INTERNATL MICROBIOL (1998) 1: 45-51

RESEARCH ARTICLES

Mercedes Ríos
Joaquín J. Nieto
Antonio Ventosa

Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, Spain

Numerical taxonomy of heavy metal-tolerant nonhalophilic bacteria isolated from hypersaline environments

Received 15 September 1997
Accepted 15 January 1998

Correspondence to:
Antonio Ventosa.
Departamento de Microbiología y Parasitología.
Facultad de Farmacia. Universidad de Sevilla.
41012 Sevilla. Spain.
Tel: +34-954556765. Fax: 34-954628162.
E-mail: ventosa@cica.es

Summary A total of 232 metal-tolerant bacterial strains were isolated from water and sediment samples collected in different hypersaline environments located in Cádiz, Huelva and Morón de la Frontera (Spain). They were isolated on a medium containing mercury, chromium, cadmium, copper or zinc. These halotolerant isolates were analyzed by numerical taxonomy techniques by using the simple matching (S_{SM}) and Jaccard (S_J) coefficients; clustering was achieved using the unweighted pair group method with averages (UPGMA) algorithm. At the 81% and 83% similarity level, different numbers of phenons were obtained for Gram-negative and Gram-positive halotolerant microorganisms. Most of the 48 Gram-negative metal-tolerant strains studied were grouped into nine phenons, representing the genera *Pseudoalteromonas*, *Alteromonas*, *Xanthomonas*, *Pseudomonas*, *Alcaligenes* and Enterobacteria. The 72 Gram-positive metal-tolerant strains grouped into eight phenons, with only 15 strains left unassigned. Most of the isolates were assigned to the genus *Bacillus* (seven phenons), and one phenon comprised microorganisms with phenotypic characteristics similar to those of the genus *Celullomonas*.

Key words Halotolerant bacteria · Heavy metal tolerance · Numerical taxonomy · Salterns · Hypersaline environments in Spain

Introduction

Hypersaline environments are extreme habitats in which two main groups of microorganisms are well adapted to grow: the extremely halophilic archaea and the moderately halophilic bacteria [19]. The halotolerant bacteria have not yet been studied in detail, and it is generally accepted that they do not play a major role in these environments [14, 17]. Besides their high salinity, hypersaline environments may have high concentrations of other compounds, such as heavy metals, that strongly influence microbial populations growing there. To thrive in such environments, microorganisms must be adapted to both high salt and metal concentrations.

So far, very little information is available on the mechanisms of tolerance to heavy metals in bacteria adapted to live in hypersaline habitats. However, such information is desirable, as some of these metal-resistant bacteria might be used as bioassay indicator organisms in saline aquatic polluted environments or as biological detoxicants. Besides, the isolation and study of metal-resistant microorganisms might provide useful information about the ecological role of these bacteria in heavy metal transformations in natural hypersaline environments.

High levels of heavy metals can affect both the qualitative and the quantitative structure of microbial communities. Several studies have reported that metals influence microorganisms by affecting adversely their growth, morphology, and biochemical activities, resulting in decreased biomass and diversity [18]. However, microorganisms have evolved resistance mechanisms to deal with metal toxicity, which include volatilization, extracellular precipitation and exclusion, binding to the cell surface, and intracellular sequestration [20]. The isolation and identification of microorganisms capable of efficient uptake of metals of environmental, economical significance is the pragmatic goal of current research on biological metal removal from treatable sources [25].

The aim of this study was to isolate and characterize taxonomically heavy metal-tolerant bacteria from hypersaline environments polluted by heavy metals which are common industrial pollutants.

Materials and methods

Isolation of strains A total of 232 metal-tolerant bacterial strains were isolated from water of solar salterns and hypersaline soils located in Cádiz, Huelva and Morón de la Frontera (Spain). Samples were collected in sterile plastic containers and were plated not later than 6 h after collection.

