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GENETIC REGULATION INVOLVED IN THE GEOMICROBIOLOGY OF MANGANESE IN THE MARINE ENVIRONMENT

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ABSTRACT - Recent reports in the literature suggest a plasmid-encoded mechanism for manganese oxidation by bacteria. To elucidate the role, if any, of plasmids in bacterial manganese oxidation in the marine environment, manganese-oxidizing bacterial strains, isolated from manganese nodules, sediment and seawater samples collected from the Pacific Ocean and Mediterranean Sea were examined for the presence of plasmids and their association with manganese oxidation. Twenty-two of 47 strains of the genera *Alcaligenes, Pseudomonas,* and *Vibrio* harbored single plasmids of various sizes even after prolonged storage. Fourteen of the plasmid-bearing manganese- oxidizing strains were *Pseudomonas* spp. and one was a *Vibrio* sp. Plasmid DNA was not detected in the remaining manganese-oxidizing strains of *Alcaligenes* and *Vibrio.* One of the isolates, *Pseudomonas* strain 57, which oxidizes manganese enzymatically, produced manganese oxide from Mn^{2+}, in a buffered reaction mixture at pH 7.4, when cell-free extracts were used. This strain harbors a single 9Kb plasmid that, upon curing, displayed a sensitivity to manganese and copper. Also, the cured variant demonstrated a reduced rate of manganese oxidation, suggesting the mechanism of manganese oxidation to be, in part, plasmid-associated. The cured variant showed no apparent change in phenotype or overall DNA base composition when compared with its parental strain. It can be concluded that the enzymatic oxidation of manganese by some marine bacteria may not be exclusively encoded by plasmid DNA. Functions ascribable to the plasmids remain cryptic, although evidence obtained from the study of *Pseudomonas* strain 57 suggests that the plasmid is associated with enzymatic manganese oxidation, perhaps by encoding a detoxification mechanism for manganese and copper. It is hypothesized that self-transmissible plasmids provide marine bacteria with an ecologically significant strategy for survival in the natural environment since they are able to transfer plasmid DNA coding for ecologically advantageous functions, such as the ability to oxidize manganese and detoxify heavy metals.

Key words : plasmids, manganese oxidation, marine bacteria.

RÉSUMÉ - Des rapports récents dans la littérature suggèrent que l'oxydation du manganèse par les bactéries serait liée à un mécanisme de codage plasmique. Pour éclaircir le rôle, s'il en existe un, des plasmides dans l'oxydation du manganèse en milieu marin, des souches bactériennes oxydant le manganèse sont isolées à partir de nodules de manganèse, de sédiment et d'eau de mer prélevés dans l'océan Pacifique et en mer Méditerranée ; la présence de plasmides et leur intervention dans l'oxydation du manganèse sont étudiées. Vingt deux des 47 souches du genre *Alcaligenes, Pseudomonas* et *Vibrio* possèdent un seul plasmide de taille variée, même à la suite d'une conservation prolongée. Quatorze des souches porteuses d'un plasmide et oxydant le manganèse sont des *Pseudomonas* spp. ; une est un *Vibrio* sp. L'ADN plasmique n'est pas détecté chez les autres souches d'*Alcaligenes* et de *Vibrio* qui oxydent le manganèse. Un des isolats, un *Pseudomonas* souche 57, qui oxyde le manganèse de façon enzymatique, produit de l'oxyde de manganèse à partir de Mn^{2+} dans un mélange réactionnel tamponné à PH 7.4, par utilisation d'extraits de cellules libres. Cette souche possède un plasmide de 9 Kb, qui sur traitement, manifeste une sensibilité au manganèse et au cuivre. De plus, le taux d'oxydation du manganèse par le variant traité est réduit, suggérant que le mécanisme d'oxydation du manganèse serait en partie associé à un plasmide. Le variant traité ne montre pas de changement apparent de phénotype ou de composition de base de l'ensemble de l'ADN, par comparaison avec sa souche d'origine. Il peut être conclu que l'oxydation enzymatique du manganèse par certaines bactéries marines pourrait ne pas être exclusivement

codée par l'ADN plasmidique. Les fonctions attribuées aux plasmides restent énigmatiques, bien qu'il ait été mis en évidence à partir de l'étude du *Pseudomonas* souche 57, que le plasmide serait associé à l'oxydation enzymatique du manganèse, peut-être par codage d'un mécanisme de détoxification du manganèse et du cuivre. Il est supposé que les plasmides, transmissibles par eux-mêmes, confèrent aux bactéries marines une efficace stratégie écologique de survie en milieu naturel, puisqu'ils codent pour des fonctions écologiques avantageuses telles que la capacité à oxyder le manganèse et à détoxifier les métaux lourds.

