APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1987, p. 1199–1202 Vol. 53, No. 5
0099-2240/87/051199-04$02.00/0

Susceptibility of Halobacteria to Heavy Metals

J. J. NIETO,* A. VENTOSA, AND F. RUIZ-BERRAQUERO
Department of Microbiology, Faculty of Pharmacy, University of Seville, Seville, Spain

Received 8 December 1986/Accepted 18 February 1987

Sixty-eight halobacteria, including both culture collection strains and fresh isolates from widely differing geographical areas, were tested for susceptibility to arsenate, cadmium, chromium, cobalt, copper, lead, mercury, nickel, silver, and zinc ions by an agar dilution technique. The culture collection strains showed different susceptibilities, clustering into five groups. *Halobacterium mediterranei* and *Halobacterium volcanii* were the most metal tolerant, whereas *Haloarcula californiae* and *Haloarcula sinaiiensis* had the highest susceptibilities of the culture collection strains. Different patterns of metal susceptibility were found for all the halobacteria tested, and there was a uniform susceptibility to mercury and silver. All strains tested were multiply metal tolerant.

Halobacteria are aerobic microorganisms which require high NaCl concentrations in a medium to grow and survive (9) and which are included, together with methanogens and thermoacidophiles, in the archaebacteria (22). This microbial group has been extensively studied with regard to their physiology, biochemistry, ecology and, recently, genetics (2, 9, 10). Although the susceptibility to antibiotics and, in some instances, the mechanisms of action of some antimicrobial agents have been studied in halobacteria (3, 4, 12), to date there are no reports concerning their natural susceptibility to heavy metals and organometallic compounds, let alone their ability to develop heavy metal resistances. The latter subject might provide useful information about ways of developing antibiotic resistance, since in nonhalophilic bacteria genes determining resistance to antibiotics and certain metals are often carried on the same plasmids (20). It was found recently that megaplasmids of unknown function are harbored by the majority of halobacteria (6). A knowledge of metal tolerance may reveal some functions for some of these plasmids and, in addition, the possible heavy metal resistances could be used in halobacteria as genetic markers. On the other hand, metal tolerance could be relevant to the ecology and physiology of halobacteria, since they usually grow in habitats such as solar salterns or hypersaline lakes containing some waters which have recently been found to be polluted with heavy metals (unpublished data).

The aim of the present study was to examine the susceptibility of a large number of halobacteria, including both culture collection strains and freshly isolated strains, to several heavy metals. A total of 68 halobacterial strains were selected for this study. The 13 culture collection strains are listed in Table 1, and the other 55 strains have been characterized in a previous taxonomic study and identified as members of the genus *Halobacterium* (19). These latter strains represent a very heterogeneous group of halobacteria that can be commonly isolated from hypersaline habitats in Spain.

The cultures were grown in a medium (referred to as SWYE) containing a final total salts concentration of ca. 25% (wt/vol) and 0.5% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.). The composition of the salts solution was as follows (percent [weight/volume]): NaCl, 19.4; $MgCl_2$, 1.6; $MgSO_4$, 2.4; $CaCl_2$, 0.1; KCl, 0.5; $NaHCO_3$, 0.02; and NaBr, 0.05 (6). The pH was adjusted to 7.2. Incubation was done at 37°C in an orbital shaker (Lab-Line Instruments, Inc., Melrose Park, Ill.) at 200 strokes per min.

The 10 heavy metals tested were provided from standard commercial sources as Na_2HAsO_4, $AgNO_3$, $CdCl_2$, $CoCl_2$, $CuSO_4$, K_2CrO_4, $HgCl_2$, $NiSO_4$, $Pb(NO_3)_2$, and $ZnSO_4$. Stock solutions were made in distilled water, sterilized by filtration through 0.22-μm-pore membrane filters (Millipore Corp., Bedford, Mass.), and kept at 4°C for no longer than 1 day.

