BACILLUS SPECIES IN THE OCEANIC WATERS ADJACENT TO CUBA: ASSOCIATION BETWEEN THEIR DISTRIBUTION AND METABOLIC ACTIVITY

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ABSTRACT

The aim of the present work was to identify spore-forming Gram-positive *Bacillus* strains isolated from oceanic waters adjacent to Cuba and to establish a possible relationship between their distribution and the metabolic activity of the isolates. Total protein patterns, derived from SDS-PAGE, were used to build a non-rooted dendrogram where the strains appeared clustered in three nodes. No direct relationship was observed between a node and particular species. In contrast, according to the physical and chemical characteristics of the zones, node I was different from node II and III, since it comprises strains from the farthest zones of the coast, poorer in nutrients. On the other hand, nodes II and III mainly collect strains isolated from nutrient and organic matter-enriched zones. Associating the node clustering with metabolic activities, it was found that in node I the ratio: number of positive activities/strain was 2.3, followed by node II with a ratio of 3.3, and finally node III exhibiting a ratio equal to 3.7. This could suggest that different total protein patterns in bacteria belonging to the same specie, but coming from environments with different degree of nutrient richness, could be an indicator of the capacities of these microorganisms to adapt and live in different environments.

Keywords: *Bacillus,* distribution, metabolic activity, oceanic waters, Cuba.

RESUMEN

El objetivo del presente trabajo fue la identificación de cepas de bacilos Gram-positivos esporulados aislados de las aguas oceánicas adyacentes a Cuba y el establecimiento de la posible relación entre la distribución y las actividades metabólicas de los aislados. A partir del patrón de proteínas obtenido mediante electroforesis SDS-PAGE, se construyó un dendrograma no enraizado, lo que permitió la agrupación de las cepas en tres nodos. No se observó una relación directa entre un nodo y especies particulares, sin embargo, se encontraron diferencias entre los nodos al considerar las características físicas y químicas de las zonas; el nodo I fue diferente a los nodos II y III. En ese nodo se agruparon bacterias aisladas de zonas alejadas de la costa, pobres en nutrientes. Por otro lado, los nodos II y III agrupan principalmente a cepas aisladas de aguas más enriquecidas en nutrientes y materia orgánica. Con respecto a la asociación de los aislados en los nodos con las actividades metabólicas, se encontró que la razón entre el número de actividades positivas y el número de aislados fue de 2.3 en el nodo I, seguida del nodo II con 3.3 y por último el nodo III con 3.7. Estos resultados sugieren que, los patrones diferentes de proteínas totales en bacterias que pertenecen a la misma especie, pero que provienen de ambientes con diferente grado de riqueza en nutrientes, podrían ser un indicador de las capacidades de estos microorganismos para adaptarse y vivir en diferentes ambientes.

Palabras claves: Distribución, *Bacillus,* actividad metabólica, aguas oceánicas, Cuba.

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INTRODUCTION

Marine microorganisms comprise a complex and diverse group of microscopic forms of life that over evolution have developed metabolic and physiological capacities ensuring survival in extreme environments, endowing them the potential to produce metabolites that have not been found in terrestrial isolates (Fenical, 1996; Faulkner, 2002).

Microorganisms inhabiting the marine ecosystem which is characterized by particular and extremely changing conditions have a complex and finely tuned metabolic system allowing them to adapt to constant changes. Therefore, distribution and genus composition of marine microbiota differ according to the precise habitat being influenced by factors such as temperature, salinity, organic matter concentration and depth, among others (Pinet, 1998). The specificity of some microbial groups of related related to some biotic and/or abiotic factors confer them the possibility to be used as indicators of water masses, as in the case of luminescent bacteria (Pérez-Nieto, 2001). Bacterial abundance and diversity of species in the water column vary at a millimetric scale, apparently in response to the heterogeneity in distribution of organic matter. Furthermore, interaction bacteria-bacteria may also contribute to variations in the community at a microscale (Long & Azam, 2001). The aim of the present work was the identification of spore-forming Gram-positive bacillus strains isolated from oceanic waters adjacent to Cuba, and to establish a possible relationship between their distribution and the metabolic activity of the isolates.

MATERIALS AND METHODS

Samples were collected in March-April 2005 and February 1997 in the oceanic waters around Cuba. In both cruisers, 80 stations, 40 in the north and 40 in the south, were sampled (Fig. 1).