All strains were isolated on a medium containing 0.5% (w/v) yeast extract (Difco) and the corresponding concentration of the each heavy metal [15]. The pH of each medium was adjusted to 7.2. The medium was solidified by adding 20 g per liter of Bacto-Agar (Difco). The inoculated samples were incubated at 28°C for 7 days. The five heavy metals tested were purchased from Sigma Chemical Co. (St. Louis, MO, USA) as cadmium chloride, potassium chromate, copper sulfate, mercuric chloride and zinc chloride. Stock solutions were made in distilled water and were sterilized by filtration through 0.22 µm size membrane filters (Millipore Corp., Bedford, MA, USA). These solutions were kept at 4°C for no longer than 1 day. The concentrations of the metals used were as follow (mM): Hg: 0.1; Zn: 2.5; Cd: 5; Cu: 2.5 and Cr: 20. These concentrations have been suggested in previous studies for the isolation of metal-tolerant strains [15]. Colonies were isolated from the plates and successively subcultured in the same isolation medium to ensure purity.

Reference strains The following reference strains were also included in this study: *Pseudomonas nautica* ATCC 27132^T, *Halomonas marina* ATCC 25374^T, *Vibrio mediterranei* ATCC 43341^T, *Vibrio splendidus* ATCC 33789, *Alteromonas macleodii* ATCC 27126^T, *Pseudoalteromonas haloplanktis* ATCC 14393^T, *Bacillus insolitus* ATCC 23299^T, *Bacillus licheniformis* ATCC 14580^T, *Bacillus pumilus* ATCC 7061^T, *Bacillus coagulans* ATCC 7050^T, *Bacillus pantothenticus* ATCC 14576^T, *Bacillus circulans* ATCC 4513^T, *Bacillus subtilis* ATCC 6051^T, *Bacillus megaterium* ATCC 14581^T and *Bacillus marinus* DSM 1277^T.

Characterization of isolates A total of 107 phenotypic characteristics were determined for each strain. The characteristics were as follows: Gram reaction, cell morphology, endospore formation, motility, growth at 0, 0.5, 3, 5, 10, 15, 20, 25 and 30% (w/v) salts concentrations, anaerobic growth, catalase and oxidase production, H_2S production, hydrolysis of gelatin, casein, starch and Tween 80 [24]. A total of 95 nutritional tests were performed by using the Biolog automatic identification system (Biolog Inc., Hayward, USA). Gram-negative strains were grown on isolate medium and the Gram-positive on the BUGM complex medium, at 28°C for 24 h, and suspended in prewarmed sterile saline solution (0.85% NaCl), within the density range specified with a Biolog photometer model 21101. Immediately after suspending the cells in the saline solution, the suspensions were transferred into sterile multichannel pipetter reservoirs and the Biolog GP (for Gram-positive) and GN (for Gram-negative) MicroPlates inoculated with 125 µl of the cell suspension per well by means of an 8-channel repeating pipetter. The inoculated Biolog plates were incubated at 28°C for 24 h and the results were read with a MicroPlate Reader using a Microlog 3.50 computer software to perform automated reading.

Numerical analysis The 104 differential features obtained were used for a numerical analysis. Positive and negative results were coded as 1 and 0, respectively; noncompatible or missing data were coded as 9. Strain similarities were estimated separately for each physiological group with both simple matching [23] and Jaccard [11] coefficients, and clustering was achieved by unweighted average linkage [22]. Cophenetic correlation was also obtained in each method [22]. The test error was evaluated by examining 10 strains in duplicate [21]. These computations were performed with the NT-SYS program of F. J. Rolf, State University of New York, Stony Brook, NY (USA), using a VAX-785 computer in the Computer Centre, University of Sevilla, Spain.

Results and discussion

Since the Biolog plates for the nutritional tests are different for Gram-negative and Gram-positive bacteria, we selected randomly 48 Gram-negative and 72 Gram-positive strains for the numerical analysis.

