The Reduction of Particulate Manganese Oxides by *Carnobacteria* in Lake Mendota, Wisconsin

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ABSTRACT

Temperature, oxygen and soluble manganese concentrations were measured at various depths in Lake Mendota, a eutrophic lake in southern Wisconsin. Lake Mendota was found to have elevated levels of soluble manganese in the oxic portion of the water column as was seen in previous studies in Lake Vanda, an antarctic lake. Some microbes are able to reduce particulate manganese oxides to soluble manganese. Manganese-reducing microbes isolated from Lake Vanda's oxic zone were unexpectedly found to belong to the genus *Carnobacteria.* We have seventeen manganese reducing isolates from the oxic portion of Lake Mendota where soluble manganese was present. Additional characterization is underway. In an attempt to look for evidence of *Carnobacteria*, a 16S ribosomal RNA-based oligonucleotide probe specific to the genus *Carnobacteria* was developed. This probe was used as a primer for polymerase chain reaction (PCR) studies with nucleic acid from our 17 isolates. Preliminary results indicate that four of these isolates may be *Carnobacteria*.

INTRODUCTION

Particulate manganese oxides may play an important role in the scavenging, transport, and release of metals in lakes (1). Since some types of bacteria have the ability to reduce particulate manganese, these bacteria are vital to aquatic geochemical cycles. In Antarctic lakes, it was discovered that members of the genus *Carnobacterium* may have this talent. This was a surprising revelation, as *Carnobacterium* has complex nutritional requirements (4), and Antarctica has very simple and pristine lakes that would seemingly not support such a demanding bacterium.

The purpose of this research was to see if manganese cycling by *Carnobacterium* is unique to Antarctic lakes. Lake Mendota, a eutrophic temperate lake was selected for study. The presence of manganese reducing *Carnobacteria* was tested for using both traditional isolation methods and 16S ribosomal RNA (rRNA) based molecular techniques. Four potential *Carnobacteria* have been isolated from water collected at a depth of 11 meters in Lake Mendota.

METHODS

Sampling. Samples were collected from Lake Mendota, Madison, Wisconsin, on August 26, 1999. Oxygen and temperature were measured at various depths throughout the water column using a YSI oxygen meter. Water samples were collected using tubing and a peristaltic pump. A portion of the water sample (10 ml) was acidified with 20 µl concentrated

trace metal grade nitric acid to preserve it for a manganese assay. The rest of the water sample was used to inoculate 3 plates via the spread plate technique. The 3 plates consisted of 2 R2A plates (Difco) and one manganese overlay plate (1).

Manganese assay. The colorimetric assay of Brewer was used to determine the concentration of soluble manganese in the acidified water samples (2). This allowed us to determine the manganese profile of the lake's water column.

Isolation and characterization. For each water depth sampled, colonies on the R2A agar were transferred with sterile toothpicks to AMR plates overlaid with manganese oxides (1) to ascertain their ability to reduce manganese. Those colonies able to reduce manganese, indicated by a clear halo around the colony, were purified from contaminants by successive platings on R2A agar. Once pure, they were Gram stained, rechecked for manganese reduction, and stored at -80°C in 35% glycerol.

***Carnobacterium* probe development.** The *Carnobacterium* probe was radiolabelled and a T_d profile determined as discussed in Tsien, et. al. (5). Probe specificity was tested against a membrane filter containing bound nucleic acid extracted from pure cultures (*Bacillus subtilis, Aerococcus viridans, Lactobacillus plantarum*, *Carnobacterium funditum*, *Carnobacterium* LV62W1). The membrane was hybridized with the probe at 42°C and washed at the newly determined T_d, 49°C, as described previously (5).

Polymerase chain reaction (PCR). Portions of individual colonies were suspended in 10 µl of Lyse-N-Go (Epicentre, Madison, WI) and then fragments of the 16S ribosomal RNA (rRNA) gene were amplified using PCR (6). The 16S rRNA forward primer corresponded to positions 8 to 27 of the 16S rRNA of *Escherichia coli* while the reverse primer corresponded to positions 163 to 181 (3). Reaction results were determined using gel electrophoresis and 1% agarose gels (6).

RESULTS

Oxygen concentration and water temperature was measured throughout the water column of Lake Mendota, Madison, Wisconsin, at the time of sample collection. Soluble manganese concentration was later determined via colormetric assay (2). These data enabled us to create a profile of the water column tested (Fig 1). Oxygen levels decreased dramatically after 10 meters, creating an oxic/anoxic interface at approximately 11 meters. Two peaks in the levels of soluble manganese were observed in the oxic portion of the water column. One was located just above the interface and the other at 9 meters.

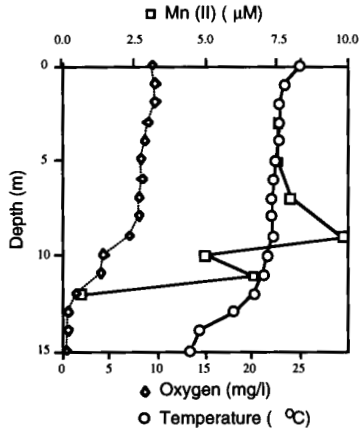

Figure 1. Profiles for oxygen concentration, water temperature, and soluble manganese concentration in Lake Mendota, Madison, Wisconsin.