Water samples were collected at a sub-superficial level (50 cm) with Nansen oceanographic bottles (1.5 L) and cultured by spread plate technique in Petri dishes containing Marine agar 2216 E (Oppenhaimer & ZoBell, 1952). After incubation for 72 h at 28 ± 2°C, the most representative colonies of each sampled zone were selected according to the number and frequency of appearance and isolated by the exhaustion procedure in the same agarized culture medium. Purity of cultures was verified by Gram staining (Harrigan & Mc. Cance, 1968). Strains were conserved by regular subcultures under sterile mineral oil and refrigeration (5°C-10°C), as described by Malik (1992).

The taxonomic position of the strains was determined according to the morphological and cultural characteristics of the colonies (Harrigan & Mc. Cance, 1968), as well as to the physiological and biochemical properties of the strains (Harrigan & Mc. Cance, 1968; Buchanan & Gibbons, 1974; Kreig & Holt, 1984; Sneath *et al.* 1986; Barrow & Feltham, 1993, DSMZ (2004).

To understand how the sporulated Gram-positive bacteria (G+) could be grouped, the total-protein pattern was analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (Laemmli, 1970).

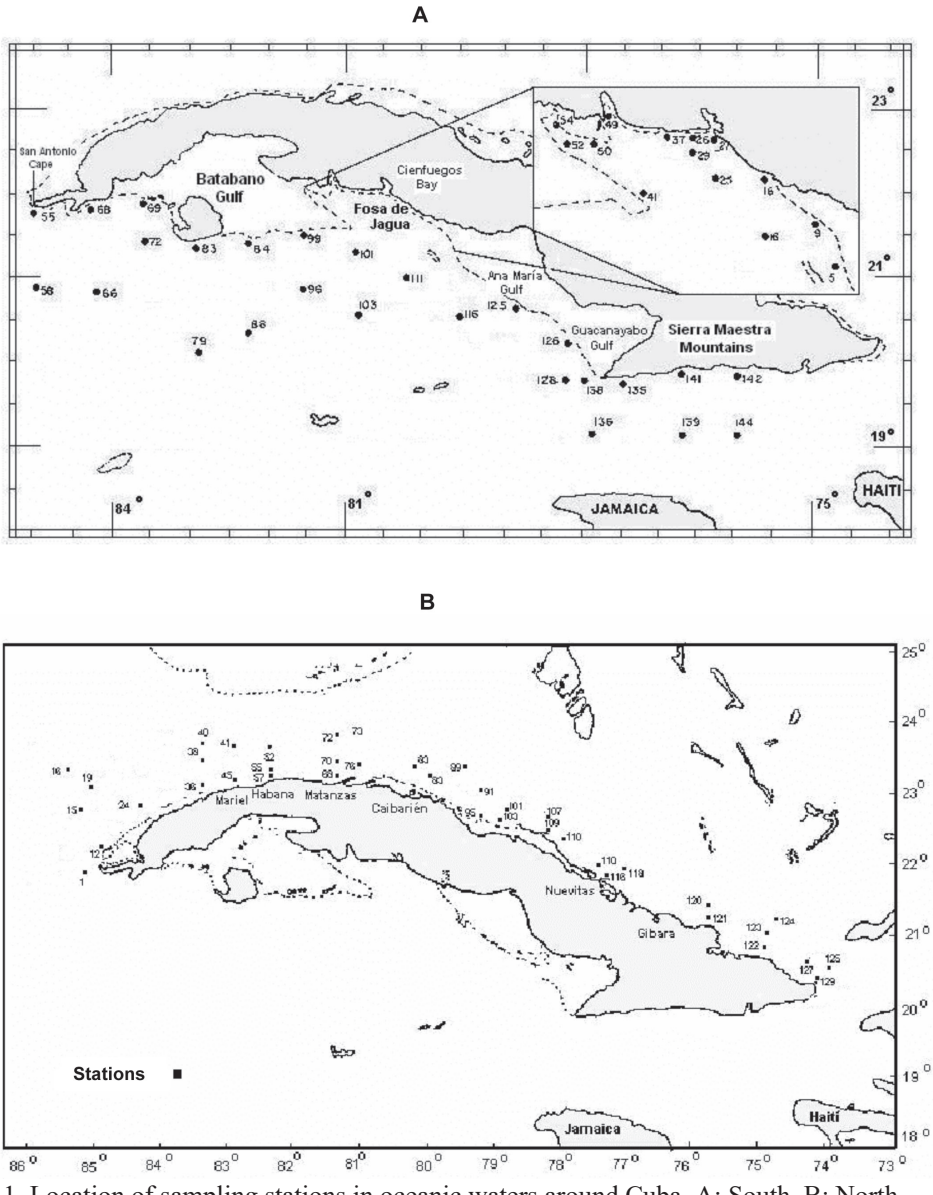

Fig. 1. Location of sampling stations in oceanic waters around Cuba. A: South, B: North

Fig. 1. Ubicación de las estaciones de muestreo en las aguas oceánicas adyacentes a Cuba. A: Sur, B: Norte

Analysis of the electrophoretic pattern was carried out using the arbitrary index:

SI = 2 (number of common bands)/ number of total bands among the compared species. It was assumed that total protein bands of similar mobility were equivalent (Vaughan & Denford, 1968; Espinosa *et al.* 1990). Similarity indexes were converted into standard distances by analogy with the genetic distances discussed by Nei (1972) using the following transformation: D = -ln SI.

Construction of the radial tree with distance measurements was performed

using UPGMA algorithm included in the statistical package PHYLIP 3.5 (Felsenstein, 1993); trees were visualized using the program TREE VIEW (Page, 1996).