We have isolated 232 strains tolerant to at least one of the heavy metals tested. Cr and Zn were the most tolerated heavy metals: 43% of strains were tolerant to Cr, and 21% to Zn. Hg was tolerated by 19% of strains, Cu by 10% and Cd by 8%. In metal-stressed media, the total numbers of bacteria were 10^3 to 10^4 times lower than without metal stress. This effect had been already reported by others authors [7, 10, 18]. Microorganisms can be classified into different categories on the basis of their optimal growth rates at different salinities [13]. According to this classification, the 232 isolated strains are halotolerant microorganisms (they grow optimally in media containing less than 0.2 M salt, but can tolerate high concentrations of salts).

Of the 232 isolates, 136 strains were Gram-positive and 96 Gram-negative. In nature, metal tolerance is more difficult to define because, in mixed microbial communities, a metal concentration which requires tolerance for one given group of organisms may fall within the normal physiological range of another. Gram-negative bacteria show more tolerance to heavy metals than their Gram-positive counterparts and tend to be the predominant prokaryotes in metal-polluted environments, due to the structure and composition of their cell walls [6]. However, in this study Gram-positive was the predominant group (58,6%). The high percentage of endospore-forming bacteria that we have isolated might account for this.

The numerical study of the 48 Gram-negative selected strains using the simple matching (S_{SM}) coefficient, clustered by the unweighted average linkage method (UPGMA), gave the dendrogram shown in Fig. 1. At 81% similarity level, nine

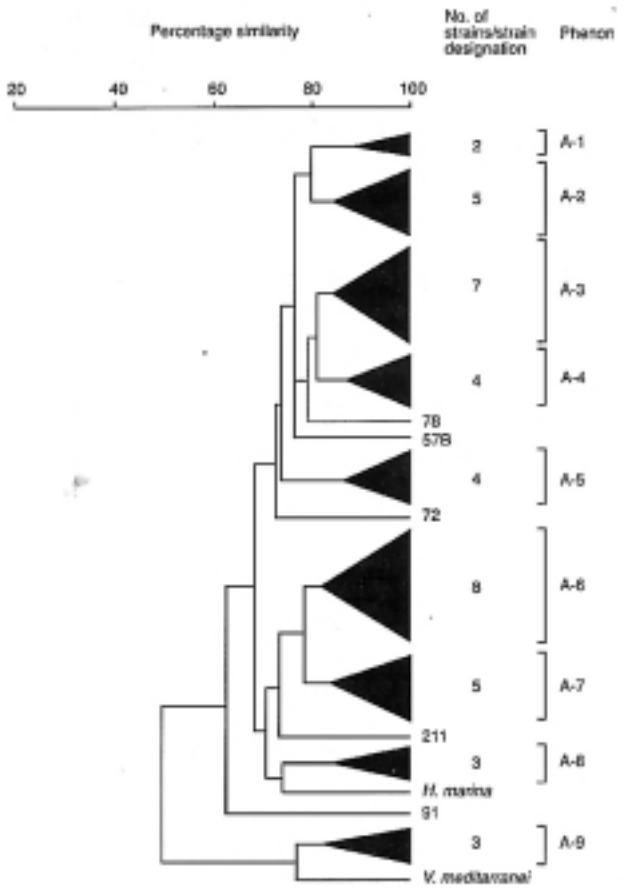

Fig. 1 Simplified dendogram showing the clustering of strains into 9 phenons based on the S_{SM} coefficient and unweighted average linkage clustering (UPGMA), for 48 non-halophilic metal-tolerant Gram-negative bacteria

phenons were defined; 7 strains did not cluster at this similarity level. The estimated test error was lower than 3.5%, which would not affect significantly the cluster analysis. The cophenetic correlation was 0.825. The Jaccard coefficient (S_J) was also used, but the cluster composition was not markedly affected. Table 1 shows the tests applied and the frequencies of positive characters that differentiate the nine phenons of Gram-negative non-halophilic metal-tolerant bacteria.

Phenon A-1 The two strains included in this phenon clustered at a similarity level of 86%. The reference strain *Vibrio splendidus* biotype 2 ATCC 33789 was included in this phenon. They were motile, facultatively anaerobic and produced H_2S. Gelatin, casein, starch and Tween 80 were hydrolized. The main differences between *Vibrio splendidus* ATCC 33789 and our isolate was the different utilization of organic compounds tested and the positive result of the oxidase test for *V. splendidus*, which was negative for our isolate. Recently, Ortigosa [16] reported that *V. splendidus* is very abundant in marine habitats.

