mass spectrometry techniques for detection of perchlorate and its degradation products in environmental samples are desribed by the U.S. USEPA methods 6850 (HPLC/ESI/MS) and 6860 (IC/ESI/MS) for groundwater analysis. The methods can also be used for drinking water, wastewater and soil samples. USEPA methods 331.0 (Liquid Chromatography Electrospray Ionization Mass Spectrometry) and 332.0 (Ion Chromatography Electrospray Ionization Mass Spectrometry) have been recommended for drinking water analysis. In addition to chemical detection and quantification of perchlorate in the environment, it is also important to assess the changes in the microbial community structure and function as a result of contamination. Previous research has reported molecular methods that can be used to detect and quantify perchlorate reducing bacteria by targeting either of the two central enzymes (chlorite dismutase and perchlorate reductase) or their encoding genes that are involved in the perchlorate degradation pathway (Figure 1). O'Connor *et al*. (2002) and Bender *et al*. (2004) described a detection method for perchlorate reducers by using immunodetection and genetic probes targeting the chlorite dismutase enzyme and gene, respectively. A

quantitative detection method using real-time PCR (qPCR) and targeting the perchlorate reductase gene (*PcrA*) was reported by Nozawa-Inoue *et al*. (2008). The chlorite dismutase gene is known to be highly conserved among the perchlorate reducing bacteria; however, our recent research indicated that there may be microorganisms that have evolved differently and may have unique chlorite dismutase genes and enzyme sequences to perform within the same metabolic pathway (Bansal et al. 2011). Also, it is known that chlorite dismutase is found in chlorate reducing microorganisms and is not unique to perchlorate reducers. Use of perchlorate reductase as an indicator of perchlorate degradation alone can also lead to inaccurate results as the *pcr*/PR sequences have been found to be less conserved in perchlorate reducing microorganisms than *cld*/CD. Perchlorate reductase also is highly similar to other enzymes from the DMSO reductase family, such as nitrate reductase (Bansal et al., 2011). Therefore, to obtain reliable results we recommend concomitant monitoring of both the genes (*pcrABCD* and *cld*) and their encoded enzymes (chlorite dismutase and perchlorate reductase; see below) for both qualitative and quantitative analyses of the dynamics of perchlorate reducing bacterial systems.

Proteomics is a widely used approach to gain insight into the dynamics and metabolic state of biological systems. It provides qualitative and quantitative information about proteins being expressed at any one point in time under a set of specific microbial growth or environmental conditions. In our research we employed a liquid chromatography/mass spectrometry (LC/MS)-based method to qualitatively and quantitatively detect perchlorate pathway enzymes as functional biomarkers in pure bacterial cultures and environmental samples (Bansal et al., 2011). We used a novel time-course lysis method to extract the metaproteomes from our samples. This method is based on the fact that environmental samples contain a variety of microorganisms, some of which may be more resistant than others to chemical or enzymatic lysis. Therefore, a step-wise lysis allows detection of proteins from hard-to-lyse cells which can otherwise be masked by more abundant proteins released from easily lysed cells. Our work on proteomics indicated that signature peptides from enzymes like chlorite dismutase and perchlorate reductase are useful for monitoring expression of perchlorate reducing enzymes in pure and mixed cultures, including environmental samples. We have identified universal protein biomarkers (signature peptides) from chlorite dismutase and the perchlorate reductase *alpha* and *beta* subunits which are conserved in a majority of perchlorate reducing species and consortia (Bansal et al., 2011). The biomarkers can be measured quantitatively and thus readily used to indicate both the perchlorate contamination status and the dynamics of perchlorate degradation processes in microbial cultures or perchlorate contaminated environments. Such proteome-based methods rely on the presence and functionality of microbial enzymes and are more specific and physiologically meaningful than monitoring static genes using PCR or qPCR that are often used to determine the natural attenuation potential. For example, proteomics-based techniques are not affected by artifacts such as PCR bias due to primer specificity. They also are very sensitive (femtomolar detection levels for target peptides), reasonably rapid, and can be used for both pure bacterial cultures and environmental samples.