Considering all the isolates, the proportion of strains exhibiting positive biological activities were classified in three qualitative categories: *higher*, *equal* or *lower* resulting from comparing nodes II vs. I, III vs. II and III vs. I using a Chi^2 test of independence through the program CHIRXC (Zaykin & Pudovkin, 1993).

RESULTS AND DISCUSSION

In order to integrally characterize an ecosystem it is essential to assess both its structure and functioning, to this end, this research has analyzed several aspects of bacterial diversity. From the Economical Exclusive Zone (EEZ) of Cuba, 80 bacterial strains representative of the oceanic waters in the studied season were isolated, 56 of them from the **S** and 24 from the **N**. The largest proportion of the total isolated strains (68.7%) corresponded to Gram-positive (G+) bacteria and according to the results of the physiological-biochemical characterization, 39 strains belong to the family *Bacillaceae*, particularly to the genus *Bacillus* (Table 1 and Fig. 2).

From the isolates (80), 48.7% belong to *Bacillus* genus; from this genus, 13 isolates were identified to species level and 8 were only identified as *Bacillus* spp. (Table 1). Studies of bacterial taxonomy in sea samples have demonstrated that from the total isolates, the proportion of G+ bacteria is relatively high (> 5%) (Cavallo *et al.* 1999; Siefert *et al.* 2000; Miravet *et al.* 2001; Gontang *et al.* 2007).

The most represented species of the genus *Bacillus* were: *B. licheniformis, B. firmus,* and *B. pumilus* (Fig. 2). Siefert *et al.* (2000) in a study of bacterial taxonomy in the Gulf of Mexico also found that *Bacillus* was the most represented genus, however, there was a lower specie diversity since only *Bacillus firmus, B. megaterium, B. pumilus* and *B. sphaericus* were reported.

The species *Bacillus cereus, B. circulans, B. firmus*, *B. mycoides, B. pumilus* are described for the first time in the EEZ waters and so far have not been reported for waters of the insular shelf (Miravet & Lugioyo, 2007).

Taking into consideration the large representativeness of *Bacillus* species in the oceanic waters adjacent to Cuba, it was

Table 1. Taxonomic position of *Bacillus* strains isolated from oceanic waters adjacent to Cuba
Cuadro 1. Posición taxonómica de las cepas de *Bacillus* aisladas de las aguas oceánicas adyacentes a Cuba

