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Marine heterotrophic bacteria associated with enrichment culture of nitrifying bacteria planned for closed aquaculture systems

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Abstract

Ammonium and nitrite oxidizing bacteria were enriched in separate cultures using a primary inoculum of water from an enclosed shrimp rearing facility. Each enrichment was continued by transfer to increasing volumes of medium up to 15 l. Seventy four strains of heterotrophic bacteria were isolated; seventeen from each primary inoculum, and twenty from each of the 15 l enrichments at the time when all the ammonium or nitrite had been utilized. All the isolates, described by 101 characters, were subjected to numerical taxonomy. During the enrichment for ammonium oxidizers, a strong selective pressure led to the disappearance of some strains, mostly enterobacteria. Two taxonomic groups composed of pseudomonads and asporogenous Gram positive rods were associated with the ammonium oxidizers. The population was characterized by an inability to use amino acids as carbon and energy sources. In contrast, the strains associated with nitrite oxidizers were able to use all the carbon and energy sources tested. Many of these strains were Gram positive, asporogenous aerobic rods, others were Gram negative pseudomonads.

Keywords : Heterotrophic bacteria, nitrifying bacteria, rearing seawater.

Bactéries marines hétérotrophes associées à des cultures d'enrichissement de bactéries nitrifiantes destinées à des systèmes clos d'aquaculture.

Résumé

Des cultures d'enrichissement, destinées à installer les processus de nitrification dans des systèmes d'aquaculture sans renouvellement de l'eau, ont été réalisées à partir d'eau d'élevage de crevettes pénéides. Les volumes des cultures d'enrichissement croissent de 50 ml à 15 l de milieu pour bactéries ammonio-oxydantes et bactéries nitrite-oxydantes. 74 souches de bactéries hétérotrophes ont été isolées, 17 provenant de l'eau d'élevage ayant servi d'inoculum, et 20 de chaque culture d'enrichissement de 15 l, lorsque la nitrosation et la nitratation sont effectives et que le substrat de départ (ammonium pour la première et nitrite pour la seconde ont été utilisés). La culture d'enrichissement des bactéries ammonio-oxydantes conduit à une forte sélection des bactéries présentes dans l'eau d'élevage, principalement à une disparition des entérobactéries associées aux animaux. Deux principaux groupes taxonomiques sont associés aux bactéries nitrosantes : des pseudomonades et des bâtonnets Gram positif asporulés. Cette communauté est caractérisée par son impossibilité à utiliser les acides aminés comme source de carbone et d'énergie. Au contraire, les bactéries nitratantes sont capables d'utiliser un grand nombre de composés organiques comme source de carbone et d'énergie. La plupart de ces bactéries sont des bâtonnets asporulés à Gram positif, associés à quelques pseudomonades.

Mots-clés : Bactéries hétérotrophes, bactéries nitrifiantes, aquaculture marine.

INTRODUCTION

In pelagic waters biogenic nitrogen input is in the reduced form, usually as ammonium, and the bacterial nitrification processes oxidize these reduced compounds to the stable state of nitrate. Nitrifiers, responsible for ammonium oxidation, are chemolithotrophic slow growing bacteria. Even in simple inorganic media these bacteria are always accompanied by rapidly propagating heterotrophic microorganisms (Kuenen and Gottschal, 1982). Chemolithotrophs such as nitrifying bacteria are known to excrete organic compounds during growth (Cohen *et al.*, 1979) and may contribute to the organic substrate supplies of surrounding heterotrophic bacteria.

On the other hand, the coculture with a heterotroph is able to increase the nitrifying activity of an autotrophic *Nitrosomonas* strain, or to reduce its lag phase (reviewed by Kuenen and Gottschal, 1982), and Kaplan, 1983). In spite of the physiological and ecological importance of mutualistic interactions developed by nitrifying bacteria with some heterotrophic bacteria, few studies have been published in the past decade on this topic.

Enrichment cultures of nitrifying bacteria in seawater were done in order to test the possibility to use frozen cells of nitrifiers to initiate and stimulate ammonium and nitrite oxidation processes in aquaculture systems (Perfettini and Bianchi, 1990). The purpose of this study was to examine the phenotypical characteristics (morphological, physiological and nutritional) of heterotrophic bacterial strains associated with ammonium-oxidizers and nitrite-oxidizers.

