GERBAM — Deuxième Colloque International de Bactériologie marine — CNRS, Brest, 1-5 octobre 1984
IFREMER, Actes de Colloques, 3, 1986, pp. 589-595

70

CONSTRUCTION AND USE OF A DIALYSIS CHAMBER FOR INVESTIGATING *IN SITU* THE TOXICITY OF HEAVY METALS ON BACTERIA

T.L. TAN

Bakteriologische Abteilung, Institut für Meeresforschung ; Am Handelshafen 12, D-2850 BREMERHAVEN (FRG)

ABSTRACT - The Teflon dialysis chamber consists of a cylinder with two screw-on rings on both its ends. Two sampling holes are in opposite ends of the cylinder wall ; the holes are fitted with silicone plates and screw-on caps. The chamber has a volume of 32 ml. For use in the sediment, the membrane has to be protected with polypropylene supporting disks and a glass fibre filter. These chambers have been employed to study the toxicity of $ZnCl_2$ on a cadmium-sensitive bacterium, *Acinetobacter* CS 13, in an aquarium with running seawater. A concentration of 10mg $Zn^{++}l^{-1}$ did not influence the cell densities in the chambers, but cell densities decreased at zinc concentrations of 15 and 20 mg l^{-1}. In seawater with 20 mg $Zn^{++}l^{-1}$, the bacteria accumulated zinc from 827 to 2813 $\mu g\ g^{-1}$ cells dry wt after 1 d. After 2 d the zinc concentration increased to 4625 $\mu g\ g^{-1}$ dry wt. Three chambers were filled with *Acinetobacter* CS 13 cells added to sterile sediment at a concentration of 1 x 10^8 cells ml^{-1} wet sediment. To test the security, these chambers were buried in the sediment of a mud flat in the Weser Estuary and recovered after 7 d. The cultures were not contaminated and cell densities increased to 3 - 4 x 10^8 cells ml^{-1}.

Key words: dialysis culture, zinc toxicity, *Acinetobacter*.

RÉSUMÉ - La chambre à dialyse en Téflon est constituée d'un cylindre bouché à ses deux extrémités par des anneaux vissés. Deux sas permettant d'échantillonner sont disposés aux extrémités opposées de la paroi du cylindre ; ils sont pourvus de plaques en silicone et de couvercles vissés. La chambre a un volume de 32 ml. Pour son utilisation dans le sédiment, la membrane doit être protégée par des disques de polypropylène et un filtre de fibre de verre. Ces chambres sont employées pour étudier la toxicité du $ZnCl_2$ sur une bactérie sensible au cadmium, *Acinetobacter* CS 13, dans un bac à eau de mer circulante. Une concentration en ions Zn^{++} de 10 mg l^{-1} n'affecte pas la densité des cellules dans la chambre, mais elle diminue à des concentrations en Zn^{++} de 15 et 20 mg l^{-1}. Dans une eau de mer comportant 20 mg l^{-1} de Zn^{++}, les bactéries accumulent de 827 à 2813 μg de zinc par gramme de poids sec après une journée. Après deux jours la concentration en zinc augmente jusqu'à 4625 $\mu g\ g^{-1}$ de poids sec. Trois chambres sont remplies de sédiment stérile auquel est ajouté *Acinetobacter* CS 13 à une concentration de 1.10^8 cellules ml^{-1} de sédiment humide. De façon à tester leur fiabilité, ces chambres sont enfouies dans le sédiment d'un marécage envasé de l'estuaire de la Weser, et récupérées 7 jours plus tard. Les cultures ne sont pas contaminées et les densités cellulaires atteignent 3 à 4.10^8 cellules ml^{-1}.

Mots clés : culture en dialyse, toxicité du zinc, *Acinetobacter*.

INTRODUCTION

The first application of dialysis sacs with cells of *Vibrio cholerae* implanted in the peritoneal cavity of animals was reported in 1896, as reviewed by Schultz and Gerhardt (1969). This review dealt with the design, theory and technique of dialysis cultures of microorganisms, mainly for medical purposes. Meanwhile, this technique of culturing microorganisms in dialysis chambers has been used to investigate the biochemistry and ecology of marine phytoplankton (Sakshaug and Jensen, 1978), the fungus-nematode and the fungus-fungus interactions (Nordbring-Hertz, 1983), and the survival of enteric

bacteria in natural waters (McFeters and Stuart, 1972; Vasconcelos and Swartz, 1976; Rhodes *et al.*, 1983; Lessard and Sieburth, 1983). *In situ* diffusion chambers have also been used to study the dynamics of microbial plankton communities (Landry *et al.*, 1984).

