Northern Illinois University

Assessment of Arsenate Resistance

in

Haloalkaliphilic Archaea

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by

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ABSTRACT:

Haloalkaliphilic Archaea, *Natronococcus occultus* is one of a few organisms which survive in soda lakes. It grows optimally in 3.4 M NaCl and pH 9. It is suspected that this organism has resistances to heavy metals because its distant relative, haloneutrophilic Archaea, had shown resistance to several different heavy metals. Although no haloneutrophilic Archaea had shown resistance to arsenate, several eubacteria had been tested positive for arsenate resistance. *Natronococcus occultus* was tested for arsenate resistance, partially because arsenate stays in solution even in 3.4 M NaCl and at pH9. The uptake rates of phosphate and arsenate are compared to investigate the mechanism of the resistance, because arsenate is an analog of phosphate and is known to be transported into the cell by a phosphate transport system.

CONTENTS

Introduction

Although the saline oceans dominate the terran ecosphere, few organisms inhabit extremely saline niches. There niches occur within add or semiarid regions, where prevailing conditions favor the concentration of salts by evaporation. Hypersaline environments differ in the diversity of life they can support, depending on the presence or absence of anions and cations in addition to the predominant sodium and chloride ions. The most extreme of the hypersaline environments are the desert soda lakes, formed in areas where dissolved carbonates elevate the pH of these salt lakes to a pH above 9.

Whereas salt lakes, such as the Great Salt Lake in northern Utah, can be highly productive ecosystem, highly alkaline salt lakes (soda lakes) are among the most sparse of ecosystems. A wide range of eukaryotes including the photosynthetic alga *Dunaliella*, brine shrimp, and brine flies inhabit the Great Salt Lake; in contrast, no eukaryotes inhabit soda lakes. Only three genera of organisms have been cultured from soda lakes: the anaerobic purple sulfur bacterium, *Ectothiorhodospira*, and two haloalkaliphilic Archaea, *Natronobacterium* and *Natronococcus* species.

The haloalkaliphiles *Natronobacterium* and *Natronococcus* are distantly related to the photosynthetic haloneutrophiles, including *Halobacterium* and *Haloferax* species, which synthesize bacteriorhodopsin, a protein that catalyzes photophosphorylation coupled with proton transfer across their purple membrane.

Although the haloalkaliphiles are not known to catalyze photophosphorylation and appear to be heterotrophs, they offer several advantages for study relative to the haloneutrophiles. Like the haloneutrophiles, they grow rapidly on plates under laboratory conditions. Unlike the haloneutrophiles, at least the haloalkaliphilic species, *Natronococcus occultus*, does not lyse upon exposure to distilled water (Kobayashi et al., 1992), suggesting that this species may be manipulated by electroporation, among the most versatile methods for genetic transfer. This species is also sensitive to the antibiotics tetracycline and erythromycin (Kobayashi et al., 1992), antibiotics which can be used for negative selection in eubacterial hosts. This finding raises the possibility that a new generation of shuttle vectors for the genetic analysis of the haloalkaliphilic genome may be propagated in both archael and eubacterial hosts. Thus, the haloalkaliphiles offer significant advantages as hosts for bioengineering relative to their less hardy haloneutrophilic sisters.

Many strains of haloneutrophiles have shown resistance to several heavy metals such as Pb, Cr, and Cu; Indeed some strains have resistance to as many as eight different heavy metals: As, Cd, Co, Cr, Cu, Ni, Pb, and Zn (Nieto et al., 1987).

