POSSIBLE CONTRIBUTION OF λ PROPHAGE GENES TO LETHALITY OF *Shewanella Oneidensis* IN RESPONSE TO CR (VI) STRESS

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ABSTRACT

Shewanella oneidensis is a Gram – bacteria which can utilize various organic and metal compounds, like uranium, chromium, technetium, nitrates and various others. *S. oneidensis* genome has a λ phage integrated into it (prophage). The prophage can be induced to enter the lytic cycle on exposure to various stresses, like Cr (VI) treatment. Cr (VI) is a carcinogen and mainly enters the body via inhalation. *S. oneidensis* converts Cr (VI) to Cr (III), which is not a carcinogen. To deduce whether the prophage genes were induced in response to Cr (VI) stress, acute (90 min.) and chronic (24 hrs.) treatments were carried out. Expression levels of two structural phage genes, Prophage LambdaSo major tail protein V (PTP) and Prophage LambdaSo major capsid protein (PCP) were examined. It was found that PTP has a basal expression levels while PCP is induced in response to chronic Cr (VI) stress.

INTRODUCTION

Similar to a human breathing in oxygen and exhaling carbon dioxide, *Shewanella oneidensis* has the ability to 'inhale' certain metals and compounds and 'exhale' these metals in an altered state. For example, *S. oneidensis* can convert chromium (VI) dissolved in contaminated groundwater to Cr (III), which is incapable of dissolving in water, preventing Cr (VI) (Lovley et al. 1991) from spreading as the groundwater flows. This ability makes *Shewanella* an important factor in confining and cleaning up contaminated areas. It can grow naturally almost anywhere, and does not cause disease in humans or other organisms. These properties make it an ideal bacterium for bioremediation of contaminated environments, while reducing the risk of harmful side effects often associated with traditional methods of site cleanup. *S. oneidensis* is related to *Escherichia coli,* a bacterium well known to microbiologists. Tools and techniques developed over the past 30 years for *Escherichia* work with *S. oneidensis*.

S. oneidensis is classified as “Dissimilatory Metal Reducing Bacteria (DMRB)” because of its ability to utilize a variety of organic compounds and metals as terminal electron acceptors for respiration (Beliaev et al. 2005, Heidelberg et al. 2002). Compounds used include those of uranium, chromate, technetium, and nitrates which can be toxic to humans and other organisms.

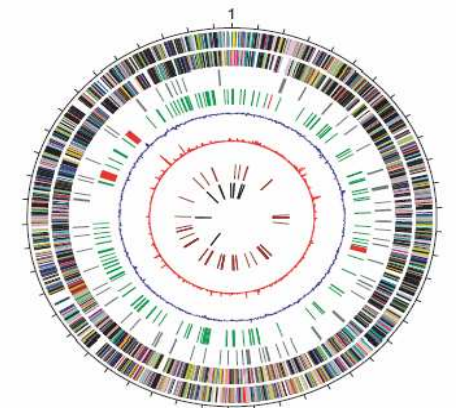

Fig 1: The S. Oneidensis genome (~ 5,00,000 bp) (Heidelberg et al 2002). 4th circle – RED – phage related genes.

The metal oxyanion chromate (CrO_4^{2-}) is a widespread environmental contaminant due to its prevalent use in industrial and defense applications such as tanning, electroplating, paint pigment manufacturing, stainless steel welding, and nuclear weapons production (James. 1996; Langard. 1980). The hexavalent form of chromium, Cr (VI), is highly soluble and toxic, with chronic exposure leading to mutagenesis and carcinogenesis (Mazdak et al. 2009). Cr (VI) induced apoptosis, for example, was demonstrated in human bronchoalveolar cells (Ruso et al. 2005, Liu & Dixon. 1996). The other most stable, common form of chromium, trivalent Cr (III), is not a carcinogen and also cannot cross the cell membrane (Plaper et al. 2002).

λ phage has two potential life cycles; lytic and lysogenic. Depending upon the pre-existing state of the bacteria it may enter into either of those two life cycles. If it goes into the lytic cycle it lyses the bacterial cell. On the other hand if it proceeds to the lysogenic cycle, it integrates into the bacterial chromosome and replicates along with it, without causing harm to the bacteria (Dodd et al. 2005). In this form λ phage is also

called a lysogenic prophage. *S. oneidensis* genome has a λ phage integrated into it. The classical way of inducing the lysogenic prophage is UV stress. Prophage also gets induced in response to other stresses such as Cr (VI) exposure. On induction of the lytic pathway, it forms the phage particles and lyses the bacteria (Friedman. 2005).