McFeters and Stuart (1972) described a membrane filter diffusion chamber made from Plexiglass. Their membrane filter chamber was later improved by the insertion of O-rings to prevent leakage and contamination, and a polycarbonate plastic to withstand repeated autoclaving was used instead of Plexiglass (Rhodes *et al.*, 1983). The development of membrane filter chambers and its applications for studies *in situ* was the subject of the review article by McFeters and Stuart (1981).

A Teflon dialysis chamber has been used for investigating the toxicity of $ZnCl_2$ on a cadmium-sensitive bacterium, *Acinetobacter* CS 13, in seawater aquariums. *Acinetobacter* CS 13 was also cultivated in sterilized sediment samples in three dialysis chambers, and the chambers were buried in the sediment of a mud flat in the Weser Estuary. The results are reported here.

MATERIALS AND METHODS

Dialysis chamber

The Teflon dialysis chamber consists of a cylinder, measuring 40 x 56 x 20 mm, with two screw-on rings on both its ends. Teflon gaskets, 47 x 50 mm, are inserted in grooves inside the rings to ensure a tight seal of the membranes. Two sampling holes are in opposite ends of the cylinder wall; the holes are fitted with silicone plates and screw-on caps (Fig. 1). The chamber has a volume of about 32 ml.

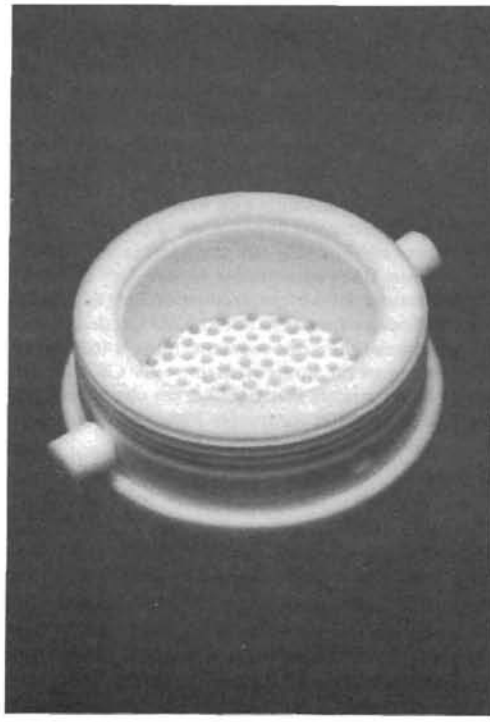

Figure 1 : Dialysis chamber without supporting disks and screw-on ring at the upper side.

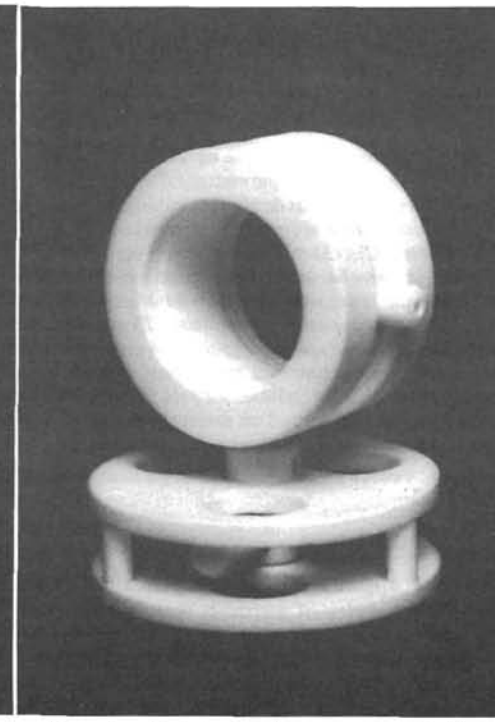

Figure 2 : Dialysis chamber on a magnetic stirrer holder.