Arsenic is one of many toxic metal ions. Its water soluble forms are As^{3+} (arsenite), and As^{5+} (arsenate). Arsenate is taken up by cells through a phosphate transport system because of its comparable pKa values to that of phosphate (Cervantes et al., 1994). Arsenite is taken up by cells passively or by organic compound carrier because it is mostly unionized at neutral pH (Cervantes et al., 1994). Arsenic resistance is found in a wide range of microorganisms including Gram-negative and Gram-positive eubacteria where resistance is determined by a chromosomal or plasmid borne gene (Silver, 1994; Cervantes et al., 1994). Chromosomally- determined resistances can encode for: reduced uptake due to a defective non-specific phosphate transport system (phosphate specific transport system must be present in order to take up phosphate which is necessary for growth), oxidation of As^{3+} to less toxic As^{5+} by arsenite oxidase, an efflux system, an intracellular metabolic pathway, or combination of these. Plasmid- mediated resistance results in reduced uptake of arsenic resulting from an ATP dependent efflux system. This system has efflux pumps for As^{3+} and an arsenate reductase which convert As^{5+} to As^{3+}.

In general, little is known about heavy metal resistance in Archaea, and it was the purpose of this study to investigate the resistance of *Natronococcus occultus* to arsenate.

Methods

Assessment of Arsenate Resistance: *Natronococcus occultus* was grown in Defined Haloalkaliphile (DH) medium containing various concentrations of As^{5+} (0 mM, 20 mM, and 80 mM). DH medium consisted of (gram per liter): 200 g NaCl, 1 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 5 g Casamino acids, and 18 g Na_2CO_3. One milliliter of a trace element solution was also added. Growth was monitored spectrophotometrically at 540 nm, twice a day for 6 days.

Assessment of Arsenate and Phosphate Transport: *Natronococcus occultus* was grown to mid-log phase and harvested by centrifugation (5,000 x g, 15 min., 4 °C). The cell pellet was washed twice with 3.4 M NaCl and stored on ice. To assess uptake , either radiolabeled phosphate (^{32}P) or arsenate (^{73}As) was added back to cells resuspended in 3.4 M NaCl. At specific intervals, 100 µl of the assay mixture was removed, filtered through a 0.45 µM Millipore nylon filter, and washed twice with 5 ml of 3.4 M NaCl (to remove exogenous rediolabel). The filter was placed in a glass Scintillation vial containing 5 ml of Scintillation cocktail. Each vial was counted on the appropriate channel using a Beckman Scintillation counter.

Results & Discussion

Little is known about the biochemistry or physiology of the haloalkaliphilic Archaea. It was the purpose of this study to determine if *Natronococcus occultus* was resistant to the phosphate analog, arsenate. Assessment of growth spectrophotometrically indicated that *Natronococcus occultus* was able to grow in the presence of high concentration of As^{5+} (Fig. 1.). Not only was this surprising, but also unusual since the haloneutrophilic Archaea show a marked sensitivity to arsenate (Nieto et al., 1987). In addition, cells grown in the presence of either 20 or 80 mM As^{5+} showed the same doubling time (14 h) as cells grown without A^{5+}.

Further experiments demonstrated that *Natronococcus occultus* could grow even in the presence of 300 mM As^{5+}! These levels are not only extremely high, but have only been seen in chemostat-grown cultures of *Thiobacillus ferroxidans* (Simon Silver, personal communication). At this point, the mechanism of As^{5+} resistance in *Natronococcus occultus* was unknown. To determine if a cells can transport a particular compound, a radiolabeled version of that compound can be used. Briefly, cells are incubated with the radiolabeled substrate. After a set amount of time, cells are filtered through the membrane and are immediately washed with an appropriate buffer to rinse off any exogenous radiolabel. Enumeration of the radiolabel will then be a direct representation of the compound transported by the cell.

Natronococcus occultus rapidly uptakes phosphate, however, arsenate does not appear to be readily transported (Fig. 2.). It is possible, however that cells may be rapidly transporting and effluxing arsenate. Further studies are underway to determine the exact mechanism of resistance.