Table 1. Manganese reducers isolated from an 11 m water sample taken from Lake Mendota, Wisconsin[1]

Strain designation	Gram stain	Cell morphology	Colonial morphology
A	-	rods	smooth, white with fringe
C-y	+	rods	mucoid, circular, yellow
C-w	-	rods	white, fringed, 1-7 mm
D	-	slender rods	thin golden sheet
F	-	slender rods	swarming yellow slime
G	-	slender rods	swarming yellow slime
J-w	-	coccobacilli	diffuse, white, 1-10 mm
L	-	cocci	circular, shiny, white
N-w	-	rods	diffuse, clear to white, 1mm core
Q	-	rods	diffuse, white to clear, 3 mm

[1]Grown on R2A plates at room temperature for 1-3 days.

Water samples from Lake Mendota were used to inoculate R2A and manganese overlay agar plates in an attempt to discover bacteria with the ability to reduce manganese. Both types of media were utilized for a greater level of recovery in the event some manganese reducing bacteria may have been out competed or otherwise unable to grow on the minimal manganese overlay agar. Growth was observed on both types of media, and ultimately 17 isolates were identified as manganese reducing bacteria; as demonstrated by a clear halo surrounding the colony on the manganese overlay plates. Thus far, 10 of the manganese reducing bacteria from Lake Mendota have been characterized (Table 1). All the isolated bac-

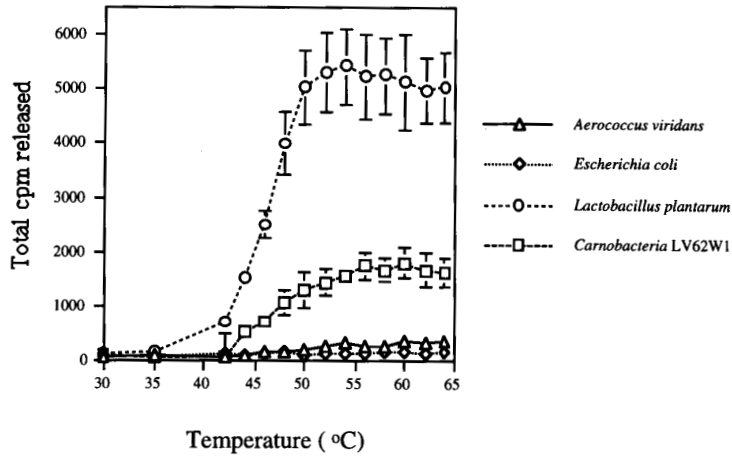

Figure 2. Thermal denaturation profile for the *Carnobacterium*-specific probe hybridized to nucleic acid from four different organisms.

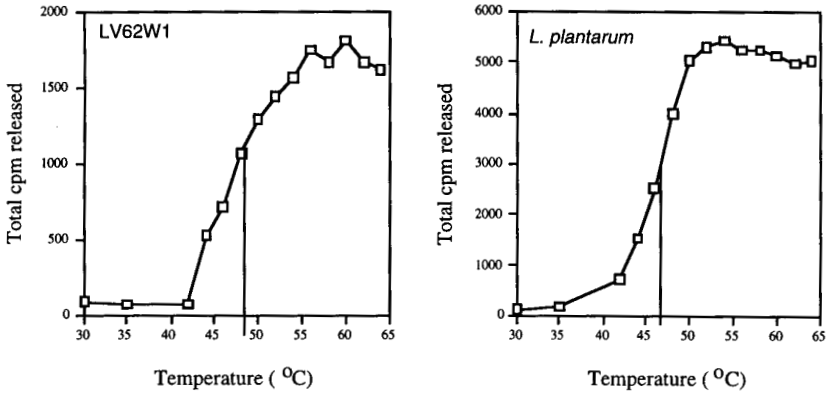

Figure 3. Using the inflection point of the curve, where 50% of the bound probe has been released, to determine the T_d of the probe bound to either *Carnobacterium* LV62W1 or *Lactobacillus plantarum*.

teria are from 11 meters, just above the oxic/anoxic interface, where a peak in soluble manganese was observed.

A *Carnobacteria*-specific probe was used to determine whether any of our 17 manganese reducing isolates belonged to the genus *Carnobacterium*, as was seen in a previous study in an Antarctic lake. However, while the *Carnobacteria*-specific probe had been designed, its conditions for use had never been optimized. Therefore, to determine experimentally the denaturation temperature (T_d), a study of the probe itself was conducted.