For experiments in seawater aquariums, the chamber was put on a magnetic stirrer holder (Fig. 2) and rotated by means of a magnetic stirrer. Polycarbonate membrane filters (Nuclepore Corp., Pleasanton, Calif.), pore width 0,1 μm and 50 mm in diameter, were used for all experiments. For application in the sediment, the membrane has to be protected with polypropylene supporting disks and a glass fibre filter (Fig. 3).

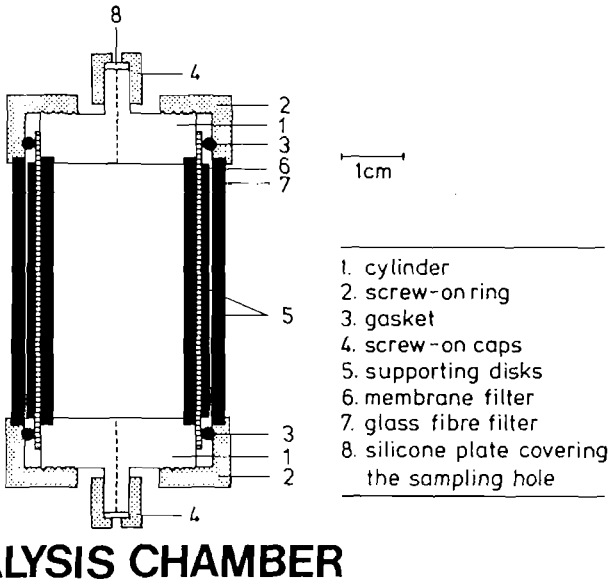

Figure 3 : Diagram of the dialysis chamber for use in the sediment.

Preparation of cell inoculum

The main characteristics of the cadmium-sensitive bacterium, *Acinetobacter* CS 13, have been reported earlier (Tan, 1983). Growth of *Acinetobacter* CS 13 on agar media containing 0.5 g peptone and 0.1 g yeast extract per liter was completely inhibited by 0.6 mg l^{-1} of Cd. Slant cultures of *Acinetobacter* CS 13 were kept in seawater agar containing 2 g Bacto-peptone, 0.5 g Bacto-yeast extract, 0.01 g $FePO_4 \cdot 4H_2O$, and 15 g agar per liter of aged seawater. A loopful from a slant culture was inoculated into a test tube containing 5 ml of a seawater solution with the same constituents as seawater agar. After 2 d of incubation at 24°C, two drops from the cell suspension were used to inoculate another test tube. After another 2 d of incubation the second culture was diluted in a solution containing 23.476 g NaCl and 10.610 g $MgCl_2 \cdot 6H_2O$ in 1 l of bidistilled water ($NaCl$-$MgCl_2$ solution). A dilution of 10^{-3} was necessary to obtain a cell density of about 1 x 10^5 cells ml^{-1}. The cell suspensions were filled into autoclaved dialysis chambers by means of a plastic 50 ml-syringe. It is important to get fill the chamber free of trapped air bubbles, and for this purpose the second hole in the cylinder wall is necessary.

A 5d-old slant culture of *Acinetobacter* CS 13 was harvested by washing the cells with 3 ml of $NaCl$-$MgCl_2$ solution. From this cell suspension 0.6 ml was thoroughly mixed with 60 ml of a sterile sediment sample in a 100 ml-flask. Sediment samples were sterilized at three successive days by autoclaving for 30 min at 121°C. The silty sediment sample was taken from a mud flat in the Weser Estuary. Sediment-cell suspensions were filled under a

laminar flow clean bench into dialysis chambers, prepared without supporting disks, filters and screw-on ring at the upper side. After filling the chamber, the upper side was covered by the supporting disks and filters, and tightened by the screw-on ring. Finally, the chamber was securely closed by using a pair of pincers.

Determination of cell densities

At the beginning of the seawater experiments, a sample was withdrawn from the chamber with a syringe, and the chamber was exposed again in the aquarium. We observed that the air space left by the sample volume taken out remained in the chamber. It was therefore decided to get the whole cell suspension from the chamber. From this suspension a 0.5 ml subsample was diluted in sterile seawater, and 0.1 ml of appropriate dilutions were plated on seawater agar. The plates were incubated at 24°C for 4 - 5 d. Three plates per dilution were prepared and the mean values presented. The deviations from the mean values amounted to 9 - 18 %.