4.2 Proteomics Methods

We have developed proteomics-based methods to detect and quantify perchlorate/chlorate-degrading enzymes in environmental systems and/or pure cultures. A number of microorganisms are known that can grow by anaerobic reductive dissimilation of perchlorate and chlorate. Perchlorate reductase and chlorite dismutase are the two central enzymes involved in the reduction pathway. In our research we used qualitative and quantitative proteomics methods to obtain insight into the dynamics of microbial perchlorate and chlorate degradation in pure and mixed cultures. We employed a novel time-course cell lysis technique and a label-free LC-MS based mass spectrometry method that allowed simultaneous protein identification and quantification. Using these methods we are quantitatively assessing the changes in expression levels of the enzymes involved in perchlorate and chlorate degradation in several pure bacterial cultures (*Dechloromonas hortensis*, *Dechloromonas agitata*, *Dechlorosoma* sp. KJ, *Azospira oryzae*, *Pseudomonas chloritidismutans*, and *Pseudomonas stutzeri*) and environmental consortia grown under similar conditions. Samples were spiked with 50 fmoles of bovine serum albumin as an internal

standard. Quantification of the perchlorate reducing enzymes from different microbes can help to determine the most efficient pure strain(s) or natural consortia for use in perchlorate bioremediation processes. We used a proteomics-based approach to identify biomarkers that could be used to measure the exposure of the given environment to perchlorate contamination and determine the current perchlorate degradation dynamics of a site. Unique enzyme-derived peptide sequences were detected and quantified even at very low concentrations (10^{-15}M). We believe that this technique will be useful for developing protein biomarkers of bioremediation processes in general.

The approach used focused on quantification and detection of perchlorate and chlorate reductase and chlorite dismutase proteins in the pure bacterial cultures and mixed environmental consortia. Pure cultures used included *Dechlorosoma* sp. KJ, *Dechloromonas hortensis, Dechloromonas agitata*, *Azospira oryzae, Pseudomonas chloritidismutans* ASK-1, and *Pseudomonas stutzeri*. Mixed-culture-based environmental samples that were examined included an anaerobic sludge enrichment culture from a sewage treatment plant in the city of Moscow (Idaho, USA), a sample of a biomass-covered activated carbon matrix taken from a bioreactor used for treating perchlorate-contaminated drinking water (Bioreactor-enrichment) (Brown *et al*., 2007), and a waste water effluent sample obtained from a paper mill (Idaho, USA). All the cultures were grown in triplicate with the same concentrations of electron donor (acetate), electron acceptor (perchlorate or chlorate) and under similar growth conditions. Growth of the samples was measured by monitoring absorbance at 600nm (OD_{600}). A novel time-course lysis method developed previously in our lab was used to extract whole cell proteins from the samples (Bansal *et al*., 2009). The sample hydrolysate was analyzed in duplicate by LC/MS^E using a nano ACQUITY Ultra Performance Liquid Chromatograph (UPLC) and Q-Tof Premier mass spectrometer equipped with a nano-electrospray ionization source and (0.075 mm X 200mm) (I) BEH 130 C18 analytical column.