Isolates	Classification
S-94	*Bacillus badius* Batchelor, 1919, 23.[AL]
S-139	*Bacillus brevis* Migula, 1900, 583.[AL]
N-532	*Bacillus brevis* Migula, 1900, 583.[AL]
N-541	*Bacillus brevis* Migula, 1900, 583.[AL]
S-92	*Bacillus cereus* Frankland y Frankland, 1887, 257.[AL]
S-212	*Bacillus cereus* Frankland y Frankland, 1887, 257.[AL]
S-104	*Bacillus circulans* Jordan, 1890, 821.[AL]
S-221	*Bacillus circulans* Jordan, 1890, 821.[AL]
S-217	*Bacillus circulans* Jordan, 1890, 821.[AL]
S-242	*Bacillus firmus* Bredemann y Werner in Werner, 1933, 446.[AL]
S-247	*Bacillus firmus* Bredemann y Werner in Werner, 1933, 446.[AL]
S-299	*Bacillus firmus* Bredemann y Werner in Werner, 1933, 446.[AL]
S-Cl26	*Bacillus firmus* Bredemann y Werner in Werner, 1933, 446.[AL]

Isolates	Classification
S-110	*Bacillus licheniformis* Chester, 1901, 287.[AL]
S-89	*Bacillus licheniformis* Chester, 1901, 287.[AL]
S-91	*Bacillus licheniformis* Chester, 1901, 287.[AL]
S-129	*Bacillus licheniformis* Chester, 1901, 287.[AL]
S-180	*Bacillus licheniformis* Chester, 1901, 287.[AL]
S-186	*Bacillus licheniformis* Chester, 1901, 287.[AL]
S-312	*Bacillus licheniformis* Chester, 1901, 287.[AL]
S-170	*Bacillus marinus* Rüger y Ritcher 1983, 157[VP]
N-544	*Bacillus megaterium* de Bary, 1884, 499.[AL]
S-204	*Bacillus mycoides* Flügge, 1886, 324.[AL]
S-240	*Bacillus mycoides* Flügge, 1886, 324.[AL]
S-87	*Bacillus polymyxa* Macé, 1889, 588.[AL]
S-191	*Bacillus pumilus* Meyer y Gottheil in Gottheil, 1901, 680.[AL]
S-Cl24	*Bacillus pumilus* Meyer y Gottheil in Gottheil, 1901, 680.[AL]
S-130	*Bacillus pumilus* Meyer y Gottheil in Gottheil, 1901, 680.[AL]
S-132	*Bacillus pumilus* Meyer y Gottheil in Gottheil, 1901, 680.[AL]
S-93	*Bacillus* sp. Cohn 1872, 174[AL]
S-243	*Bacillus* sp. Cohn 1872, 174[AL]
S-Cl23	*Bacillus* sp. Cohn 1872, 174[AL]
S-Cl29	*Bacillus* sp. Cohn 1872, 174[AL]
S-111	*Bacillus* sp. Cohn 1872, 174[AL]
N-529	*Bacillus* sp. Cohn 1872, 174[AL]
N-536	*Bacillus* sp. Cohn 1872, 174[AL]
N-538	*Bacillus* sp. Cohn 1872, 174[AL]
N-549	*Bacillus sphaericus* Meyer y Neide in Neide, 1904, 337.[AL]
S-261	*Bacillus subtilis* Cohn, 1872, 174.[AL]
N-548	*Bacillus subtilis* Cohn, 1872, 174.[AL]

N: strains isolated from northern of EEZ. S: Strains isolated from southern of EEZ.
AL: designation included in the approved list of bacterial names (1980); VP: validated name by DSMZ (http://www.dsmz.de/bactnom/bactname.html, updated May 2010).

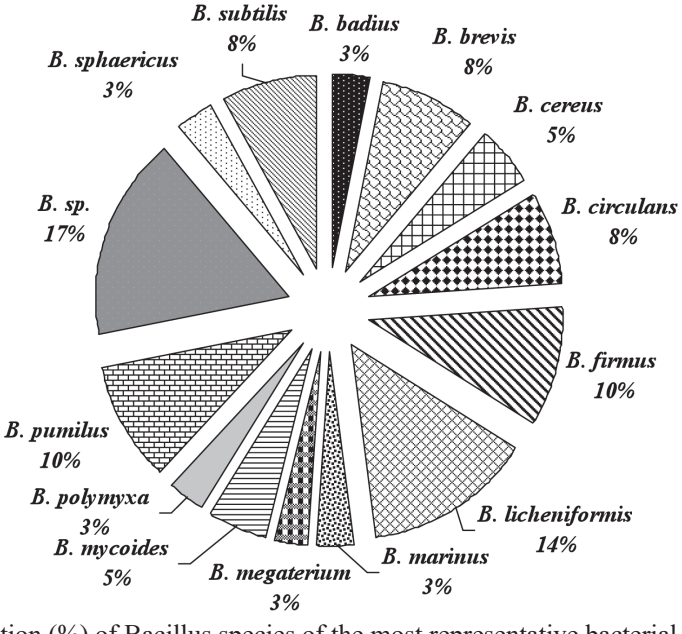

Fig. 2. Distribution (%) of Bacillus species of the most representative bacterial strains from the EEZ of Cuba