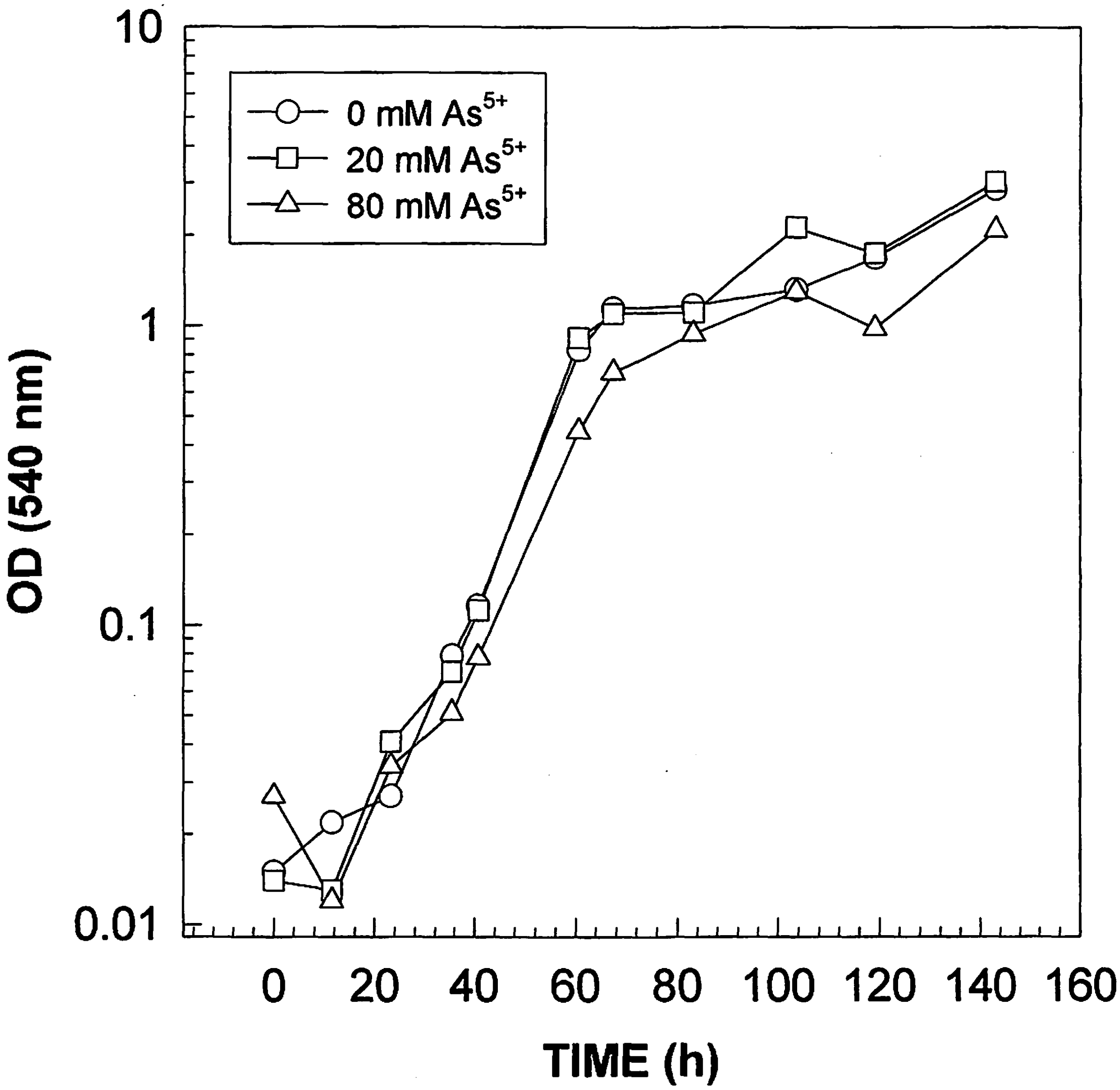

Fig. 1. Growth of *Natronococcus occultus* in the presence of varying concentrations of arsenate