The presence of plasmids in bacteria can extend the metabolic capability of the host and alter phenotypes such as resistance to anti-bacterial agents, toxin production, surface adhesiveness, and ability to catabolize unusual substrates. The ubiquitous occurrence of manganese oxidation mediated by a variety of microorganisms in fresh water (Ghiorse and Hirsch, 1979), on the surfaces of marine manganese nodules, and in seawater and sediment surrounding manganese nodules (Ehrlich, 1966 ; Nealson, 1978 ; Schuett and Ottow, 1978) and the observation of spontaneous loss of the ability to oxidize manganese by fresh water isolates during prolonged storage (Gregory and Staley, 1982) allows the hypothesis that bacterial manganese oxidation is, in part, plasmid encoded. A study by Lidstrom *et al.*, (1983) offered evidence for plasmid encoded manganese oxidation by a marine *Pseudomonas* strain 63-B.

The objective of the study reported here was to investigate the association of plasmids with manganese oxidation in bacterial strains isolated from sediment, manganese nodules and seawater from the Pacific Ocean and Mediterranean Sea. Thus, an understanding of the ubiquitous occurrence of manganese-oxidizing bacteria in the marine environment was sought.

To elucidate the association of plasmids with manganese-oxidizing bacteria, 40 manganese-oxidizing bacterial strains, isolated from manganese nodules and samples of seawater and sediment collected at the sediment-seawater interface of the Pacific Ocean, and an additional seven strains from water samples collected from the Mediterranean Sea were included in this study. The Pacific isolates had been stored for up to 8 years at 4°C, without spontaneous loss of ability to produce manganese oxide. Of the strains, 26 were identified as *Pseudomonas* spp. These had been isolated either from manganese nodules (25 strains) or the top layer of sediment (1 strain). Ten were *Alcaligenes* spp., isolated from manganese nodules and water samples collected at deep ocean sites (4 strains) or sediment (1 strain), five strains were identified as *Vibrio* spp., these having been isolated from the top layer of a sediment sample (Schuett, 1979).

A yeast extract-proteose peptone medium (YP medium), prepared with artificial seawater, was used for growth and storage of the culture. The medium contained (g/l) yeast extract (Difco Laboratories, Detroit, MI) 2.0 ; and proteose-peptone (Difco) 2.0 ; pH adjusted to 7.2. A medium containing Mn^{2+} and artificial seawater (M-medium) was used for manganese oxide detection and was composed (in g/l) of yeast extract (Difco) 2.0 , tryptone (Difco) 2.0 ; Na-acetate (Difco) 1.0 ; $MnSO_4.H_2O$ (Baker Chemical Co., Phillipsburg, NJ) 0.2 ; and Bacto-agar (Difco) 15.0 ; pH adjusted to 7.2. Artificial Sea Water (ASW) was composed of (g/l) NaCl 11.7 ; KCl 0.35 ; and $MgSO_4.7H_2$ 3.5.

Oxidation of Mn^{2+} to Mn^{4+} was measured using o-tolidine reagent (Sigma) according to the methods of Morgan and Stumm (1965) and also employed as a spot test.

Plasmid deoxyribonucleic acid (DNA) was extracted and analyzed using a modification of the rapid screening method of Kado and Liu (1981). Modifications were as follows : To obtain complete lysis, cells were incubated before lysis in the presence of 5 mg/ml lysozyme (Sigma, St. Louis, MO). After lysis with Sodium dodecilsulfate (SDS), plasmid DNA was prepared by agarose gel (0.7 %, BBL, Cockeysville, MD) electrophoresis for

3.5 h and 125 V (≈53 mA) in a watercooled flat bed electrophoresis apparatus (Savant Inc., Hicksville, NY, model No. HGE 1312). Gels were stained with ethidium bromide (1 μg/ml) for 30 mn, destained overnight in distilled water, and photographed using a transilluminator (Fotodyne model 3-4400) fitted with type 665 film and MP-4 Camera (from Polaroid Corp.). The film was exposed using Wratten No. 23 A and 2 B gelatin filters (Eastman Kodak Co., Rochester, NY). In all plasmid DNA experiments, *Escherichia coli* strain V 517, which harbors 8 plasmids of different sizes (Macrina *et al.*, 1978), was used as a control and molecular weight standard. Size estimates of the plasmids were generated by linear regression analysis.