Phenon A-2 This group included five strains which were Gram-negative, strictly aerobic (except one strain), and both oxidase and catalase positive. The strains were able to hydrolyze gelatin, casein and starch and produced H_2S. Each strain was tolerant to a different single metal: mercury, chromium or copper. According to their phenotypic features, the strains of this group could be assigned to the genera *Pseudoalteromonas* or *Alteromonas* [9].

Phenons A-3 and A-5 These phenons included seven and four strains, respectively. They produced colonies strongly yellow-pigmented. They were Gram-negative motile rods, strictly aerobic (except one strain) and oxidase negative. Most of the strains were isolated from samples collected in an inland saltern located in Morón de la Frontera (Sevilla). Gelatin and casein were hydrolyzed but neither starch nor Tween 80 were. The strains included in phenon A-5 did not produce H_2S (whereas those of phenon A-3 produced H_2S) and they showed more nutritional versatility than the strains of phenon A-3. According to their characteristics, the strains of those phenons could be placed in the genus *Xanthomonas* [3]; however, this genus comprises plant pathogens and their relation with marine environments is not yet known. Further research is in progress to define properly their taxonomic position.

Phenon A-4 This phenon contained four related strains at a similarity level of 86%. They were tolerant to chromium, motile, facultatively anaerobic and oxidase negative. Gelatin and starch were hydrolyzed. They were neither caseinolytic nor lipolytic, and used many organic compounds for growth. All the isolates of this phenon were considered to be members of the *Enterobacteriaceae* [4]. These strains were isolated from sediments collected in the area of San Fernando (Cádiz). The high volume of sewage discharged by the urban area directly or indirectly into marine environments would explain the presence of these microorganisms in hypersaline environments that receive seawater from these coastal areas.

Phenon A-6 Eight strains were clustered in this group, which included the reference strain *Pseudomonas nautica* ATCC 27132^T. Most strains were tolerant to mercury. They were motile, oxidase positive and strictly aerobic (except strain 373), and showed a low, heterogeneous nutritional response. The main difference between the reference strain and our isolates was that the former did not produce H_2S, and used only 8 of the 95 compounds tested, which were different from those used by our isolates. This group does not fit in any previously described bacterial genus.

Phenons A-7, A-8 and A-9 These phenons included five, three, and three strains, respectively, which showed very similar phenotypic characteristics. They were motile, oxidase positive and strictly aerobic (except one strain); gelatin was not hydrolyzed, except for strains of the phenon A-8. All could use the following compounds as sole carbon and energy source: Tween 40, Tween 80, methylpyruvate, acetic acid, α-ketobutyric acid, L-alanine, L-glutamic acid and L-proline. Most strains of phenon A-7 were tolerant to mercury; all strains of phenon A-8 were tolerant to zinc and two of the three strains of phenon A-9 were tolerant to zinc and the other one to mercury. On the basis of their features, these strains could be assigned to the

Table 1 Percentages of positive phenotypic characteristics found in each phenon which groups 41 non-halophilic metal-tolerant Gram-negative strains

Characteristic	Phenon	A-1	A-2	A-3	A-4	A-5	A-6	A-7	A-8	A-9
	No. strains	2	5	7	4	4	8	5	3	3
Motility		100	80	100	100	100	100	100	100	100
Oxidase		50	100	0	17	0	100	100	66	100
Anaerobic growth		100	20	14	83	0	12	0	33	0
H_2S production		ND	100	43	83	0	75	100	100	100
Hydrolisis of:										
Starch		100	100	14	100	0	12	0	0	0
Gelatin		100	100	71	83	100	62	60	100	0
Casein		ND	100	57	83	100	25	20	66	0
Tween 80		100	80	0	17	0	12	20	0	0
Utilization of:										
Dextrin		100	100	86	100	100	0	0	33	100
Gentibiose		100	0	86	100	100	0	0	33	0
α-D-glucose		100	100	100	83	100	12	0	33	100
Maltose		100	100	86	0	100	0	0	33	100
Acetic acid		0	20	0	0	0	12	80	100	100
Malonic acid		0	0	0	0	0	0	0	100	0
Succinic acid		0	0	0	0	0	0	0	100	100
L-alanine		100	100	0	0	0	0	100	100	100
L-alanyl-glicine		100	100	0	0	100	12	0	100	100
L-histidine		0	0	0	0	0	0	0	100	100
Putrescine		0	0	14	0	100	0	0	0	0
D,L-α-glicerol phosphate		0	0	0	0	25	0	0	0	100
Glucose-1-phosphate		0	0	0	0	0	0	0	0	100
Glucose-6-phosphate		0	0	0	0	0	0	0	0	100