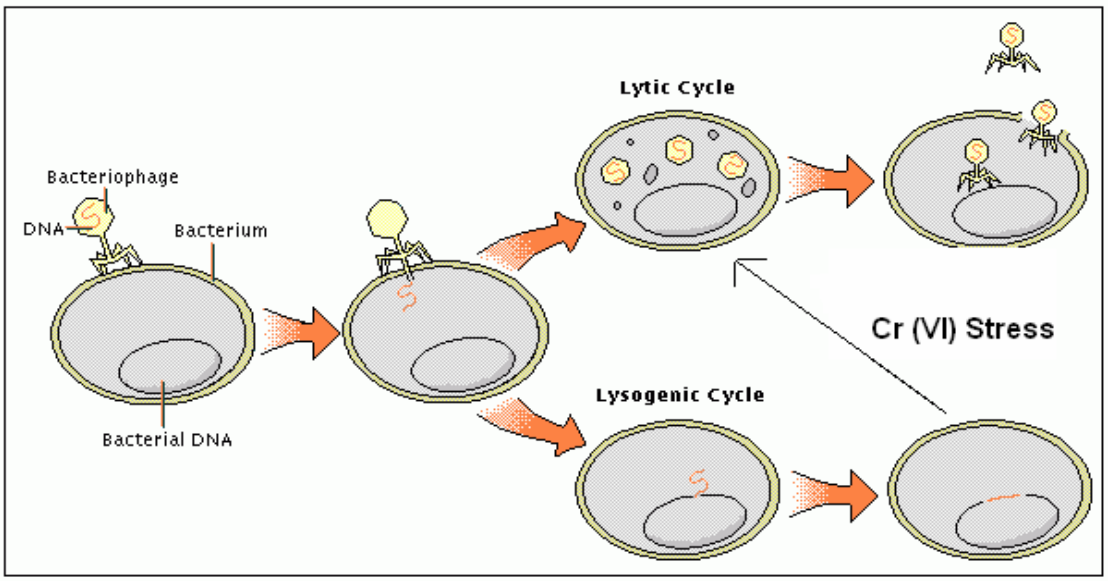

Fig 2: λ phage lytic vs. the lysogenic cycle and induction of lytic cycle by stress (like Cr (VI)).

The primary goal of this project was to gain insight into the changes in the mRNA expression patterns of λ prophage structural genes in response to either acute (90 min.) or chronic (24 hrs.) Cr (VI) (Chourey et al. – 2006) stress in *S. oneidensis* cells. Prophage LambdaSo major tail protein V (PTP) and Prophage LambdaSo major capsid protein (PCP) were chosen as genes of interest since they would be significantly upregulated if the lysogen goes into the lytic cycle.

MATERIALS AND METHODS

Bacterial growth and Cr (VI) treatment:

S. oneidensis was obtained from ATCC (ATCC® Number: BAA-1096™). The freeze-dried culture was grown in Luria Bertani (LB) Medium (pH 7.2) at 30.0°C with shaking at 250 rpm in an Excella E 25 incubator (New Brunswick Scientific, NJ) incubator. For the Cr (VI) treatment, a starter culture was prepared by inoculating LB medium with *S. oneidensis* and allowing the cells to grow overnight at the above mentioned conditions. An aliquot of the overnight culture was used to set the initial O.D. to ~0.15 at 600 nm using a SmartSpec 3000 (BioRad). The culture was then allowed to grow in 250 ml Erylene Meyer flasks containing 50 ml of LB medium at the above mentioned conditions till the O.D. reached ~1.5 at 600 nm (log phase) (Brown et al. 2005). Once the culture reached log phase, the medium was supplemented with K_2CrO_4 [Cr (VI)] (Sigma - Aldrich). Acute treatment (90 min) was carried out using 1mM of Cr (VI) and chronic treatment (24 hrs.) with 0.3mM of Cr (VI) as final concentration. Flask with no Cr (VI) was used as control.

mRNA isolation and DNase–I treatment:

Following the Cr (VI) treatments; the mRNA was stabilized using RNeasy® Protect Bacteria Reagent (QIAGEN) using the manufactures protocol. The total RNA was extracted using RNeasy® Plus Mini Kit (QIAGEN), according to the manufacturer's instructions. To protect against possible DNA contamination, the isolated mRNA samples were treated with DNase – I (Invitrogen) using the manufactures instruction.