Three chambers were buried in the sediment of the same mud flat with the marked upper sides upright. The upper sides of the chambers were covered with 1 cm of sediment layer. The chamber was secured by two stainless steel wire clamps and flags were attached to the wire for detecting the position of the chamber. After recovery the chambers were transported in a cool box to the laboratory. The outside wall of the chamber was first rinsed with tap water and dried with a cellulose tissue. Subsequently the upper side was opened under a laminar flow clean bench and 1 cm^3 of surface sediment was suspended in 4.5 ml of sterile seawater. The suspension was homogenized with an Ultra-Turrax 18 KG blender (IKA, Staufen) at 20 000 rpm for 1 s. The Ultra-Turrax shaft was then cleaned in 4.5 ml of sterile seawater at 20 000 rpm to get the remaining cells and sediment particles off. Both suspensions were combined and further dilutions were made in sterile seawater. Cell densities were also determined on seawater agar.

Experimental arrangement for culturing in seawater aquariums

The Teflon dialysis chambers were cleaned in concentrated nitric acid and rinsed several times with bidistilled water before use. The chambers on magnetic stirrer holders were kept in an aquarium and rotated by means of magnetic stirrers. The aquarium was built from Lexan, a polycarbonate from General Electric Inc., New York. A 1 m^3 glass fibre tank was used as reservoir. The tank and aquarium were cleaned with a brush, and rinsed with 2 M HCl and seawater before starting the experiment. The two aquariums were covered with black plastic sheets to retard algal growth in the seawater. A schematic representation of the experimental arrangement is shown in Figure 4. This arrangement was used for dialysis cultures in seawater without zinc addition. In toxicity experiments with $ZnCl_2$, a closed system with the seawater tank as reservoir was chosen. A $ZnCl_2$ stock solution, 1000 fold in concentration, was made in 1 l of bidistilled water, acidified with 0.5 ml of concentrated nitric acid. This stock solution was mixed to 1000 l of seawater in the reservoir, giving the desired final concentration of $ZnCl_2$.

Zinc analyses

About 30 ml of the cell suspension was filtered on 0,4 μm Nuclepore filter. The cells were washed twice with 5 ml of NaCl-$MgCl_2$ solution. Wet cells were dried at 80°C for 3 d, and the dry weights determined. Dried cells on the filter were digested with a mixture of 3 ml HCl and 1 ml HNO_3 (both concentrated suprapure acids from Merck, Darmstadt) in a Teflon autoclave at 150°C for 45 min. Zinc determinations were done with an atomic absorption spectrophotometer 300 (Perkin-Elmer, Überlingen) equipped with a D_2-

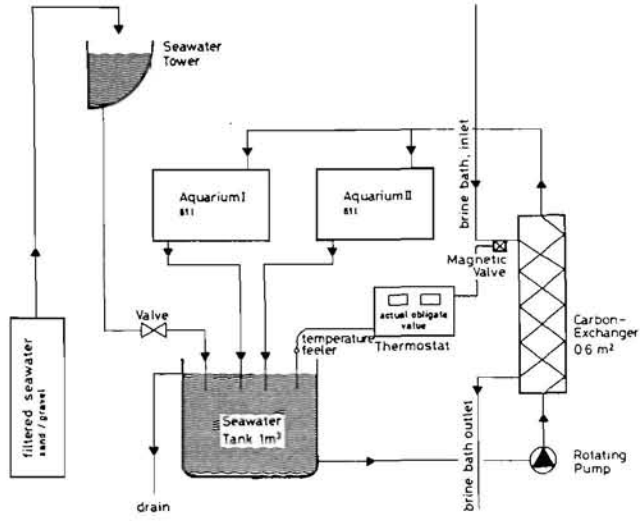

Figure 4 : Schematic representation of the experiment with dialysis cultures in an aquarium with running seawater at a flow rate of 14 l min^{-1}.

compensator and a graphite furnace HGA-72. Zinc was determined according to the instructions given by the manufactorer. Digested samples and standard solutions have to be diluted in 0,1 M HCl suprapure in polypropylene 2 ml-containers (Eppendorf, Hamburg), in order to get lower Zn concentrations for atomic absorption analyses. The containers were cleaned with concentrated H_2SO_4 (Merck, suprapure) and rinsed several times with bidistilled water before use. Appropriate dilutions from the samples and standards were pipetted with a 20 μl-pipettor (Eppendorf) into the graphite tube. The pipette tips were precleaned in concentrated HCl and rinsed 10 times with suprapure HCl before pipetting the sample. The following temperature programme was chosen for sample treatment in the tube: drying at 89°C for 60 s, gradual temperature increase to 450°C at rate 5, isothermic treatment for 30 s and atomizing at 2019°C for 10 s. After atomization the furnace was cleaned by heating at maximal temperature for 4 s. The standard addition procedure was used for determining the zinc contents.