Susceptibility was determined by an agar dilution method (21) with a Steers replicator (17). Plates containing 20 ml of SWYE medium solidified with 2% (wt/vol) Bacto-Agar (Difco) and several different concentrations of the metal inhibitors were prepared on the days of the experiments. The concentrations of all the metals were as follows (millimolar): 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2.5, 5, 10, 20, 40, and 80. This range covered those concentrations usually used in studies of metal resistance in eubacteria (7, 20). Before use, the plates were dried at 37°C for 30 min; they were then inoculated with 10^4 to 10^5 microorganisms from exponentially growing cultures per spot. Twenty-one spots (each from a different bacterial strain) could be conveniently tested per plate. The plates were then incubated at 37°C for 10 days. Agar plates without the antimicrobial agents and inoculated with the corresponding test microorganisms were used as controls. Similar experiments, in which the salt concentration in the test medium was 15, 20, or 30% (wt/vol), were also carried out. After incubation, the MIC was determined as the lowest concentration of metal which prevented growth. The MICs for all the strains were the same when the strains were tested in at least three different experiments. For the purpose of defining metal tolerance, those strains which were not inhibited by 10 mM As; 1 mM Ag, Cd, Co, Cr, Cu, Ni, Pb, and Zn; and 0.1 mM Hg were regarded as tolerant. These concentrations have been used for this purpose in the majority of studies carried out with eubacteria (11, 14, 20). It is important to point out that the availability of the respective metals can be influenced by chlorides or other kinds of salts contained in the culture

* Corresponding author.

TABLE 1. MICs of 10 metal ions tested against 13 halobacterial culture collection strains

Microorganism	MIC (mM) of[a]:									
	As	Ag	Ni	Co	Pb	Cd	Cr	Hg	Zn	Cu
H. mediterranei ATCC 33500	**20**	0.5	**2.5**	1	**20**	**2.5**	**5**	0.01	0.5	1
H. volcanii DS2	**20**	0.5	**2.5**	1	**20**	0.5	**5**	0.01	0.5	1
H. hispanicum ATCC 33960	10	0.5	**2.5**	1	**10**	0.5	**5**	0.01	0.5	1
H. vallismortis ATCC 29715	10	0.5	**2.5**	1	**10**	0.5	**5**	0.01	0.5	1
H. gibbonsii ATCC 33959	10	0.5	**2.5**	1	**10**	0.5	**5**	0.01	0.5	1
H. saccharovorum ATCC 2952	10	0.05	1	0.5	**20**	0.5	**2.5**	0.01	0.5	**2.5**
H. halobium CCM 2090	10	0.05	1	0.5	**20**	0.5	**2.5**	0.01	0.5	**2.5**
H. trapanicum NCMB 767	10	0.05	1	0.5	**5**	0.5	**2.5**	0.01	0.5	**2.5**
H. salinarium CCM 2084	**20**	0.05	1	0.5	**5**	0.1	**2.5**	0.01	0.05	1
Haloarcula sp. strain WS-1	10	0.05	0.1	1	**5**	0.05	**2.5**	0.05	0.05	**2.5**
Haloarcula sp. strain GN-1	10	0.05	0.1	1	**5**	0.05	**2.5**	0.05	0.05	**2.5**
H. californiae ATCC 33799	10	0.05	1	0.5	**10**	0.1	1	0.05	0.5	**2.5**
H. sinaiiensis ATCC 33800	10	0.05	1	0.5	**5**	0.05	**2.5**	0.05	0.05	1

[a] Boldface type indicates concentrations involving tolerance of the corresponding metal ion.

medium used in the present study. In addition, the formation of metal complexes in this culture medium may determine the true soluble metal concentrations, and, indeed, the toxicity of some metals could be attributed to a metal complex rather than a metal cation.