RT-PCR and PCR:

RT-PCR was performed using QIAGEN® OneStep RT-PCR Kit following the manufacture's protocol. 30ng RNA was added to each RT-PCR reaction. The primers designed for the control gene and genes of interest are as follows. Gap–A1 (loading control); forward - 5'-GTTATCCTGTGACGGCGAAT-3', reverse, 5'-TCTTCTTTCACGGGAGCAGT-3'. Prophage LambdaSo major tail protein V (PTP); forward: 5'-CAGCAGGCACTAACACAGGA-3', reverse: 5'-ACATTGATCCACGTATCGCA -3' and Prophage LambdaSo major capsid protein (PCP); forward: 5'- TGCACGGATACTGACCATGT-3', reverse: 5'-CTGAGAGCAACACCATCGAA -3'. Their respective product length was 208 bp, 264 bp and 665 bp.

Amplification conditions for PTP and PCP reactions were as follows: 50°C for 30min; 95°C for 15min; 40 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 30s; 72°C for 10min. The amplification conditions for Gap-A1 were the same except that 35 cycles were used. The PCR products were separated on a 1% agarose gel and visualized by ethidium bromide in a UV trans-illuminator (BioRad) using the Quantity One software (BioRad). All experiments were repeated in triplicates for statistical significance.

PCR was performed with PCP and PTP specific primers using the GoTaq PCR Kit (Promega). Amplification conditions were similar to the ones used for RT-PCR except that the initial RT part was not performed. Visualization conditions were also same as those used for RT-PCR. To verify whether the PCR reaction was working, DNA was used to perform PCR control.

RT-PCR product Purification and Sequencing:

The RT-PCR products were purified using QIAquick PCR Purification Kit. The sequenced products were BLASTed.

RESULTS:

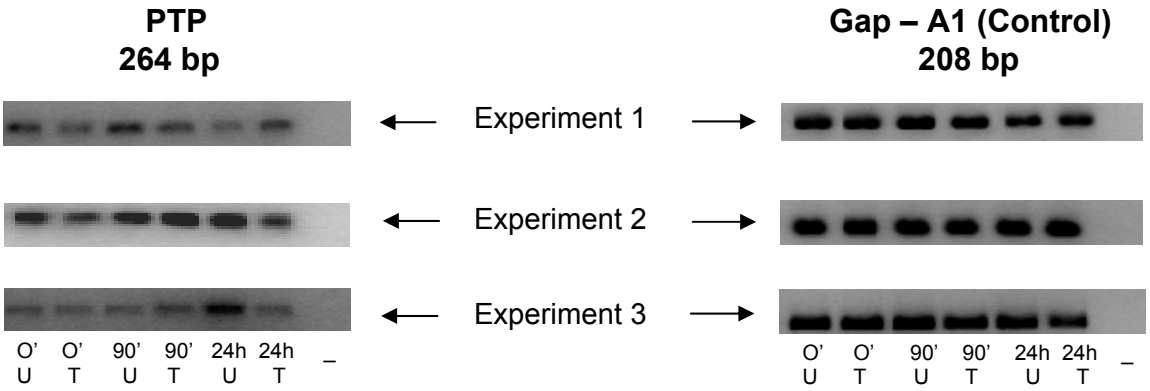

Fig 3: Gel pictures of RT-PCR products for PTP. Lanes 1, 3 and 5 are untreated (U) and lanes 2, 4 and 6 are treated (T) with Cr (VI) for different times. Lane 7 is negative control. Experiment was repeated 3 times for statistical significance and represented as Experiment 1, 2 and 3. Gap-A1 was used as loading control.

Results suggest that the Cr (VI) treatment does not increase the expression level of PTP. Control (untreated) sample show that there is a basal level of PTP transcript present. The growth phase of the bacteria (log vs. stationary) has no effect on the expression level of PTP.