Results

Densities of *Acinetobacter* CS 13 in dialysis chambers in an aquarium with running seawater are presented in Figure 5. Growth and survival of *Acinetobacter* CS 13 in seawater were dependent on the incubation temperature and the cell densities of the inoculum. A concentration of 10 mg $Zn^{++}l^{-1}$ did not influence the cell densities in the chambers, but cell densities decreased at zinc concentrations of 15 and 20 mg l^{-1}. In seawater with 20 mg $Zn^{++}l^{-1}$, the bacteria accumulated zinc from 827 to 2813 μg g^{-1} cells dry wt after 1 d. After 2 d the zinc concentration increased to 4625 μg g^{-1} dry wt.

Density determinations of *Acinetobacter* CS 13 from three dialysis chambers buried in the sediment for 7 d revealed that the cultures were not contaminated, and cell densities increased from 1 x 10^8 to 3 - 4 x 10^8 cells ml^{-1} wet sediment. These pure cultures of *Acinetobacter* CS 13 showed that the chambers were secured against bacterial contamination. Our results about heavy metal toxicity on *Acinetobacter* CS 13 in dialysis chambers in the sediment will be reported later.

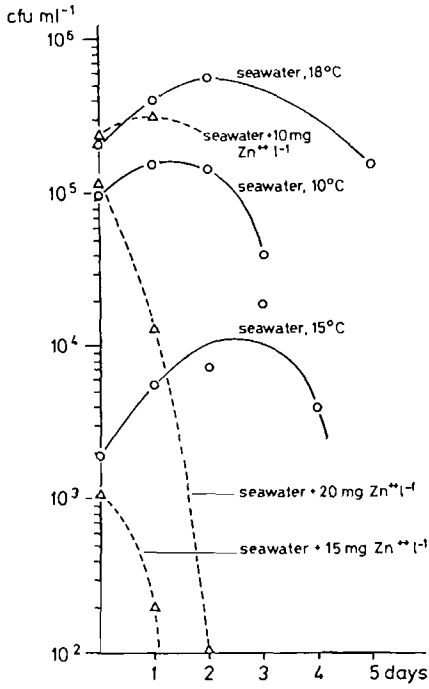

Figure 5 : Cell densities of *Acinetobacter* CS 13 in dialysis chambers in an aquarium with running seawater. Toxicity experiments were performed at 15°C.

Discussion

Teflon has been chosen as material for the dialysis chamber because of its heat stability up to 250°C and its resistance to concentrated acids. It is recommended to clean the chambers with concentrated acid, before investigations with heavy metals begin. Rhodes *et al.* (1983) used dialysis chambers made from polycarbonate, but polycarbonate is not resistant to concentrated acid. The chamber used by Landry *et al.* (1984) resembles the chamber described here. For our experiments in the sediment, however, the dialysis membranes have to be protected against mechanical damage by polypropylene supporting disks. A similar device has been used for the interstitial water sampler reported by Winfrey and Zeikus (1977).

Pickett and Dean (1976 and 1979) investigated the sensitivity and tolerance of zinc and cadmium in bacteria. They found that minimum Zn^{2+} concentrations of 0.12, 1.53 and 0.2 mM if completely inhibit the growth of *Pseudomonas* sp., *Bacillus subtilis* subsp. *niger*, and *Klebsiella pneumoniae*. The minimum Cd^{2+} concentrations completely inhibiting the growth of the same three organisms were 0.13, 0.62 and 0.25 mM, respectively. Growth inhibition experiments were performed in minimal liquid medium with glucose as organic substrate. On solid agar medium, however, the *Pseudomonas* sp. was the least sensitive of the three strains to Cd^{2+} and Zn^{2+}. Changing the growth limiting nutrient in chemostat cultures had a profound effect on the subsequent survival of the cells on Cd^{2+} -and Zn^{2+} - agar. In glucose-limited chemostats, in the presence of zinc, the tolerance of *Pseudomonas* sp. was associated with a decrease in uptake of the metal ion. After 3 h of continuous culture Zn uptake was 0.93 % of dry wt, and after 93 h only 0.47 % of dry wt was measured (Pickett and Dean, 1979). Growth and survival of *Acinetobacter* CS 13 in seawater submerged dialysed chambers were influenced by Zn^{2+} concentrations of 15 and