The MICs of the heavy metals tested against the halobacterial collection strains used in the present study are shown in Table 1. On the basis of the similar MICs of all the metals tested, these strains can be grouped into five major groups. (i) *Halobacterium mediterranei* and *Halobacterium volcanii* appeared to be the least susceptible. (ii) *Halobacterium hispanicum*, *Halobacterium vallismortis*, and *Halobacterium gibbonsii* had the same response to each metal and were equally affected by three metal ions (Cr, Ni, and Pb). (iii) *Halobacterium halobium*, *Halobacterium saccharovorum*, *Halobacterium trapanicum*, and *Halobacterium salinarium* had a similar response to Ag, Cr, Co, Hg, and Ni ions. However, the MICs of Cd, Cu, and Zn ions for *H. salinarium* were lower, but this strain was the only one in this group for which the MIC of As ion was higher. Otherwise, all these strains were relatively tolerant of Cr and Pb ions (MICs, 2.5 and 5 mM, respectively), and three of them were also tolerant of Cu ion (MIC, 2.5 mM). (iv) *Haloarcula* sp. strain WS-1 and *Haloarcula* sp. strain GN-1 had the same response to each of the metals tested and were tolerant of Cr, Cu, and Pb ions. (v) *Haloarcula californiae* and *Haloarcula sinaiiensis* were inhibited by the lowest concentrations of most of the metals tested.

TABLE 2. Susceptibility of 68 halobacterial strains to 10 metal ions

Metal ion	Cumulative % of strains susceptible to the following metal ion concn (mM):									
	0.005	0.01	0.05	0.1	0.5	1	2.5	5	10	20
As	0	0	0	0	0	5	12	41	85	100
Ag	0	28	35	71	90	100	100	100	100	100
Cd	0	0	0	28	66	91	100	100	100	100
Co	0	0	0	7	38	75	100	100	100	100
Cr	0	0	0	0	0	32	41	85	100	100
Cu	0	0	0	0	5	20	98	100	100	100
Hg	0	70	100	100	100	100	100	100	100	100
Ni	0	0	0	7	10	55	86	100	100	100
Pb	0	0	0	0	0	0	6	20	66	100
Zn	0	0	10	43	84	94	100	100	100	100

All the culture collection strains were relatively tolerant of lead and chromium (except for *H. californiae* for the latter metal). Only the strains belonging to group iii (except for *H. salinarium*) and the *Haloarcula* strains (except for *H. sinaiiensis*) were tolerant of copper. Only three strains (*H. mediterranei*, *H. volcanii*, and *H. salinarium*) were tolerant of arsenate. *H. mediterranei* showed the highest tolerance of cadmium of all the strains tested.

The cumulative percentages of strains susceptible to various concentrations of metal ions are shown in Table 2. A wide range of concentrations was tested because of the paucity of information on the susceptibility of halobacteria to heavy metals. It was hoped that an inhibitory concentra-

TABLE 3. Patterns of tolerance of 10 heavy metal ions in 68 halobacterial strains

No. of different tolerances	Types of tolerance	No. (%) of strains
8	As, Cd, Co, Cr, Cu, Ni, Pb, Zn	1 (1.5)
7	Cd, Co, Cr, Cu, Ni, Pb, Zn	1 (1.5)
6	Cr, Co, Cu, Ni, Pb, Zn	1 (1.5)
5	Co, Cr, Cu, Ni, Pb	7 (10.3)
	As, Cr, Cu, Ni, Pb	2 (2.9)
	As, Cd, Cr, Ni, Pb	1 (1.5)
	As, Cr, Co, Cu, Pb	1 (1.5)
	Cd, Cr, Cu, Ni, Pb	1 (1.5)
	Co, Cr, Cu, Ni, Pb	1 (1.5)
4	Cr, Cu, Ni, Pb	6 (8.8)
	Co, Cu, Ni, Pb	4 (5.9)
	As, Cr, Ni, Pb	1 (1.5)
	Cd, Cr, Cu, Pb	1 (1.5)
	Cr, Cu, Pb, Zn	1 (1.5)
	Cd, Co, Cu, Pb	1 (1.5)
3	Cr, Cu, Pb	12 (17.6)
	Cr, Ni, Pb	4 (5.9)
	As, Cr, Pb	3 (4.5)
	As, Cu, Pb	1 (1.5)
	Cu, Ni, Pb	1 (1.5)
2	Cu, Pb	12 (17.6)
	Cr, Pb	5 (7.5)

tion would be obtained which could be used as a guide for further investigations, despite the fact that the toxicities of some metals could be attributed to metal complex formation. On the other hand, in experiments in which the salt concentration in the test medium was 15, 20, or 30% (wt/vol), instead of the standard 25%, no significant differences in the MICs were observed in the range of 20 to 30% salts. However, at the lowest salt concentration, an enhanced toxicity of the heavy metals tested was found. This result may have been due to a higher availability of cells to take up the metal ions in the medium with the lowest salt concentration (closely related to physiological cellular changes which take place at this low salinity) or to a lower degree of interaction between the free metal ions and the salts present in the medium.