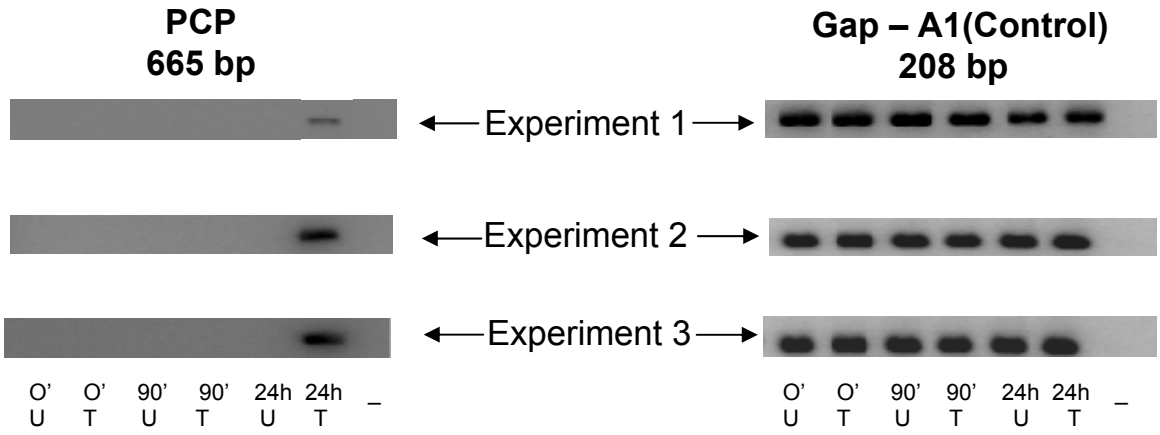

Fig 4: Gel pictures of RT-PCR products for PCP. Lanes 1, 3 and 5 are untreated (U) and lanes 2, 4 and 6 are treated (T) with Cr (VI) for different times. Lane 7 is negative control. Experiment was repeated 3 times for statistical significance and represented as Experiment 1, 2 and 3. Gap-A1 was used as loading control.

There is an induction of PCP after chronic (24 h) but not acute (90 min) Cr (VI) treatment. The growth phase does not seem to be the deciding factor since 24 hrs. untreated sample shows no PCP band.