Fig. 2. Distribución porcentual de las especies del género *Bacillus* de los aislamientos de cepas bacterianas más representativas de la ZEE de Cuba

interesting to explore if there was any relationship among the protein electrophoretic pattern, the phenotypical characteristics of the strains and the environment from where they were isolated (Avise, 2004). To this end, the electrophoretic pattern of every strain was acquired showing a noticeable coincidence between a group of bands (Fig. 3) that could be the expression that all the strains belong to the same genus (*Bacillus*), probably differing only in some of their biochemical capacities as previously described (Buchanan & Gibbons, 1974). The non-rooted dendrogram obtained by comparing the electrophoretic patterns allowed to cluster the strains into three well defined nodes, even though, there is no a direct relationship of these nodes with particular species; notice, for instance, that *B. cereus* and *B. licheniformis* were present in nodes I and II (Fig. 4).

Analysis of clustering as a function of the physical and chemical characteristics of the sampling zone reveals, in general, that node I, differently from node II and III, gathers those strains isolated from the farthest zones of the shelf (Fig. 5). These stations are very poor in nutrients (Fernández *et al.* 1990), this is the case of the zone located in the central region of the EEZ (stations: S 101, S 103 and S 111), of stations S 79 and S 88 situated in the western region limiting the EEZ in the **S**, and station N 78 in the **N** of the EEZ (Fig. 1).

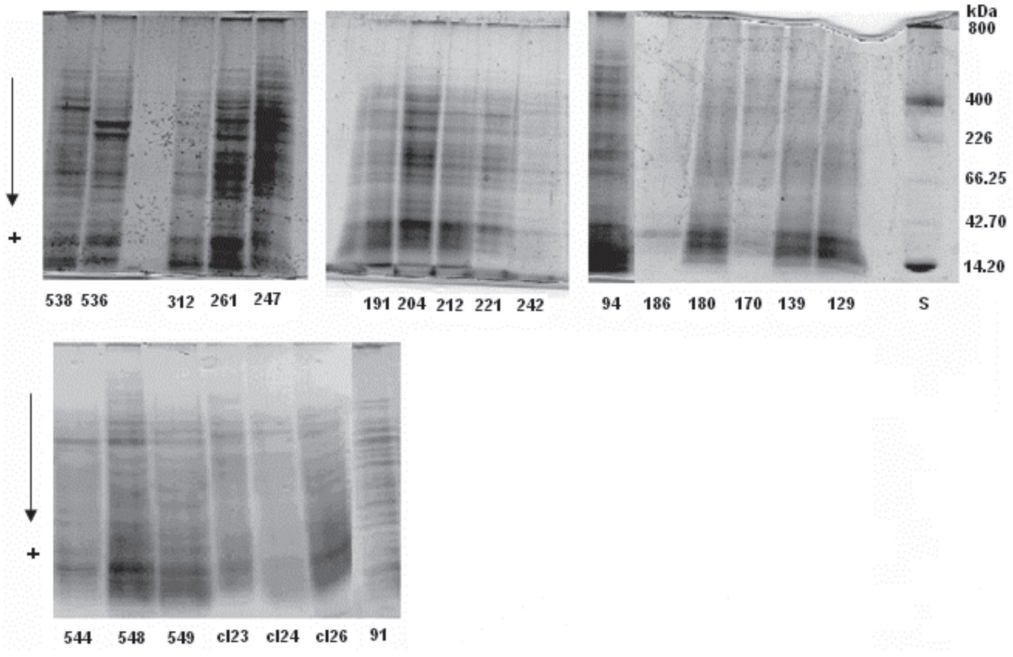

Fig. 3. SDS-polyacrylamide (5%) gel electrophoresis of total proteins of the isolates from the oceanic waters adjacents to Cuba. Numbers under each lane identify the strain analysed

