



Isolation and Identification of Bacteria from Mercury-Contaminated Soils

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INTRODUCTION

Anthropogenic activity has resulted in localized ecosystem contamination with metals that serve no known biological purpose such as mercury, arsenic, cadmium, and lead (1). Mercury is a heavy metal and known neurotoxin that is a byproduct of coal and petroleum combustion, and can enter the waste stream because it is used in a variety of manufactured items such as thermosensors and fluorescent lights (7, 8). In nature, mercuric compounds accumulate and magnify when an ecosystem is unable to process or remove them (2). This study focuses on the acquisition and identification of isolates from bacterial communities able to survive such contaminated conditions. The samples were taken from Barnum Court, an abandoned hat factory in Danbury, Connecticut. This mercury-contaminated site served as host to a set of genetically modified trees planted as part of a phytoremediation study. Samples were taken from soil around the roots of genetically modified trees (the rhizosphere) and from control plots with no trees. In order to identify the 'most numerous culturable heterotroph' (MNCH) from each sample, the soil was diluted and bacteria cultivated using standard enrichment and isolation techniques, and according to the design of successful bioprospector Ralph Tanner (5). Five types of agar were used, alone and with the addition of mercury. Colony forming units were counted, and isolates taken from the highest successful dilutions of each sample were identified using the Biolog system in addition to genetic (PCR) 16S rDNA identification.

MATERIALS AND METHODS

1. Soil sampling and extraction:

Soil samples were obtained in the field and sieved (8mm). 5g aliquots were diluted into 50ml sterile 0.85% sodium chloride (saline) and shaken at 250rpm for 20 minutes. Sample was then centrifuged at 1000 for 3 minutes and supernatant was either plated directly or used for further dilution.

2. Dilution and Plating:

One milliliter of extract was used directly and further diluted into sterile 0.85% saline from 101 to 108 in 10-fold increments. An aliquot of 0.1ml of each dilution was then streaked onto plates with and without added mercuric chloride at 150uM concentration, of following types: Nutrient Agar (NA), Actinomycete Agar (AC), Pseudomonas Isolation Agar (PA), R2 Agar (R2A), and Starch Agar (SA). Starting sample for SA was first subjected to heat treatment of 85°C for 10 minutes to select for endospore-forming cells of *Bacillus* species. Plates were examined after 8 day incubation at 25°C, and colony forming units (cfu) recorded. Isolates were selected at highest dilutions for further isolation and identification.

3. Identification:

Ultimately, 18 isolates representing a cross-section of the sample zones were chosen for identification. DNA was extracted using the bead beating protocol of PowerSoil DNA Extraction Kit (MoBio, Inc.). 16S rDNA was amplified (63F-1406R) and sequenced in order to identify the isolates genetically.

4. Isolate A3P-4

Particular attention was paid to one microbe that created a highly unusual crystalline growth pattern on PA from the rhizosphere of zone A3. In order to investigate the organism, it was restreaked not only onto PA, but also onto NA, AC, R2A and PA with mercury. After a week of incubation, mucoid growth on all plates without Hg had occurred, and again on PA, crystals formed. Heightened interest prompted a genetic identification, using the above mentioned PCR and Biolog processes, as well as a chemical characterization of the crystals, performed by colleagues at Keene State College in New Hampshire (Dr. Denise Junge).

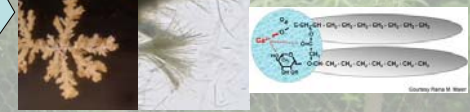
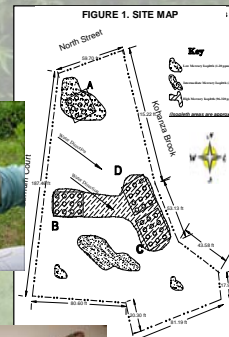
RESULTS, DISCUSSION