MATERIALS AND METHODS

General protocol

Two separate enrichment cultures were made for ammonium and nitrite-oxidizing bacteria in appropriate simple inorganic media (Perfettini and Bianchi, 1990). The inocula consisted of two samples of seawater collected at two week interval in a shrimp rearing tank in which nitrification had already been observed. The heterotrophic strains were isolated after 2 or 3 transfers in mineral medium. The goal of this work was to compare heterotrophic bacterial populations developing during ammonium oxidizers and nitrite oxidizers enrichment cultures respectively. Hence the same medium (marine nutrient agar) was used to count and isolate heterotrophic bacteria, although it was not a universal growth medium.

Nitrifying bacteria enrichment cultures. For both enrichments the inocula were seawater samples from a closed aquaculture system of *Penaeus vanamei*. The simple inorganic culture media were from Schmidt *et al.*, (1973) and the incubation temperature was 30°C. The culture flasks were kept on a shaking table to provide aeration, except for the 15 l cultures aerated with sterile air bubbling.

Ammonium-oxidizing bacteria enrichment:

Step 1: 1 ml seawater inoculum + 50 ml culture medium

Step 2: 10 ml step 1 + 1 l culture medium

Step 3: 300 ml step 2 + 3 l culture medium

Step 4: 1.5 l step 3 + 15 l culture medium

Nitrite-oxidizing bacteria enrichment:

Step 1: 20 ml seawater inoculum + 1 l culture medium

Step 2: 300 ml step 1 + 3 l culture medium

Step 3: 1.5 l step 2 + 15 l culture medium

20 $g.l^{-1}$ of glass powder was added into each culture flask to provide a surface for the settlement of nitrite-oxidizing cells.

Cultures were continued until ammonium or nitrite totally disappeared.

Chemical parameters. Inorganic nitrogen was determined, in the culture vials, at intervals using the method of Solarzano (1969) for NH_4-N, of Bendschneider and Robinson (1952) for NO_2-N, and Technicon analyser for NO_3-N.

Total bacterial counts. Samples were preserved using 0.2% (v/v) formaldehyde. Before counting, the samples were sonicated in order to remove cells from the glass particles in nitrite-oxidizing bacteria samples (Vianna Doria and Bianchi, 1982). Epifluorescence microscopical counts (Hobbie *et al.*, 1977) were done using an image analysis System III (Van Wambeke, 1988).

Viable counts of heterotrophic bacteria. Serial dilutions of water samples were prepared to 10^{-5} in 9 ml amounts of sterile seawater and were inoculated (0.2 ml) in duplicate on Marine Agar (Difco) plates. Incubation time was dependent one week at 30°C.

Qualitative study of heterotrophic bacteria

Strain collection. Strains were picked at random from colonies growing on marine agar plates containing around 150 colonies. 17 strains from each seawater inoculum and 20 strains from each of the 15 l culture for ammonium and nitrite-oxidizer enrichments (*i.e.* day 62 and day 49, respectively) were isolated and purified.

Characterization of strains and cluster analysis. The strains were described using 101 phenotypical characters including cellular morphology, Gram staining, colonial morphology and colour; production of catalase, oxidase, phosphatase, urease, gelatinase, esculinase, lipase (Tween 80), DNAse, amylase, lecithinase; ability to oxidize or ferment glucose, ability to grow at 4, 37 or 44°C; ability to reduce nitrate and nitrite.

The nutritional tests, the growth factor (amino acid and/or vitamin) requirements and the ability to grow without NaCl, or with 10% NaCl, were studied using a microtiter plate technique as described in Van Wambeke *et al.*, (1984). The cluster analysis was performed using KHI 2 for taxonomical distance and variance for clustering (Sohier and Bianchi, 1985).

Bacterial catabolic potentialities were expressed by both: the exoenzyme average index (EAI), corresponding to the average number of positive responses for the seven exoenzymes associated with a sample or a phenon; *the average index of carbonaceous compounds utilization* (UAI) corresponding to the average number of the 38 substrates tested which could be utilized as sole source of carbon and energy by all the strains of a sample or a phenon. For each family of compounds tested (amino acids, carbohydrates, alcohols, aromatic compounds, organic acids, fatty acids) a similar index was established using the same calculation method.