The *Carnobacterium*-specific probe was hybridized to *Carnobacterium* LV62W1 (positive control), *Aerococcus viridans* and *Lactobacillus plantarum* (two close relatives whose target sequences each contain only 1 mismatch with the probe), and *Escherichia coli* (negative control). Next, a series of washes from 30-64°C were conducted to discover the wash temperature necessary to attain the desired specificity. Ideally, this is the temperature where at least 50% of the originally bound probe remains hybridized to the positive control,

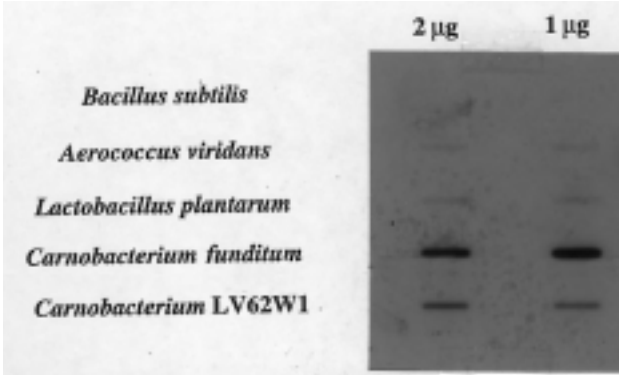

Figure 4. Probe specificity was tested by hybridizing the probe to a membrane filter containing bound nucleic acid extracted from pure cultures of *Bacillus subtilis, Aerococcus viridans, Lactobacillus plantarum, Carnobacterium funditum, and Carnobacterium* LV62W1. The membrane was stringently washed at the newly determined T_d of 49°C.

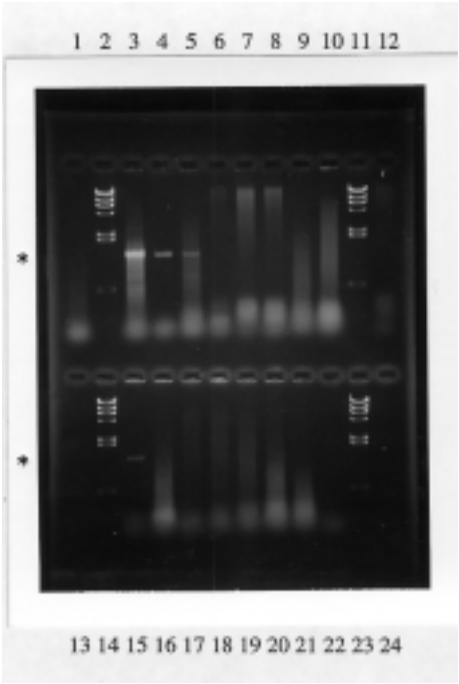

Figure 5. Seventeen isolates from 11 meters in Lake Mendota were tested for the presence of *Carnobacteria* 16S rRNA genes using PCR. The amplified fragments are marked (*). The gel was loaded as follows: Lanes 2, 11, 14, and 23, $\lambda_{HindIII}$ ladder; Lane 1, A; Lane 3, B-f; Lane 4, C-y; Lane 5, C-w; Lane 6, D; Lane 7, F; Lane 8, G; Lane 9, H-2; Lane 10, I-w; Lane 12, B-l; Lane 15, J-y; Lane 16, J-w; Lane 17, L; Lane 18, M; Lane 19, N; Lane 20, P; Lane 21, Q; and Lane 22, negative control.

while all probe that hybridized to the other bacterial nucleic acids has been dissociated. The amount of probe that became unbound as the temperature of the wash was incrementally increased was measured using a scintillation counter (Fig. 2). The *Carnobacterium*-specific probe hybridized to both *Lb. plantarum* and *Carnobacterium* LV62W1. Looking at the two curves individually (Fig. 3), it is possible to identify the inflection point where approximately 50% of the originally bound probe was released by the increase in temperature. The lines drawn on the graphs show a T_d of 48.5°C for *Carnobacterium* LV62W1 while that of *Lb. plantarum* is 46.5°C. Using a wash temperature of 49°C, therefore, should be sufficient to avoid cross-hybridization of the probe to *Lb. plantarum* while still allowing some probe to remain bound to *Carnobacterium* LV62W1 and hopefully other *Carnobacteria*.

The *Carnobacterium*-specific probe was next hybridized to a membrane filter containing bound nucleic acid extracted from pure cultures to confirm the probe specificity using a stringent wash of 49°C as determined from the melting experiment. The probe successfully bound to both *Carnobacteria funditum* and *Carnobacteria* LV62W1, but not to the closely related *A. viridans* and *Lb. plantarum* or the unrelated *B. subtilis*.

The *Carnobacteria*-specific probe was then used as one of the two primers in a polymerase chain reaction (PCR) with the 17 manganese reducing isolates. Four of the isolates (B-f, C-y, C-w, J-y) may be *Carnobacteria* as indicated by the amplified fragments seen at the expected size on the gel (Fig. 5). Further tests will be conducted to confirm these results.

SUMMARY

Four isolates recovered from Lake Mendota are potentially members of the genus *Carnobacterium*. All four are manganese reducing bacteria isolated from just above the oxic/anoxic interface, where a peak of soluble manganese was found. This correlates with manganese reducing *Carnobacteria* found in Lake Vanda, an Antarctic lake, which were also discovered in a peak of soluble manganese just above the oxic/anoxic interface (1). Further research is needed to confirm the presence of *Carnobacteria* in temperate lakes. It appears, however, that the role of manganese reducing *Carnobacteria* in aquatic geochemical cycles is not unique to Antarctica.

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