ND, not determined

Table 2 Phenotypic characteristics that differentiate the eight phenons which groups 72 non-halophilic metal-tolerant Gram-positive strains

Characteristic	Phenon	B-1	B-2	B-3	B-4	B-5	B-6	B-7	B-8
	No. strains	25	3	7	6	3	3	9	3
Endospore formation		80	66	100	100	100	100	100	33
Motility		92	66	57	100	33	33	55	66
Growth at 10% salts		52	0	25	33	0	50	22	0
Oxidase		92	66	0	0	0	0	100	100
Anaerobic growth		32	66	71	33	100	33	33	66
H_2S production		92	100	29	100	33	0	66	100
Hydrolisis of:									
Starch		68	66	71	50	100	33	44	33
Gelatin		92	100	57	100	100	33	78	66
Casein		80	100	14	66	33	66	11	33
Tween 80		12	66	14	50	0	0	33	0
Utilization of:									
D-fructose		4	0	100	100	100	100	100	100
α-D-glucose		12	0	100	100	100	100	89	100
D-mannose		8	0	100	100	100	100	100	100
β-methyl-D-glucoside		0	0	86	83	100	0	100	0
Salicin		0	0	100	100	100	100	100	100
Turanose		4	0	100	100	100	100	100	0
D-malic acid		0	100	0	0	0	0	0	100
N-acetyl-L-glutamic acid		0	100	14	0	0	0	100	100
Uridine		12	0	14	100	100	100	100	100
Uridine-5'-monophosphate		8	0	14	0	0	0	100	100
Glucose-1-phosphate		0	0	0	0	0	0	0	100

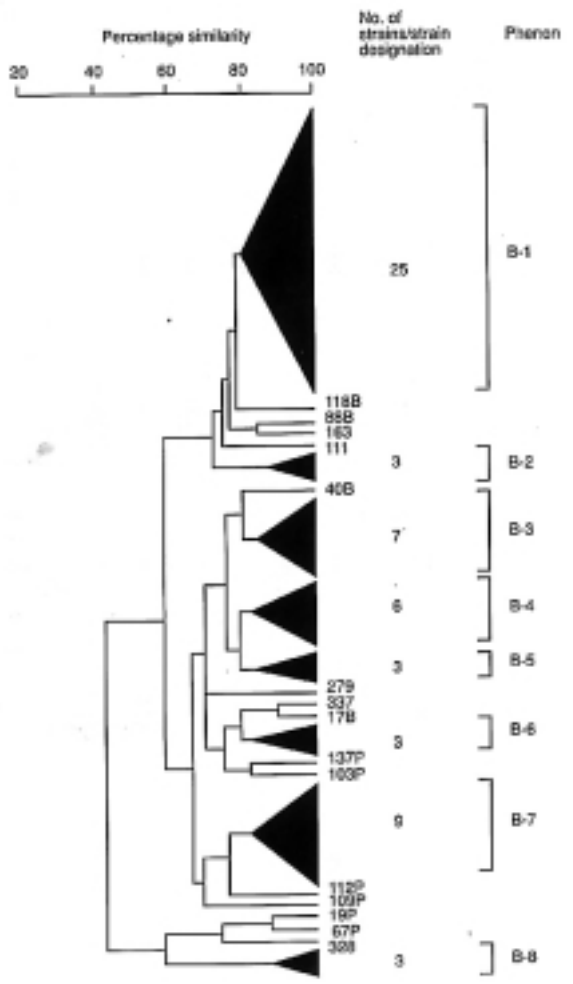

Fig. 2 Simplified dendogram showing the clustering of strains into 8 phenons based on the S_{SM} coefficient and unweighted average linkage clustering (UPGMA), for 72 non-halophilic metal-tolerant Gram-positive bacteria