The frequencies of tolerance of each metal ion in all the strains tested were as follows: As, 15%; Ag, 0%; Cd, 9%; Co, 25%; Cr, 72%; Cu, 80%; Hg, 0%; Ni, 45%; Pb, 100%; and Zn, 6%. All halobacteria tested were tolerant of lead, and a great percentage of them were tolerant of even a 10 mM concentration of this metal. Furthermore, a great fraction of the strains were also tolerant of copper and chromium. However, it should be pointed out that this high tolerance of lead, copper, and chromium could have been due to the binding of these metal ions to some of the components of the test medium, as has been reported by Ramamoorthy and Kushner (13). These investigators showed that different components of the medium (yeast extract, peptone, tryptone, etc.) exhibited high binding to lead, copper, and cadmium. Besides, lead can be accumulated in the cell wall and membrane (18). Moreover, it may be that there was much less lead available in the culture medium than initially added, since there was a great amount of sulfate present in the medium. As a result, the soluble lead would be at a lower concentration. Nevertheless, since very little research has been conducted on the genetic basis of lead resistance in bacteria, the tolerance exhibited by halobacteria appears to be an interesting field for additional research.

Otherwise, all the strains demonstrated a high susceptibility to mercury and silver and very little tolerance of zinc, cadmium, and arsenate. Mercury was the agent that showed the highest activity against the halobacteria tested; all were inhibited by a 0.05 mM concentration of this metal ion, and most of them were inhibited by 0.01 mM. The susceptibility of halobacteria to mercury has been previously used as a phenotypic feature in some taxonomic studies on halobacteria (5, 15). However, the concentration of $HgCl_2$ used in those reports was higher than 0.1 mM, a concentration which inhibited all the strains tested. It was reported (1) that, in the presence of high concentrations of NaCl, the toxicity of zinc was increased, and this was the result not of a synergistic interaction between Zn^{2+} and elevated osmotic pressures but of the formation of complex anionic $ZnCl^-$ species, which exerted greater toxicities than did cationic Zn^{2+}. This fact could be responsible for the high susceptibility of halobacteria to zinc.

All the strains tested were tolerant of multiple metal ions: 17 were tolerant of two metal ions (25%), 21 were tolerant of three (30.9%), 14 were tolerant of four (20.7%), 13 were tolerant of five (19.2%), and 1 (1.5%), 1 (1.5%), and 1 (1.5%) were tolerant of six, seven, and eight metal ions, respectively. Strains with triple tolerance were found most frequently, followed by those with double tolerance. However, strains tolerant of five or four different metal ions were significantly detected (about 40% of the total strains). The patterns of tolerance of the heavy metals tested in the 68 halobacterial strains tested are shown in Table 3.

It is well documented that many metal resistances can be governed by plasmid-encoded mechanisms in both gram-negative and gram-positive eubacteria (8, 16, 20). Since we have recently found that 75% of all halobacteria used in this study carry at least one plasmid and that in the majority of these strains three or four megaplasmids can be detected (6), additional experiments to correlate the presence of plasmids and the tolerance of some heavy metal ions in each individual strain are now in progress in our laboratory.

This investigation was supported by grants from the Comisión Asesora para el Desarrollo de la Investigación Científica y Técnica and from the Junta de Andalucía.