Fig. 3. Electroforesis en geles de poliacrilamida al 5% en presencia de SDS de proteínas totales de las cepas aisladas de las aguas oceánicas adyacentes a Cuba. Los números debajo de cada carrilera indican la cepa aplicada

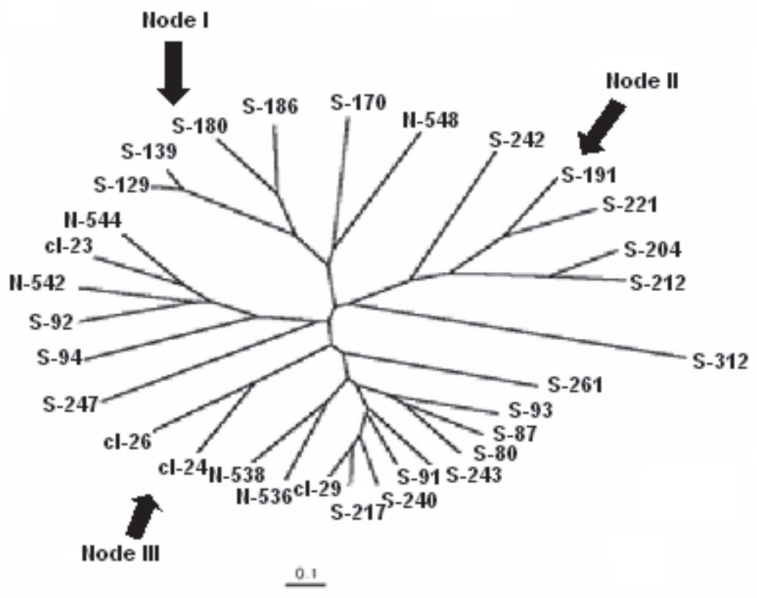

Fig. 4. Bacteria strain grouping in adjacent oceanic waters to Cuba constructed from arbitraries distances obtained from total protein electrophoresis

Fig. 4. Agrupamiento de las cepas de bacterias aisladas de las aguas oceánicas adyacentes a Cuba a partir de los valores de distancia arbitraria obtenidos a través de las electroforesis de proteínas totales

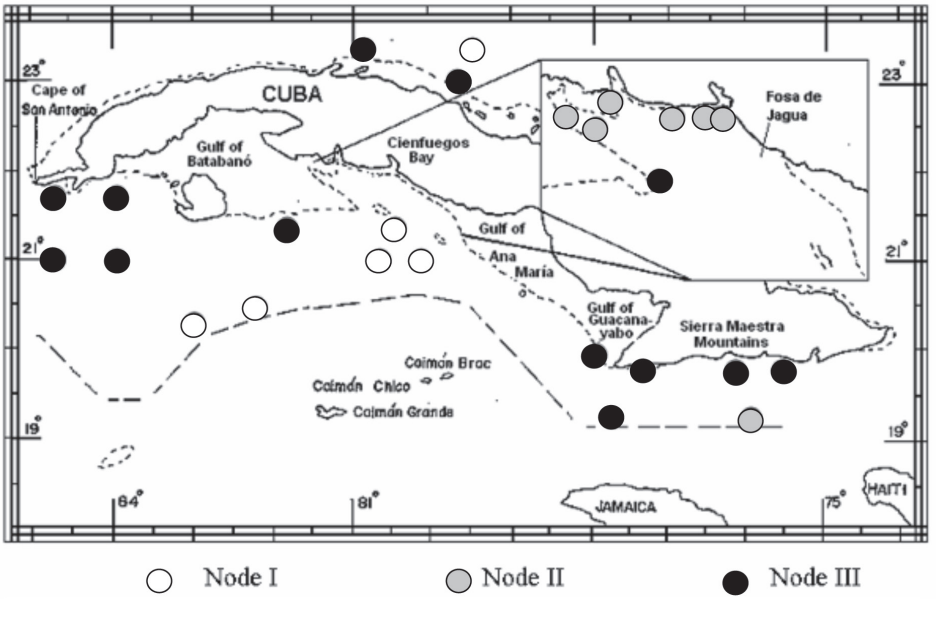

Fig. 5. Spatial distribution of bacterial isolates in the nodes resulting from the analysis of the metabolic activities assayed

Fig. 5. Distribución de los aislados de bacterias de cada uno de los nodos atendiendo a las actividades metabólicas ensayadas

Nodes II and III, in turn, mainly cluster those strains isolated from zones with high availability of nutrients and organic matter, either resulting from a higher exchange with shelf waters, as is the case of **W** of Fosa de Jagua (Fernández *et al.* 1990), **S** of Cienfuegos Bay (Areces, 1986) and **N** of Archipelago Sabana-Camagüey, or due to the water masses dynamics favoring arrival and stay of nutrients as, for instance, in the most western and eastern regions of the **S** EEZ (Victoria *et al.* 1990).