Results of the plasmid screening indicated that 22 of 47 Mn-oxidizing bacterial strains were found to contain a single plasmid. Nineteen of the 26 *Pseudomonas* spp. isolated from the Central Pacific Ocean possessed a plasmid of *ca.* 66 Mdal and two strains, 55 and 57, demonstrated a single plasmid of 8 and 9 Mdal, respectively. Strains harboring plasmids of 66 Mdal had been shown in an earlier study to have very similar biochemical properties (Schuett, 1979). A faint plasmid band was detected in each of the gels prepared for the five *Vibrio* spp. Interestingly, the *Alcaligenes* strains did not reveal detectable plasmids. Two of the seven unidentified Mn-oxidizing strains isolated from seawater samples collected in the Mediterranean contained single plasmids of *ca.* 50 and 3 Mdal. Molecular weights of plasmids harbored by selected strains are summarized in Table 1.

Genus	Strain No.	Plasmid Mol. wt. (x 10^6)
Pseudomonas	17	66
Pseudomonas	55	8
Pseudomonas	57	9
Unidentified gram negative rod	4 D-1	50
Unidentified gram negative rod	7D-1	3

Table 1. : Molecular weights of plasmids harbored by selected strains of manganese-oxidizing bacteria examined in this study.

*Plasmids were isolated by gel electrophoresis and the molecular weight estimated by linear regression analysis. Seventeen *Pseudomonas* spp., other than those listed above, were found to contain plasmids (65 Mdal). All of the *Pseudomonas* spp. demonstrated similar biochemical properties (Schuett, 1979). The *Pseudomonas* spp. were isolated from the Pacific Ocean and the unidentified strains were from the Mediterranean Sea. (Stemmler and Colwell, manuscript in preparation).

It was found that the bacteria from the Central Pacific Ocean did not lose the ability to oxidize manganese, after long storage, i.e., 5 to 8 years at 4°C. Furthermore, results of the studies reported here indicate that *ca.* 53% of forty-seven strains examined did not possess detectable plasmids, i.e., genes for oxidation of manganese and/or its regulation were not located on a plasmid, using the methods described above. Thus, plasmids are not necessarily associated with marine bacteria capable of oxidizing manganese.

To investigate the association of plasmids with a manganese oxidation mechanism, seven manganese oxidizing strains of *Pseudomonas* were used for curing experiments, of which six were from the Central Pacific Ocean and the other was an unidentified strain from the Mediterranean Sea (Tab. 2). Several curing treatments and a number of different curing agents were tested, including 50 and 100 μg acridine orange/ml (Eastman Kodak, Rochester, NY), 5,10, and 20 μg ethidium bromide/ml (EB) (Sigma Chemical Co., St.

Manganese nodules contain, besides manganese, other even more toxic heavy metals, including cobalt, nickel, and copper (Yang and Ehrlich, 1976). Heavy metal resistance of the seven manganese-oxidizing strains was determined using a modification of the method described by Austin *et al.* (1977), i.e., M-medium was used in place of the medium employed by Austin *et al.* (1977) and the following concentration of each heavy metal : $MnSO_4$. H_2O (150 μg/ml Mn^{2+}) was replaced by $NiCl_2$. $6\ H_2O$ (100 μg/ml Ni^{2+}), $CoSO_4$. $7H_2O$ (100 μg/ml Co^{2+}) and $CuSO_4$. $5H_2O$ (100 μg/ml Cu^{2+}). The inoculated test media were incubated for six days at 26°C. The resistance patterns of seven of the manganese-oxidizing isolates showed that all strains grew well in the presence of 150 μg/ml Mn^+, i.e., similar growth response on Mn^{2+} containing medium as on unamended medium (Tab. 4). Only strain 57 displayed sensitivity to Mn^{2+} . The other heavy metals inhibited growth of all strains tested, except in the case of strain 57 (pZP1) and its derivative. However, the cured strain was observed also to be sensitive to 100 μg/ml copper.