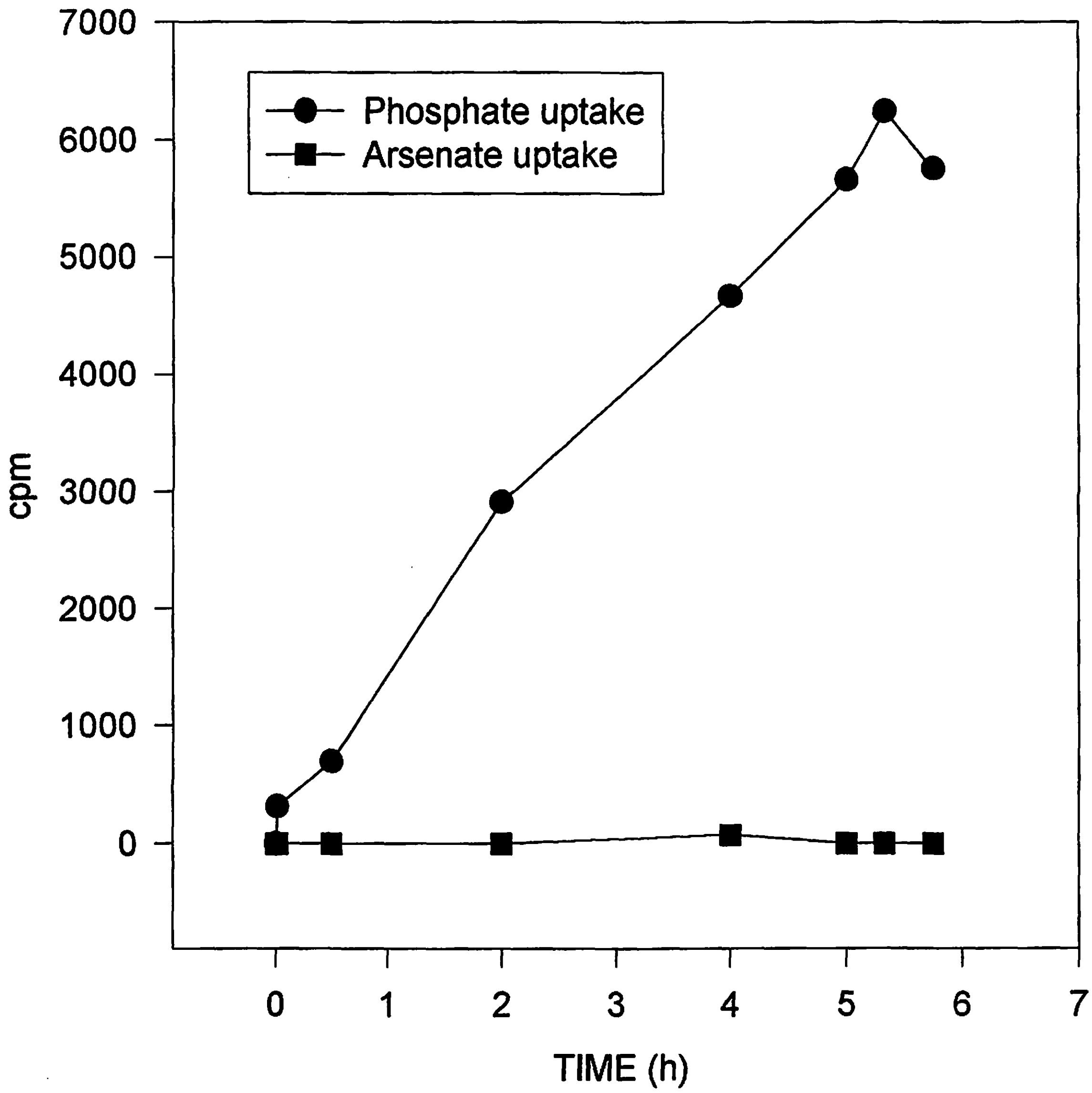

Fig. 2. Phosphate and arsenate transport by *Nc. occultus*

Conclusions

Natronococcus occultus is capable of growing in the presence of high concentration of arsenate. Cells rapidly transport phosphate, but not arsenate, a phosphate analog. It is possible that cells either do not transport arsenate efficiently or rapidly uptake and efflux this toxic heavy metal.

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