Even though no significant differences were found among the nodes, it was observed that clustering was in closely related to with the metabolic activities. In node I the ratio number of positive activities/ strain was 2.3, the lowest observed, followed by node II with a ratio equivalent to 3.3, and finally node III exhibiting a ratio of 3.7 (Table 2). We have also compared nodes II *vs.* I, III *vs.* II, and III *vs.* I considering the number of positive activities (Table 2) and classified them into three qualitative categories: higher, equal

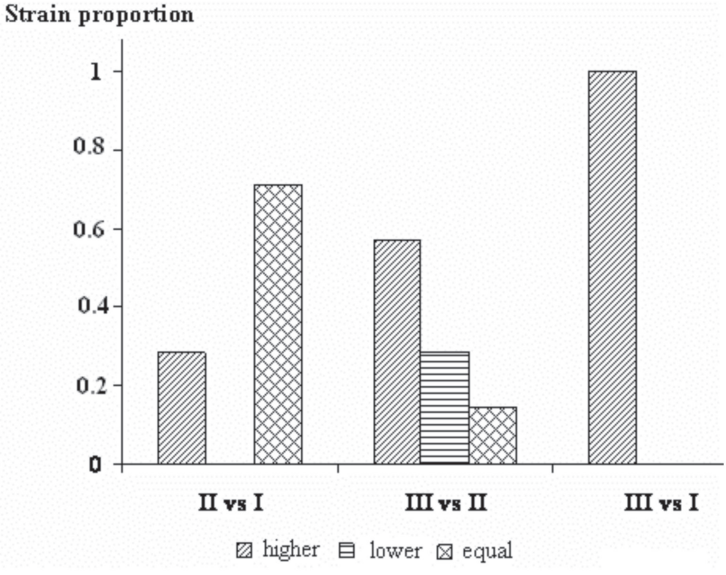

Fig. 6. Proportion of bacterial strains in a node where the number of positive activities was *higher*, *lower* or *equal* when compared to a second node. Data were compared by a Chi^2 test of independence using the program CHIRXC (Zaykin and Pudovkin 1993). The global analysis revealed that there was a significant difference among the three comparisons ($P = 0.003$), probably due to the differences between node III and I, where all the compared activities were higher in node III differently to node II vs. I ($P = 0.163$) or III vs. II ($P = 0.18$)

Fig. 6. Proporción de cepas bacterianas en un nodo donde el número de actividades positivas fue *mayor*, *menor* o *igual* cuando se le comparó con un segundo nodo. Los datos se compararon mediante una prueba de Chi^2 de independencia utilizando el programa CHIRXC (Zaykin y Pudovkin, 1993). El análisis global reveló que hubo diferencias significativas entre las tres comparaciones ($P = 0.003$), debido probablemente a las diferencias entre los nodos III y I, donde todas las actividades comparadas fueron mayores en el nodo III, de modo diferente a lo que ocurrió cuando se comparan el nodo II y el nodo I ($P = 0.163$) o el nodo III vs. el nodo II ($P = 0.18$)

Table 2. Node clustering of bacterial strains and their metabolic activities
Cuadro 2. Agrupación por nodos de las cepas bacterianas y sus actividades metabólicas