Genus	Strain	Resistance patterna			
		Mn (150)	Co (100)	Mi (100)	Cu (100)
Pseudomonas sp	7	R	S	S	S
Pseudomonas sp	12	R	S	S	S
Pseudomonas sp	17	R	S	S	S
Pseudomonas sp	18	R	S	S	S
Pseudomonas sp	55	R	S	S	S
Pseudomonas sp	57 (pZP1)	R	R	R	R
Pseudomonas sp	57	(S)	R	R	S
Unidentified gram-negative rod	4D-1	R	S	S	S

Table 4 : Heavy metal resistance pattern observed for eight manganese-oxidizing bacterial strains.

R = growth; S = no growth; (S) very weak growth on heavy metal-containing medium. The numbers in parenthesis are the final concentrations of metals (μg/ml) after addition of the metal to M-medium.

The resistance pattern observed for *Pseudomonas* 57 (pZP1) and its cured variant suggest that the curing of pZP1 results in a sensitivity of strain 57 to manganese and copper. The effect of these metals on viable cell counts is shown in Figure 1.

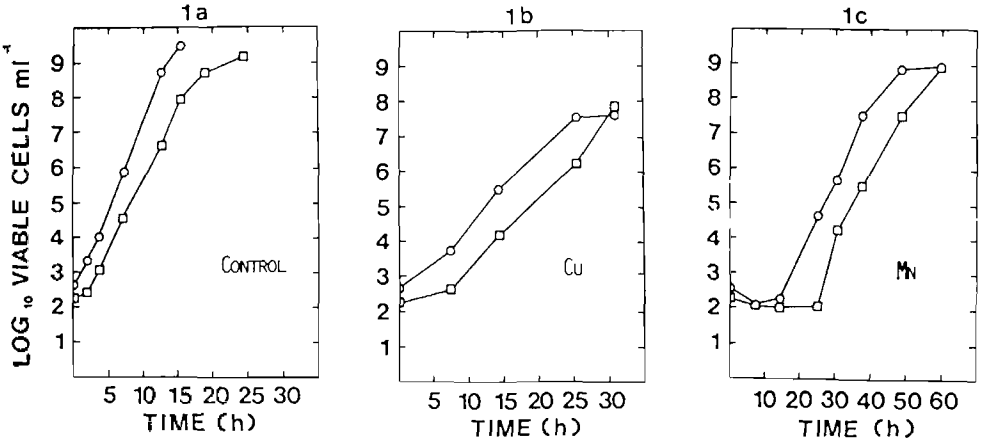

Figure 1 : Growth measurements (viable cells) of *Pseudomonas* 57 (pZP 1) (0) and *Pseudomonas* 57 (□) in modified K broth (a) unamended, (b) amended with 70 μg/ml Cu^{2+}, (c) amended with 170 μg/ml Mn^{2+}.

A buffered Mn (IV) stabilizing medium with artifical sea water (Modified K broth) amended with Cu^{2+} or Mn^{2+} was developed to measure the effect of those metals on viable counts and manganese oxidation rates. This medium was composed of (g/l) proteose-peptone (Difco) 2.0 ; yeast extract (Difco) 0.5 ; $FeSO_4 \cdot 7H_2O$ (Fisher) 0.00001 ; Hepes (Calbiochem) 11.9 (final concentration (50 mM) ; Na-pyrophosphate (Fisher) 4.4 (final concentration 0.02 M) ; pH adjusted to 7.0. After sterilization, the medium was amended with $MnSO_4 \cdot H_2O$ (0.2 M) 10 mls or $CuSO_4 \cdot 5H_2O$ (16 mM) 7.0 mls using asceptic technique.

Growth of *Pseudomonas* 57 (pZP1) and its cured derivative was measured according to methods described by Koch (1981). Cultures were grown in 100 ml of the Modified K broth and incubated during the duration of the experiment in 250 ml Erlenmeyer flasks at 25°C, in duplicate, on a rotory shaker at 80 rpm. At specified time intervals, aliquots were serially diluted in ASW and plated on YP-agar. Plates were incubated at room temperature and counts made after incubation for 4 days and also after 8 days. Only those plates containing between 30 and 300 colony-forming units (CFU) were counted. Rates of manganese oxidation were determined by measuring residual Mn^{2+} after filtration (0.2 μm Millipore) and centrifugation (3 000 rpm for 10 minutes at 4°C) by atomic absorption (Perkin-Elmer 5 000 ; air- acetylene flame, impact bead nebulizer).