20 mg l^{-1}. In seawater with 20 mg Zn^{2+} l^{-1} zinc concentration in *Acinetobacter* CS 13 after 2 d of exposure was 4625 μg g^{-1} dry wt, corresponding to 0.46 % of dry wt. In glucose-limited chemostats, a concentration of 25 μg Cd^{2+} l^{-1} already inhibited the growth of *Acinetobacter* CS 13 (Tan, 1983). It is likely that *Acinetobacter* CS 13 is more sensitive to zinc in minimal liquid medium than in seawater.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Bundesministerium fur Forschung und Technologie (MFU 0505 BO). The experiments in seawater aquariums were carried out at the Meeresstation Helgoland from the Biologische Anstalt Helgoland. I am grateful for good facilities provided and for the helpful support given by J. Holtmann and his colleagues. I am obliged to H. Westphal and K. Wohlert for preparing the figures and photographs, and to M. Gomez for manuscript correction. My thanks are also due to Erika Allhusen and Susanne Spahic for their technical assistance.

LANDRY M.R., L.W. HAAS and V.L. FAGERNESS, 1984. Dynamic of microbial plankton communities: experiments in Kaneohe Bay, Hawaii. *Mar. Ecol. Prog. Ser. 16,* 127-133.

LESSARD E.J. and J. McN. SIEBURTH, 1983. Survival of natural sewage populations of enteric bacteria in diffusion and batch chambers in the marine environment. *Appl. Environ. Microbiol. 45,* 950-959.

MC FETERS G.A. and D.G. STUART, 1972. Survival of coliform bacteria in natural waters: Field and laboratory studies with membrane-filter chambers. *Appl. Microbiol. 24, 805-811.*

MC FETERS G.A. and D.G. STUART, *In situ* studies using membrane filter chambers. In: *Membrane filtration. Applications, techniques, and problems,* pp 481-512. Ed. by B.J. Dutka, New York: Marcel Dekker, Inc. 1981.

NORDBRING-HERTZ B., 1983. Dialysis membrane technique for studying microbial interaction. *Appl. Environ. Microbiol.* 45, 290-293.

PICKETT A.W. and A.C.R. DEAN, 1976. Cadmium and zinc sensivity and tolerance in *Klebsiella (Aerobacter) aerogenes. Microbiol. 15,* 79-91.

PICKETT A.W. and A.C.R. DEAN, 1979. Cadmium and zinc sensitivity and tolerance in *Bacillus subtilis subsp. niger* and in a *Pseudomonas* sp. *Microbios 24,* 51-64.

RHODES M.W., I.C. ANDERSON and H.I. KATOR, 1983. *In situ* development of sublethal stress in *Escherichia coli*: Effects on enumeration. *Appl. Environ. Microbiol. 45,* 1870-1876.

SAKSHAUG E. and A. JENSEN, 1978. The use of cage cultures in studies of the biochemistry and ecology of marine phytoplankton. *Oceanogr. Mar. Biol. Ann. Rev. 16,* 81-106.

SCHULTZ J.S. and P. GERHARDT, 1969. Dialysis culture of microorganisms: Design, theory and results. *Bacteriol. Rev. 33,* 1-47 (1969).

TAN T.L., 1983. Unbalanced growth of *Acinetobacter* CS 13 in glucose-limited chemostats caused by sublethal $CdCl_2$ concentrations. *Mar. Biol. 76,* 247-252.

VASCONCELOS G.J. and R.G. SWARTZ, 1976. Survival of bacteria in seawater using a diffusion chamber apparatus *in situ. Appl. Environ. Microbiol. 31,* 913-920.

WINFREY M.R. and J.G. ZEIKUS, 1977. Effect of sulfate on carbon and electron flow during microbial methanogenesis in freshwater sediments. *Appl. Environ. Microbiol. 33,* 275-281.