The non-selective agars such as Nutrient Agar (designed for organisms that develop quickly and vigorously) and R2 agar (a low-nutrient agar for slower growing cells) yielded the highest colony forming units (cfu) at 6.25 x 10⁷ and 1.49 x 10⁸ respectively. The MNCH isolates identified from these agars were obtained at extremely low dilutions on both mercury- and non-mercury-containing plates in relation to the other media (A2R2-2, C10/R2-2, D7/NA-6, and D8/R2-1). More specific agars included: Pseudomonas Isolation Agar to promote the growth of *Pseudomonas* species; Actinomycete Agar to enrich for *Actinomycetes* and *Streptomyces*; and Starch agar with heat treatment to enrich for *Bacillus* species, the spore former. The metabolic flexibility of these bacteria allows them to survive in stressful conditions such as contaminated environments, while aiding in the decomposition of organic matter. As can be expected, the plates with 150uM mercury had significantly less growth, with a reduced variety of colony types. Sample zones A1, A2, and A3 (from the very low mercury zone at Barnum Court) yielded very few colonies on mercury agar, and only at low dilution. Since total bacterial yield on 5 agar types was 1.69 x 10⁹ while total for plates containing mercury was 3.26 x 10² cfu, the disparity reaffirms the lethal implications of anthropological pollution on soil ecology.

Two isolates proved to be particularly interesting in this study. The first produced unusual bundles of needle-like crystals on P agar. Investigation of the chemical makeup of these crystals and the organism's metabolic characteristics revealed it to be *Pseudomonas chlororaphis*, a fluorescent pseudomonad similar to *Pseudomonas aeruginosa* that possesses qualities needed for survival in contaminated soils (3,4). The second was a rhamnolipid producing *Pseudomonad* (see separate section).

In the late 1970s, a group of scientists at Rutgers University were intrigued by curious crystalline growth, "bright green needle-shaped crystals several millimeters in size," in the cultivation medium of a strain of *Pseudomonas aeruginosa* (4). *P. aeruginosa* is a fluorescent pseudomonad that typically produces the phenazine pigment pyocyanine. Upon dissolving the crystals and allowing them to re-form, new yellow crystalline material emerged. Employing specific melting point comparisons and other analyses, the scientists identified the green crystals as chlororaphin and the yellow crystals as oxychlororaphin. Although Kanner *et al.* admitted that other strains of *P. aeruginosa* produce chlororaphin, this growth pattern appeared to be unique, either to the isolate or the cultivation conditions.

Despite the fact that we did not find any completely new and undiscovered microbes at Barnum Court, the isolates we did obtain may yet prove to be useful in understanding the cellular processes of heavy metal metabolism and remediation in the environment.



RHAMNOLIPIDS: COMMERCIAL POTENTIAL

Rhamnolipids are biosurfactant compounds made up of rhamnose sugar molecules and β -hydroxy fatty acids, often produced by the genus *Pseudomonas* (6). Their detergent-like characteristics imply great industrial potential: for example, their ability to reduce the surface tension of water may assist in the remediation of oil spills. These naturally existing compounds demonstrate antibacterial and antifungal properties needed by the health care and food-processing industries as well (9). The vast majority of surfactant products are petroleum-based chemicals, however because of their environmental compatibility, biodegradability, and diverse nature, rhamnolipids and other natural microbial surfactants have benefits over their synthetic counterparts.

The most well known producer of rhamnolipid is *P. aeruginosa*. The organism is capable of using a wide range of carbon sources for rhamnolipid production, however the sources with the highest yield are vegetable-based oils, such as soybean, corn, canola and olive oils (6). Unfortunately, *P. aeruginosa* is a human pathogen, and it would be cost prohibitive to maintain the necessary safety conditions to produce the compound commercially.

In 2004, Gunther *et al.* set about exploring the rhamnolipid-yielding potential of *P. chlororaphis*, notably a NON-pathogenic microbe. They discovered that *P. chlororaphis*, while not as vigorous a producer as *P. aeruginosa*, demonstrated an ability to create the lipid. They also found that *P. chlororaphis* produced rhamnolipid most efficiently while static and at room temperature, as opposed to the heat and agitation requirements of *P. aeruginosa*. The researchers concluded that production costs using *P. chlororaphis* would be lower, and this benefit combined with its benign nature would make it a more plausible commercial contender. (Gunther, NW, A. Nuñez, W. Fett, and DKY Solaiman. 2005. Production of Rhamnolipids by *Pseudomonas chlororaphis*, a Nonpathogenic Bacterium. Appl. Env. Microbiol. 71 (5) 2288-2293.)

TABLE 1

Sample:	Type:	Total Hg, ppm:
A1	wild type plant rhizosphere	15
A2	control plot, no plant	16
A3	genetically modified plant rhizosphere	15
C10	wild type plant rhizosphere	463
C11	control plot, no plant	425
C12	genetically modified plant rhizosphere	576
D7	genetically modified plant rhizosphere	5264
D8	wild type plant rhizosphere	4235
D9	control plot, no plant	3688

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