Node	Strain	Proteolytic Activity	Hemolytic Activity	Antimicrobial activity					ST (mN/m)	IT/D (mN/m)	DNA Intercalating agent	Hydrocarbon degradation
				An	*Bs*	*Ca*	*Ec*	*Sa*				
	S 129	+	-	-	-	-	-	-	48.2	17.7	-	-
I	S 139	-	+	-	-	-	-	-	42.0	14.5	+	-
	S 170	+	-	-	-	+	-	nd	42.4	15.2	-	-
	S 180	-	-	+	-	-	-	-	30.5	4.5	-	-
	S 186	-	-	-	-	-	-	-	29.9	0.7	+	-
	N 548	+	+	+	+	-	+	-	32.8	5.9	-	-
	S 191	+	-	-	+	+	+	-	36.7	8.7	+	-
II	S 204	-	-	+	+	+	-	-	31.1	4.9	-	-
	S 212	-	+	-	-	+	+	nd	31.9	3.7	-	-
	S 221	+	+	-	-	-	-	-	31.8	1.0	-	-
	S 242	-	-	-	-	-	-	-	31.9	4.5	+	-
	S 312	+	-	-	-	-	-	-	35.8	6.1	-	-
	S 87	+	+	+	-	+	-	+	28.9	1.05	+	-
III	S 89	+	+	-	+	-	-	nd	29.75	1.25	-	-
	S 91	+	+	-	-	-	-	+	33.1	7.3	-	+
	S 92	+	+	-	-	-	-	+	27.3	0.85	+	-
	S 93	+	-	-	-	-	-	-	27.3	0.75	-	-
	S 94	-	+	-	-	-	+	-	48.0	21.2	-	-
	S 217	-	-	-	-	-	-	+	41.9	13.6	+	-
	S 240	-	+	-	+	-	+	-	42.7	17.8	-	-
	S 243	-	-	-	-	-	-	-	29.8	2.2	+	-
	S 247	-	-	+	+	-	-	-	29.8	2.2	nd	-
	S 261	+	+	+	+	+	+	-	30.1	3.1	+	-
	SCl23	+	nd	-	-	-	-	-	28.7	3.7	-	-
	SCl24	+	+	-	-	-	-	-	27.4	2.3	-	-
	SCl26	+	+	+	-	-	-	-	29.6	2.0	-	-
	SCl29	nd	nd	-	-	+	-	-	34.0	7.1	-	-
	N 536	+	+	-	+	-	-	-	36.2	6.2	-	-
	N 538	+	-	-	+	-	-	-	29.8	1.7	-	+
	N 544	+	+	-	+	-	-	-	51.1	6.6	-	-
	N 549	+	+	-	-	-	+	+	29.8	3.7	-	+

An: Aspergillus niger, Ec: Escherichia coli, Bs: Bacillus subtilis, Sa: Staphylococcus aureus, Ca: *Candida albicans,* ST: surface tension, IT/D: interfacial tension using diesil as oleous phase; nd: non-determined. Clustering was constructed applying the UPGMA algorithm (Felsenstein, 1993).

or lower (Fig. 6). The global analysis revealed that there was a significant difference among the three comparisons (P = 0.003), probably due to the differences between nodes III and I, where all the activities examined were higher in node III.

Bacillus species found in the oceanic waters differ from those previously reported in waters and sediments of several zones of the Cuban insular shelf (Miravet *et al.* 1983; Lugioyo *et al*. 1987; Lugioyo and Rodríguez, 1988; Coya, 1999; Miravet *et al.* 1992; Miravet *et al.* 2001; Miravet *et al.* 2003) fact that could be associated with the adaptive capacity of these species to propagate and metabolize under oligotrophic marine conditions (Oguntoyinbo, 2007). In fact, the phylogenetic clustering built from total protein data of spore-forming Gram positive bacteria resulted in three nodes in which bacteria do not associate according to species, since it was found out that more than one species was represented in different nodes. In fact, there is an apparent relationship between the environmental conditions from where bacteria and the results of clustering were isolated. In a similar way, Zuriaga *et al*. 2009, examining the genetic and bioclimatic variation in *Solanum pimpinellifolium* found out that the locality with the greatest climatic diversity, also exhibited the highest genetic diversity. In our work, those strains found in node I were isolated, generally, from the farthest zones of the coast, more impoverished in nutrients and organic matter while nodes II and III group those strains coming from zones with higher exchange with shelf waters and where dynamics of water masses promote arrival and stay of nutrients. This might suggest that the existence of different total protein patterns in bacteria belonging to the same species, but from environments with different degree of richness, could be an indicator of the capacities of these microorganisms to adapt and live in different environments. Another argument in this same direction is that the bacterial strains of these nodes also showed the highest values of the ratio positive enzymatic activity per isolate, which in turn may represent another indicator of their adaptive potentiality.

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