suprageneric grouping *Pseudomonas-Alcaligenes* [9]. However, there are many species with similar phenotypic features, which make it difficult to assign them to any specific species of these genera; their identification would require further studies.

From a total of 145 Gram-positive, non-halophilic, metal-tolerant strains, we selected randomly 72 strains for the numerical analysis. The results of the study of the features of the strains grouped by means of the S_{SM} coefficient and UPGMA clustering yielded the dendrogram shown in Fig. 2. The cophenetic correlation was 0.793. Most of the strains were grouped into eight phenons at 83% similarity level; 13 strains did not clustered in any of these phenons. Similar groupings were obtained when the S_J coefficient was used. The differential characteristics of the eight phenons are summarized in Table 2.

The strains included in phenons B-1 to B-7 were Gram-positive, spore-forming, catalase positive and strictly aerobic or facultatively anaerobic rods, and they were assigned to the genus *Bacillus* [5].

Phenon B-8 included three non spore-forming Gram-positive rods. Most strains were motile. They were facultatively anaerobic, produced H_2S and oxidase negative. All of them could use many compounds as sole carbon and energy source. These strains could not be assigned to any previously validly described genus.

A numerical taxonomy study of metal-tolerant bacteria isolated from an estuary in *Chesapeake Bay (USA)* was carried out by Austin et al. [2]. The isolation medium (glucose-tryptone-yeast extract agar prepared in an estuarine salts solution) contained Co, Pb, or Mo at a concentration of 10 mg/l. Mercuric chloride was used at a concentration of 10 mg/l. A 97% of the bacterial isolates were grouped in 12 phenons. The predominant metal-tolerant organisms were species belonging to *Bacillus*, *Erwinia*, *Mycobacterium*, *Pseudomonas* and coryneforms. Nevertheless, our results are somewhat different from those obtained by these authors, since *Pseudomonas*, *Pseudoalteromonas*, *Alteromonas*, *Bacillus*, *Vibrio* and enterobacteria were the predominant groups we found.

Microorganisms have many mechanisms for their successful adaptation to the presence of heavy metal ions [8]. Among these mechanisms, hydrogen sulfide production may act as a detoxification process through the precipitation of metal sulfides [1]. The high number of H_2S-producing isolates recovered in this study may indicate that this mechanism is involved. This finding has also been reported by Jeanthon and Prieur [12], who studied the diversity of metal-tolerant bacteria isolated from a hydrothermal vent.

Acknowledgements This study was supported in part by grants from the European Commission, Generic Projects Biotechnology of Extremophiles (BIO-CT93-0273) and Extremophiles as Cell Factories (BIO4-CT96-0488), from the Ministerio de Educación y Ciencia, Spain (PB93-0920), and from the Junta de Andalucía.

References

1. Aiking HA, Stijnman C, van Gaederen H, van Heerikhuizen, Van't Riet J (1984) Inorganic phosphate accumulation and cadmium detoxification in *Klebsiella aerogenes* NCTC 418 growing in continuous culture. Appl Environ Microbiol 47:374-377
2. Austin B, Allen DA, Mills AL, Colwell RR (1977) Numerical taxonomy of heavy metal-tolerant bacteria isolated from an estuary. Can J Microbiol 23:1433-1447
3. Bradbury JF (1984) Genus II *Xanthomonas*. In: Krieg NR, Holt JG (eds) Bergey's Manual of Systematic Bacteriology. Vol 1. Baltimore: Williams & Wilkins, pp 199-203
4. Brenner J (1984) Family Enterobacteriaceae. In: Krieg NR, Holt JG (eds) Bergey's Manual of Systematic Bacteriology. Vol 1. Baltimore: Williams & Wilkins, pp 408-516
5. Claus D, Berkeley RCW (1986) Genus *Bacillus* Cohn 1872. In: Sneath PHA (ed) Bergey's Manual of Systematic Bacteriology. Vol 2. Baltimore: Williams & Wilkins, pp 1105-1139