4.3 Results

4.3.1 Selection of protein biomarkers

The MS spectra obtained for the pure bacterial and environmental consortia samples were searched against the custom database containing amino acid sequences of known perchlorate metabolizing enzymes created in the ProteinLynx Global Server; Protein Identity 2.3 software package. The amino acid sequences (signature peptides) from chlorite dismutase, perchlorate reductase subunit A and subunit B from all 12 samples (*Dechlorosoma* sp. KJ, *Dechloromonas hortensis*, *Dechloromonas* MissR, *Dechloromonas agitata* CKB*, Dechlorospirillum anomalous, Azospira oryzae* (DSM-No. 11199), *Azospira oryzae* (DSM-No. 13638), Bior isolate, Crw isolate, Bior enrichment, Crw enrichment, and Pt enrichment culture) were compiled and analyzed. The amino acid sequences from CD, PcrA, and PcrB which were detected in a majority of samples were chosen to be used as biomarkers. The chlorite dismutase signature peptides GLETNSDFFFR, GTILTQPGVFGVFTMFK, HKDNVLVDLYLTR, and YVIVIPVKK were detected in all the samples except *Dechloromonas agitata* CKB. Thus, any or all of the chosen CD signature peptides can be used as biomarkers. In the case of the perchlorate reductase A subunit, amino acid sequence MDSTALYSDVVLPSAHWYEK was universally found in all samples except the Bior strain. Signature peptides DIAPMPNIPEYNPR, EQTDLSYLVR, and YIILWGSNPTQTR were found in every sample except *Dechloromonas agitata* CKB, Crw strain and the Pt enrichment culture. For the perchlorate reductase B subunit, peptide NVETAPGLGYPR was detected in all samples except the Crw strain. PcrB sequence IPLAQLEGLFGK was not found in *Dechloromonas agitata* CKB and the Pt enrichment culture. The signature peptides GKIPPMIDYGIPFEFDYAGR, IEQGVAPACVAQCVGR, IPPMIDYGIPFEFDYAGR, VALPLHPEFGTEPNVFYVPPVLGPR, and SAPNWDEDQGAGEYPNNSFFYLPR were detected in nine samples. The first four were absent in *Dechloromonas agitata* CKB, Crw strain and the Pt enrichment culture, while SAPNWDEDQGAGEYPNNSFFYLPR was not found in *Dechloromonas agitata* CKB, the Bior strain or the Pt enrichment culture. In the case of perchlorate reductase, a combination of any of the above signature peptides from PcrA and PcrB can be used as biomarkers. The signature peptides of chlorite

dismutase and perchlorate reductase in *Dechloromonas agitata* CKB were found to be different from the majority of other perchlorate degrading microbial cultures. Therefore, a different set of biomarkers can be used for this strain.

4.3.2 Validation and quantification of biomarkers

The presence of the selected CD and Pcr biomarkers was tested when a representative mixed-culture sample (Bior enrichment culture) was grown under different growth conditions: a) different time-points during the growth cycle; b) different concentrations of perchlorate; and c) in presence of an alternative/competing electron acceptor, nitrate. The chlorite dismutase and perchlorate reductase biomarkers detected under the aforesaid conditions were also quantified using the LC/MS^E mode of data acquisition on the Q-TOF mass spectrometer.

In the early-log phase of the growth cycle we detected all four of the selected CD signature biomarkers (GLETNSDFFFR, GTILTQPGVFGVFTMFK, HKDNVLVDLYLTR, and YVIVIPVKK) with the mean concentration of 284.9 ng/mg of total protein. We also detected all the selected PcrA (DIAPMPNIPEYNPR, EQTDLSYLVR, MDSTALYSDVVLPSAHWYEK, and YIILWGSNPTQTR) and PcrB (GKIPPMIDYGIPFEFDYAGR, IEQGVAPACVAQCVGR, IPLAQLEGLFGK, IPPMIDYGIPFEFDYAGR, NVETAPGLGYPR, SAPNWDEDQGAGEYPNNSFFYLPR, and VALPLHPEFGTEPNVFYVPPVLGPR) subunit biomarkers with the mean concentration of 1051 ng/mg and 267.8 ng/mg of the total protein, respectively. In the mid-log phase of the growth cycle the mean concentration for CD (271.3 ng/mg) and PcrB (224.5 ng/mg) peptides did not change much; however, the mean concentration for PcrA peptides (645.5 ng/mg) decreased ~38% as compared to the early-log phase. In the late-log phase the selected CD and PcrB biomarkers were detected with mean concentrations of 341.8 ng/mg and 243.2 ng/mg of total protein, respectively. Only one peptide for PcrA, YIILWGSNPTQTR, was detected with a mean concentration of 316.6 ng/mg, which is ~50% of the concentration detected at the mid-log phase.