LITERATURE CITED

1. **Babich, H., and G. Stotzky.** 1978. Toxicity of zinc to fungi, bacteria, and coliphages: influence of chloride ions. Appl. Environ. Microbiol. **36:**906–914.
2. **Bayley, S. T., and R. A. Morton.** 1978. Recent developments in the molecular biology of extremely halophilic bacteria. Crit. Rev. Microbiol. **6:**151–205.
3. **Bock, A., and O. Kandler.** 1985. Antibiotic sensitivity of archaebacteria, p. 525–544. *In* C. R. Woese and R. S. Wolfe (ed.), The bacteria, vol. 8. Academic Press, Inc. (London), Ltd., London.
4. **Bonelo, G., A. Ventosa, M. Megías, and F. Ruiz-Berraquero.** 1984. The sensitivity of halobacteria to antibiotics. FEMS Microbiol. Lett. **21:**341–345.
5. **Colwell, R. R., C. D. Litchfield, R. H. Vreeland, L. A. Kiefer, and N. E. Gibbons.** 1979. Taxonomic studies of red halophilic bacteria. Int. J. Syst. Bacteriol. **29:**379–399.
6. **Gutierrez, M. C., M. T. García, A. Ventosa, J. J. Nieto, and F. Ruiz-Berraquero.** 1986. Occurrence of megaplasmids in halobacteria. J. Appl. Bacteriol. **61:**67–71.
7. **Harnett, N. M., and C. L. Gyles.** 1984. Resistance to drugs and heavy metals, colicin production, and biochemical characteristics of selected bovine and porcine *Escherichia coli* strains. Appl. Environ. Microbiol. **48:**930–935.
8. **Kelly, W. J., and D. C. Reanney.** 1984. Mercury resistance among soil bacteria: ecology and transferability of genes encoding resistance. Soil Biol. Biochem. **16:**1–8.
9. **Kushner, D. J.** 1985. The Halobacteriaceae, p. 171–214. *In* C. R. Woese and R. S. Wolfe (ed.), The bacteria, vol. 8. Academic Press, Inc. (London), Ltd., London.
10. **Larsen, H.** 1984. Family *Halobacteriaceae*, p. 261–267. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
11. **Nakahara, H. T., T. Ishikawa, Y. Sarai, I. Kondo, and H. Kozukue.** 1978. Survey of resistance to metals and antibiotics in clinical isolates of *Klebsiella pneumoniae* in Japan. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **240:**22–29.
12. **Pecher, T., and A. Bock.** 1981. In vivo susceptibility of halophilic and methanogenic organisms to protein synthesis inhibitors. FEMS Microbiol. Lett. **10:**295–297.
13. **Ramamoorthy, S., and D. J. Kushner.** 1975. Binding of mercury and other heavy metal ions by microbial growth media. Microb. Ecol. **2:**162–176.
14. **Riley, T. V., and B. J. Mee.** 1982. Susceptibility of *Bacteroides* spp. to heavy metals. Antimicrob. Agents Chemother. **22:**889–892.
15. **Rodriguez-Valera, F., G. Juez, and D. J. Kushner.** 1983. *Halobacterium mediterranei* spec. nov., a new carbohydrate-utilizing extreme halophile. Syst. Appl. Microbiol. **4:**369–381.
16. **Silver, S.** 1981. Mechanisms of plasmid-determined heavy metal resistances, p. 179–189. *In* S. Levy, R. Glowes, and E. Koenig (ed.), Molecular biology, pathogenicity and ecology of bacterial plasmids. Plenum Publishing Corp., New York.

17. **Steers, E., L. Foltz, and B. S. Graves.** 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. **9:**307–311.
18. **Tornabene, T. G., and H. W. Edwards.** 1972. Microbial uptake of lead. Science **196:**1334–1335.
19. **Torreblanca, M., F. Rodriguez-Valera, G. Juez, A. Ventosa, M. Kamekura, and M. Kates.** 1986. Classification of non-alkaliphilic halobacteria based on numerical taxonomy and description of *Haloarcola* gen. nov. and *Haloferax* gen. nov. Syst. Appl. Microbiol. **8:**89–99.
20. **Trevors, J. T., K. M. Oddie, and B. H. Belliveau.** 1985. Metal resistance in bacteria. FEMS Microbiol. Rev. **32:**39–54.
21. **Washington, J. A., II, and V. L. Sutter.** 1980. Dilution susceptibility test: agar and macro-broth dilution procedures, p. 453–458. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd. ed. American Society for Microbiology, Washington, D.C.
22. **Woese, C. R., and G. E. Fox.** 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc. Natl. Acad. Sci. USA **74:**5088–5090.