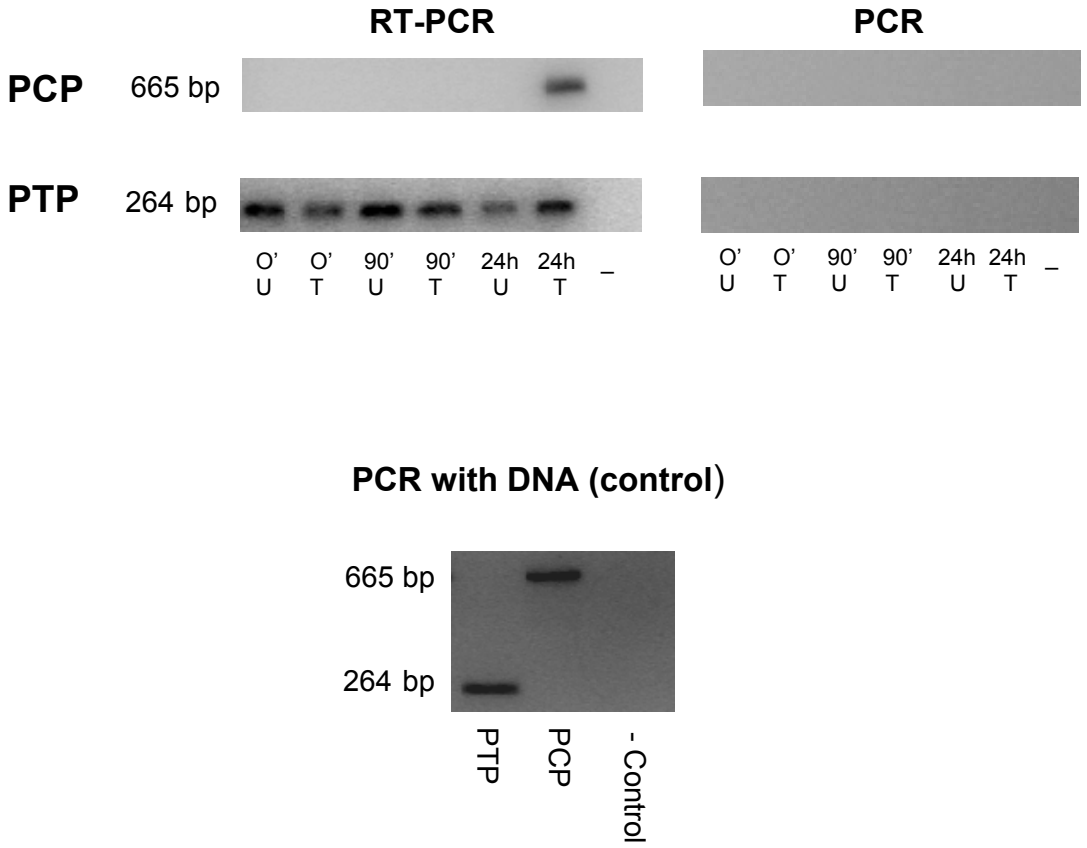

Fig 5: Gel pictures of RT-PCR vs. PCR products of Prophage LambdaSo major tail protein V (PTP) and Prophage LambdaSo major capsid protein (PCP). PCR control was performed utilizing DNA.

The above results indicate that the mRNA isolation and the subsequent DNase-I treatment was successfully performed and DNA contamination, if any, was removed. Judging by the size of the fragments obtained, the identity of RT-PCR products was suspected to be the ones of interest. The identity of the PCR products was confirmed by BLASTing their sequencing results.

NO.	SEQUENCE	BLAST RESULTS	% IDENTITY
1	ACGATCCGGCTATGCATCCCATAGTGAC CGCCGCATCTTGTACCACCAATTGCCTT GCGCCTGTGGTGAAAGTGATCC	Glyceraldehyde-3-phosphate dehydrogenase (Gap–A1)	100
2	AAAGTAAAGTTTTTAGTTGTAACCGTTG GCGCGGTGCCCACTGCATCACTAAAACA ATTGCCCATTTAATGGTCGTG	Prophage LambdaSo major tail protein V (PTP)	100
3	CTTCGATATCCATGCGGTCATAGATCTG TGCGCCCATTTGGAATGCGCCGACTAAG AACTCGTTTTGCA	Prophage LambdaSo major capsid protein (PCP)	100

Table 1: Sequenced RT-PCR products and their respective BLAST results. The identity of each product is given in the percentage (%) format.

DISCUSSION and CONCLUSIONS:

There is a basal expression level of PTP transcript but not PCP transcript. Cr (VI) treatment, neither acute (1mM, 90 min) nor chronic (0.3mM, 24 hrs.), seems to have an affect on the expression level of PTP. On the other hand PCP seems to be induced after chronic but not acute Cr (VI) treatment. The bacterial culture enters the stationary phase by ~24 hrs. (Brown et al. 2006). There is no detectable difference in the expression levels of PTP in response to different growth phases of the bacteria. PCP is

induced only due to Cr (VI) stress and not in response to the bacteria entering the stationary phase.

The phage particle cannot lyse the bacteria without the capsid protein (PCP) (Kaiser & Masuda. 1973). The tail protein alone is not enough to induce the prophage to enter the lytic cycle (Lederberg & Lederberg. 1953, Ptashne. 2005, Hendrix. 1999). Hence, on induction of the capsid protein (PCP), it could potentially combine with the tail protein (PTP) and the other phage proteins to induce lysis of the bacterial cell. To confirm this hypothesis, the respective proteins should be isolated and their levels quantified using Western blots. Also, the expression levels of various other phage genes should be quantified using semi-quantitative and quantitative RT-PCR followed by quantification of those proteins using Western blots.

If it is indeed confirmed, using the above approaches, that the prophage goes into the lytic cycle in response to chronic Cr (VI) stress then the next approach should be making mutants without the prophage gene/s. A mutant *S. oneidensis* without the prophage genes might be able to withstand Cr (VI) stress for a longer duration. This potentially reduces the amount of time and money required to clean up contamination sites. It is possible that *S. oneidensis* will respond in a similar manner to uranium stress. US - Department of Energy (DOE) is very interested in cleaning up sites contaminated with Uranium. The mutant S. *oneidensis* could potentially have applications in that area too.

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