6. Duxbury T (1986) Microbes and heavy metals: an ecological overview. Microbiol Sci 8:336-339
7. Frostegard A, Tunlid A, Baath E (1993) Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. Appl Environ Microbiol 59:3605-3617
8. Gadd GM, Griffiths AJ (1978) Microorganisms and heavy metals toxicity. Microb Ecol 4: 303-317
9. Gauthier G, Gauthier M, Christen R (1995) Phylogenetic analysis of the genera *Alteromonas*, *Shewanella* and *Moritella* using genes coding for small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (Emended) and *Pseudoalteromonas* gen. nov., and proposal of twelve new species combinations. Int J Syst Bacteriol 45:755-761
10. Hattori H (1992) Influence of heavy metals on soil microbial activities. Soil Sci Plant Nutr 38:93-100
11. Jaccard P (1908) Nouvelles recherches sur la distribution florale. Bull Soc Vaudoise Sci Nat 44:223-270
12. Jeanthon C, Prieur D (1990) Susceptibility to heavy metals and characterization of heterotrophic bacteria isolated from two hydrothermal vent polychaete annelids, *Alvinella pompejana* and *Alvinella caudata*. Appl Environ Microbiol 56:3308-3314
13. Kushner DJ (1985) The Halobacteriaceae. In: Woese CR, Wolfe RS (eds) The Bacteria. Vol VIII: Archaebacteria. Orlando: Academic Press, pp 171-214
14. Márquez MC, Ventosa A, Ruiz-Berraquero F (1987) A taxonomic study of heterotrophic halophilic and non-halophilic bacteria from a solar saltern. J Gen Microbiol 133: 45-46
15. Nieto JJ (1991) The response of halophilic bacteria to heavy metals. In: Rodríguez-Valera F (ed) General and Applied Aspects of Halophilic Microorganisms. New York: Plenum Press, pp 173-179
16. Ortigosa M (1995) Bacterias heterótrofas aerobias y facultativas asociadas a aguas y ostras en el Mar Mediterráneo: Abundancia, ciclo anual y taxonomía numérica. PhD Thesis. University of Valencia.
17. Quesada E, Ventosa A, Ruiz-Berraquero F, Ramos-Cormenzana A (1982) Types and properties of some bacteria isolated from hypersaline soils. J Appl Bacteriol 53:155-161
18. Roane TM, Kellogg ST (1996) Characterization of bacterial communities in heavy metal contaminated soils. Can J Microbiol 42:593-603
19. Rodríguez-Valera F, Ventosa A, Juez G, Imhoff JF (1985) Variation of environmental features and microbial populations with salt concentrations in a multipond saltern. Microb Ecol 11:107-115
20. Silver S (1992) Plasmid-determined metal resistance mechanisms: range and overview. Plasmid 27:1-3
21. Sneath PHA, Jonhson R (1972) The influence on numerical taxonomic similarities of errors in microbiological tests. J Gen Microbiol 72:377-392
22. Sneath PHA, Sokal RR (eds) (1973) Numerical Taxonomy. The Principles and Practice of Numerical Classification. San Francisco: Freeman
23. Sokal RR, Michener CD (1958) A statistical method for evaluating systematic relationships. Univ Kansas Sci Bull 38:1409-1438
24. Ventosa A, Quesada E, Rodríguez-Valera, F, Ruiz-Berraquero F, Ramos-Cormenzana A (1982) Numerical taxonomy of moderately halophilic Gram-negative rods. J Gen Microbiol 128:1959-1968
25. Unz RF, Shuttleworth KL (1996) Microbial mobilization and immobilization of heavy metals. Curr Op Biotechnol 7:307-310