The signature peptides from both CD and PcrA and PcrB were detected at several different perchlorate concentrations (0.1 mM, 0.5 mM, 1 mM, and 2.5 mM). The growth of the Bior enrichment culture on different perchlorate concentrations was determined by monitoring turbidity at 600 nm. At 0.1 mM perchlorate, we detected one peptide biomarker each of CD (GTILTQPGVFGVFTMFK) and PcrA (YIILWGSNPTQTR) with mean concentration of 641.2 ng/mg and 1202.9 ng/mg of total protein, respectively. The signature biomarker sequences from PcrB (GKIPPMIDYGIPFEFDYAGR, IPLAQLEGLFGK, IPPMIDYGIPFEFDYAGR, and VALPLHPEFGTEPNVFYVPPVLGPR) were also found with a mean concentration of 815.9 ng/mg of total protein. With a 0.5 mM perchlorate concentration, three biomarkers each of CD (GLETNSDFFFR, GTILTQPGVFGVFTMFK, and YVIVIPVKK) and PcrA (DIAPMPNIPEYNPR, EQTDLSYLVR, and YIILWGSNPTQTR) were identified. The concentration of CD and PcrA peptides was increased to 1236.5 ng/mg and 1459.3 ng/mg of total protein, respectively. For PcrB, we detected five peptide biomarkers (GKIPPMIDYGIPFEFDYAGR, IPLAQLEGLFGK, IPPMIDYGIPFEFDYAGR, SAPNWDEDQGAGEYPNNSFFYLPR, and VALPLHPEFGTEPNVFYVPPVLGPR) with a mean concentration of 774.3 ng/mg of total protein. At 1 mM perchlorate concentration, all of the above selected CD, PcrA and PcrB signature peptides were detected with mean concentration of 1353.2 ng/mg, 1645.1 ng/mg, and 683.3 ng/mg of total protein, respectively. Microbial cultures growing on perchlorate concentrations lower than 0.1 mM were not used for analysis as the cellular biomass was not sufficient for proteomic experiments.

Perchlorate and nitrate degradation by the Bior enrichment culture were monitored along with its growth. The decrease in the concentration of perchlorate and nitrate was measured using ESI-mass spectrometry, and the growth was determined by monitoring turbidity at 600 nm. It was observed that both perchlorate and nitrate were removed simultaneously by the Bior enrichment culture at all the different concentration

combinations of these electron acceptors used. Results indicated that the signature biomarkers from CD, PcrA and PcrB were detected even in the presence of nitrate. At 1.25 mM perchlorate and 3.75 mM nitrate (combination a) we detected CD (GLETNSDFFFR, GTILTQPGVFGVFTMFK, HKDNVLVDLYLTR, and YVIVIPVKK), PcrA (DIAPMPNIPEYNPR, EQTDLSYLVR, MDSTALYSDVVLPSAHWYEK, and YIILWGSNPTQTR) and PcrB (IEQGVAPACVAQCVGR, and NVETAPGLGYPR) biomarkers with mean concentrations of 410.4 ng/mg, 1213.1 ng/mg and 655.2 ng/mg of total protein, respectively. Similarly, CD, PcrA and PcrB biomarker peptides were obtained for 1.67 mM perchlorate and 3.33 mM nitrate (combination b) and 2.5 mM perchlorate and 2.5 mM nitrate (combination c) experimental sets. For the (b) combination set, the mean concentration for CD was found to be 725.2 ng/mg. PcrA was 1430.5 ng/mg and PcrB was 622.4 ng/mg of total protein. And for the (c) combination set, the mean concentration for CD was found to be 159.6 ng/mg. PcrA was 613.3 ng/mg and PcrB was 226.6 ng/mg of total protein. The results showed that the PcrB peptides IEQGVAPACVAQCVGR and NVETAPGLGYPR are unique to perchlorate reductase while other chosen signature peptides from PcrB (GKIPPMIDYGIPFEFDYAGR, IPLAQLEGLFGK, IPPMIDYGIPFEFDYAGR, SAPNWDEDQGAGEYPNNSFFYLPR, and VALPLHPEFGTEPNVFYVPPVLGPR) matched with *beta* subunit of respiratory nitrate reductase. At 5 mM nitrate only (control), as expected no chlorite dismutase and PcrB (IEQGVAPACVAQCVGR, and NVETAPGLGYPR) biomarkers were detected. However, one peptide from PcrA with negligible mean concentration was detected, which could be due to possible cross contamination of chromatography column from the previous sample. No similarity was found between the amino acid sequences from perchlorate reductase *beta* subunit and periplasmic nitrate reductase.