The results indicate that, under the experimental conditions tested, no significant difference in growth rate exists between strains whether with or without heavy metal amendment. However, loss of pZP1 results in a slight lag in the medium prepared without heavy metals (Fig. 1a). The viable cell counts in the medium amended with Cu^{2+} were less for both strains, relative to their respective controls (Fig. 1b). The effect of Cu^{2+} may be one of nutrient limitation for these strains at concentrations below toxic levels (70 μg/ml). The effect of Mn^{2+} on growth differs from that of copper for both strans, as shown in Fig. 1c. Both strains exhibited an increased lag growth phase when exposed to 170 μg/ml Mn^{2+}. Loss of the plasmid, pZP1, resulted in a 10 hour increase in lag time, relative to the parental strain.

The loss of Mn^{2+} from the culture medium during growth of strain 57 and strain 57 (pZP1) is shown in Figure 3. Both strains oxidized manganese at the same rate, suggesting the oxidation mechanism is not at all plasmid encoded. However, manganese oxidation was correlated with growth for each strain, suggesting that the manganese oxidation mechanism is coupled to cell respiration. Results of other experiments (Zelibor and Colwell, unpublished data) indicate that manganese oxidation does not occur in both strains when the total organic nutrient concentration is below 125 μg/ml.

In conclusion, these studies confirm the presence of plasmid DNA in manganese-oxidizing strains of deep sea bacteria, most of which had been stored for up to 8 years at 4°C prior to examination for plasmids. Spontaneous loss of the ability to produce manganese oxide was not observed. Twenty-two of 47 strains of the genera *Alcaligenes, Pseudomonas* and *Vibrio,* that had been isolated from samples of seawater collected at the sediment-water interface and from manganese nodules of the pelagic Central Pacific Ocean and from seawater samples collected in the Mediterranean Sea harbored plasmids.

Six manganese-oxidizing strains of *Pseudomonas* spp. isolated from samples collected in the Central Pacific Ocean and one unidentified strain from the Mediterranean Sea selected for curing experiments revealed only *Pseudomonas* strain 57, isolated from a manganese nodule, to be cured successfully. The plasmid was 9 mDal and designated pZP1.

In studies carried out using a marine *Pseudomonas* strain 57 (pZP1), manganese oxida-

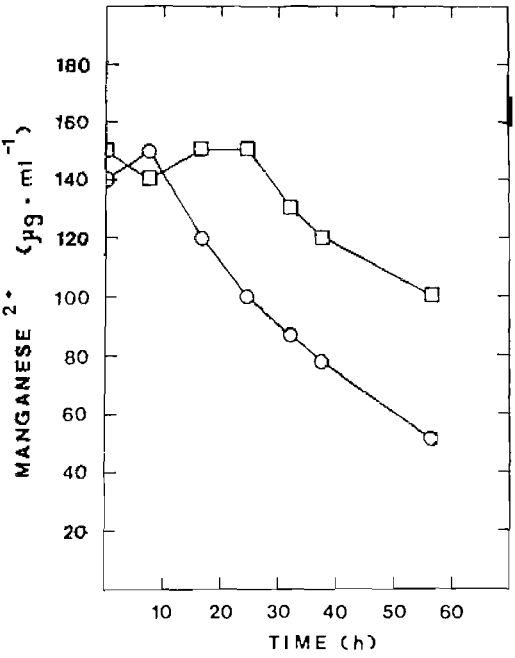

Figure 3 : Changes in Mn^{2+} concentrations by *Pseudomonas* 57 (pZP 1) (0) and *Pseudomonas* 57 (□). Residual Mn^{2+} concentrations were determined using filtered (0.2 μm millipore) aliquots centrifuged at 3000 rpm for 10 minutes and examination by Flame atomic absorption (Perkin-Elmer 5000). Total error was less than 10 % of measured Mn^{2+} values.

tion was enhanced by the presence of the plasmid, allowing the hypothesis that a function(s) related to manganese oxidation was plasmid encoded, similar to the hypothesis proposed by Lidstrom *et al.*, (1983) for *Pseudomonas* strain 63-B. Further investigation indicated that pZP1 does not encode directly for enzymatic manganese oxidation, because of the observation of similar rates of oxidation having been observed for strains 57 (pZP1) and 57. However, the plasmid does appear to encode for a resistance mechanism for manganese and copper, an ecological advantage for bacteria inhabiting the manganese nodule environment, considering the sorption of divalent cations by Mn (IV) oxide and consequent higher metal concentration. Thus, the function of the plasmid reported here, although, in fact, yet cryptic, appears to be associated with heavy metal resistance and, indirectly, with manganese oxidation.

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