4.4 Conclusions

The results demonstrated that our LC/MS^n -based proteomic method is a promising tool for both identification and quantification of perchlorate and chlorate-reducing enzyme systems in pure and mixed cultures, including those derived from environmental samples. Signature peptides from chlorite dismutase, perchlorate reductase and chlorate reductase were detected in all the samples. Quantification results show that *Dechlorosoma sp.* KJ had the highest level of expression of the enzymes involved in perchlorate reduction among the microbial samples analyzed. For example, the concentration of chlorite dismutase in *Dechlorosoma sp.* KJ was found to be approximately 10 times and 4 times higher than in the environmental sample (BR-enrichment) and *D. hortensis,* respectively. Similarly, the concentrations of perchlorate reductase subunits A, B and D were also higher in *Dechlorosoma sp.* KJ. The concentrations of perchlorate reducing enzymes were found to be higher in early-log phase as compared to the late-log growth phase in *D. hortensis*. This may be because the enzymes (CD & PR) are destroyed or inactivated in the late growth phase. In the future we will use our proteomics-based technique to obtain quantitative proteomics data from other perchlorate and chlorate reducing microorganisms. The most efficient pure strain(s) or natural consortia in terms of the expression levels and perchlorate reducing enzyme stability might then be chosen for use in perchlorate bioremediation process development. Eventually we will also develop proteomic biomarkers that can be used to measure the exposure of a given environment to perchlorate contamination and determine the current perchlorate degradation status within that environment.

All technical details related to the qualitative and quantitative proteomics methods and data of this project is described in our two peer reviewed publications:

- Bansal, R., Deobald, L. A., Crawford, R. L., and Paszczynski, A. J. 2009. *Proteomic detection of proteins involved in perchlorate and chlorate metabolism*. Biodegradation. 20 (5): 603-620.
- Bansal, R., Crawford, R. L., and Paszczynski, A. J. 2011. *Peptide biomarkers as evidence of perchlorate contamination and biodegradation*. Applied and Environmental Microbiology (In press, Published on line first, November 29, 2010).

- Bansal, R., Deobald, L. A., Crawford, R. L., and Paszczynski, A. J. 2010. *Development of Protein Biomarkers as Evidence for Perchlorate Contamination and Degradation in Environmental Samples*. American Society for Microbiology 110th General Meeting, San Diego. Poster.
- Bansal, R., Deobald, L. A., Crawford, R. L., and Paszczynski, A. J. 2009. *Quantification of perchlorate and chlorate reducing enzymes using liquid chromatography and mass spectrometry (LC-MS/MS) based methods*. SERDP and ESTCP's Partners in Environmental Technology Technical Symposium & Workshop, Washington, D.C. Poster.
- Ederer, M., Bansal, R., Crawford, J., Paszczynski, A. J., Hess, T., and Crawford, R. L. 2008. *Comparison of genes involved in perchlorate degradation*. SERDP and ESTCP's Partners in Environmental Technology Technical Symposium & Workshop, Washington, D.C. Poster.
- Bansal, R., Ederer, M., Paszczynski, A. J., Thomas, H. F., and Crawford, R. L. 2008. *Cloning and Expression of the Genes Encoding the Perchlorate Reduction Pathway from Different Bacterial Species*. American Society for Microbiology 108th General Meeting, Boston. Poster.
- Paszczynski, A. J., Hess, T., Ederer, M., Crawford, R. L., Smith, S., and Bansal, R. 2007 *Engineered intrinsic bioremediation of ammonium perchlorate in groundwater*. SERDP and ESTCP's Partners in Environmental Technology Technical Symposium & Workshop, Washington, D.C. Poster.

5.0 GENERAL CONCLUSIONS

Perchlorate (ClO_4^-) is both a natural and anthropogenic chemical that can be found in natural environments, including extraterrestrial locations such as Mars. It is often found in groundwater as a result of industrial or military activities. Since perchlorate is toxic toward humans and other organisms, its presence in the environment, especially at elevated levels, is of concern. As detailed above, much is now known about perchlorate as a toxicant and regarding its presence in the environment. Also, much is known at the genetic and enzyme levels about the abilities of microbes to degrade perchlorate by using it as an electron acceptor in a process known as dissimilatory perchlorate reduction. This knowledge is leading to ways of monitoring the fate of perchlorate in the environment and of using biological processes for remediation of perchlorate-contaminated waters and soils. State-of-the-art tools of genomics and proteomics are being developed that will allow sensitive and real-time monitoring of processes that remove perchlorate from the environment, such as facilitated remediation and monitored natural attenuation.

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