RESULTS

Ammonium-oxidizing bacteria enrichment

The ammonium oxidation started without any lag phase in the first step (*fig.* 1) as the inoculum was from an experimental rearing of *Penaeus japonicus* in which ammonium-oxidizers were already active. Bacterial numbers (total counts) showed a drastic drop, from 2×10^7 to 2×10^6 cells ml^{-1}, during the first two steps (*fig.* 1). The inoculum was made of water from a rearing batch in use for several weeks, in which the organic matter level, resulting from diet pellets and fecal pellets, permitted the growth of an abundant heterotrophic bacterial population. The inoculation of such a water sample into a simple inorganic medium (Schmidt *et al.*, 1973) provokes the decrease of this heterotrophic microflora. In addition, the nitrite increase was able to inhibit the growth of a wide range of bacteria (Yarbrough *et al.*, 1980), including those in aquaculture systems as described earlier (Sohier and Bianchi, 1985).

The phenotypic features of strains isolated on marine agar plates from the inoculum and from the last step of the enrichment procedures, were different (*table* 1). There was a clear decrease of fermentative bacteria (from 59 to 15%), and strains able to reduce nitrite (from 41 to 20%). The heterotrophic bacteria, associated with ammonium-oxidizing cells grown in mineral medium, developed a high nutritional versatility, mostly devoted to the uptake of low molecular weight compounds (Cohen *et al.*, 1979). The average number of compounds used as carbon and energy sources increased from 35 to 53%. At the same time, as noted for one strain of *Nitrosomas europa* by Kuenen and Gottschal (1982), there was an increase of growth factor requiring bacteria (23 to 30%).

Nitrite-oxidizing bacteria enrichment

There was a lag phase of 8 days, before nitrite decreased in the mineral medium (*fig.* 1). Bacterial counts increased from 19 to 40×10^5 bacteria ml^{-1} during the 8 first days of the step 1 (*fig.* 1). These values were similar to those noted in the 15 l culture for ammonium-oxidizers. This increase corresponded to the lag phase of nitrite oxidation process and could, probably, be related to the adjustment of the nitrite-oxidizing cell numbers to the volume of the culture and the nitrite concentration.

As for the ammonium-oxidizing bacteria enrichment, the numbers of colony forming units on marine agar plates stayed around 10^5 ml^{-1} (*fig.* 1). Comparing the bacteria from the inoculum and from the end of the enrichment (*table* 2), there was an increase in Gram-positive cells (from 36 to 70%). The increase of growth factors requiring strains rose from 12 to 40%, suggesting a species selection of heterotrophic bacteria (Jones and Hood, 1980; Sohier and Bianchi, 1985). With respect to the compounds used as carbon and energy source, very few changes were noted. Strains isolated from the last step possessed more exoenzymes than strains from the inoculum (*table* 2). The consistently high level of strains able to reduce nitrate to nitrite to molecular N_2 was probably maintained by anoxic microniches possible on glass powder or on the wall of the vials. This capability to reduce the nitrate to molecular N_2 was not measured in terms of activity, but our study showed the increased potentiality of such strains to contribute to the decrease of the nitrite oxidation rate (Belser, 1977), or remove nitrate, as suggested by Timberlake *et al.* (1988) who were able to combine both the nitrifying and denitrifying layer using a support biofilm of 1 mm thickness in wastewater.

Comparison of strains isolated from enrichment experiments

The 74 strains isolated from the four samples were submitted to a hierarchical classification. The dendrogram (*fig.* 2) showed three phena clustering at high taxonomical distance.

The phenon 1 (*fig.* 2) was composed of 10 strains, all isolated from the inoculum used for the ammonium-oxidizer enrichment. This phenon gathers the dominant microbial type of the T0 sample (10/17 strains). The strains showed a fermentative metabolism, only 20% were able to produce oxidase and all were able to grow at 44°C (*table* 3). These characteristics matched the family description of Enterobacteriaceae. The ability of such strains to hydrolyse starch was also noted by Sugita *et al.*, (1985) in the water of a carp rearing tank. These fishes, like our shrimps, were fed by pellet diet. The 10 strains of this phenon demonstrated the ability to use various compounds as carbon and energy sources, the average index of

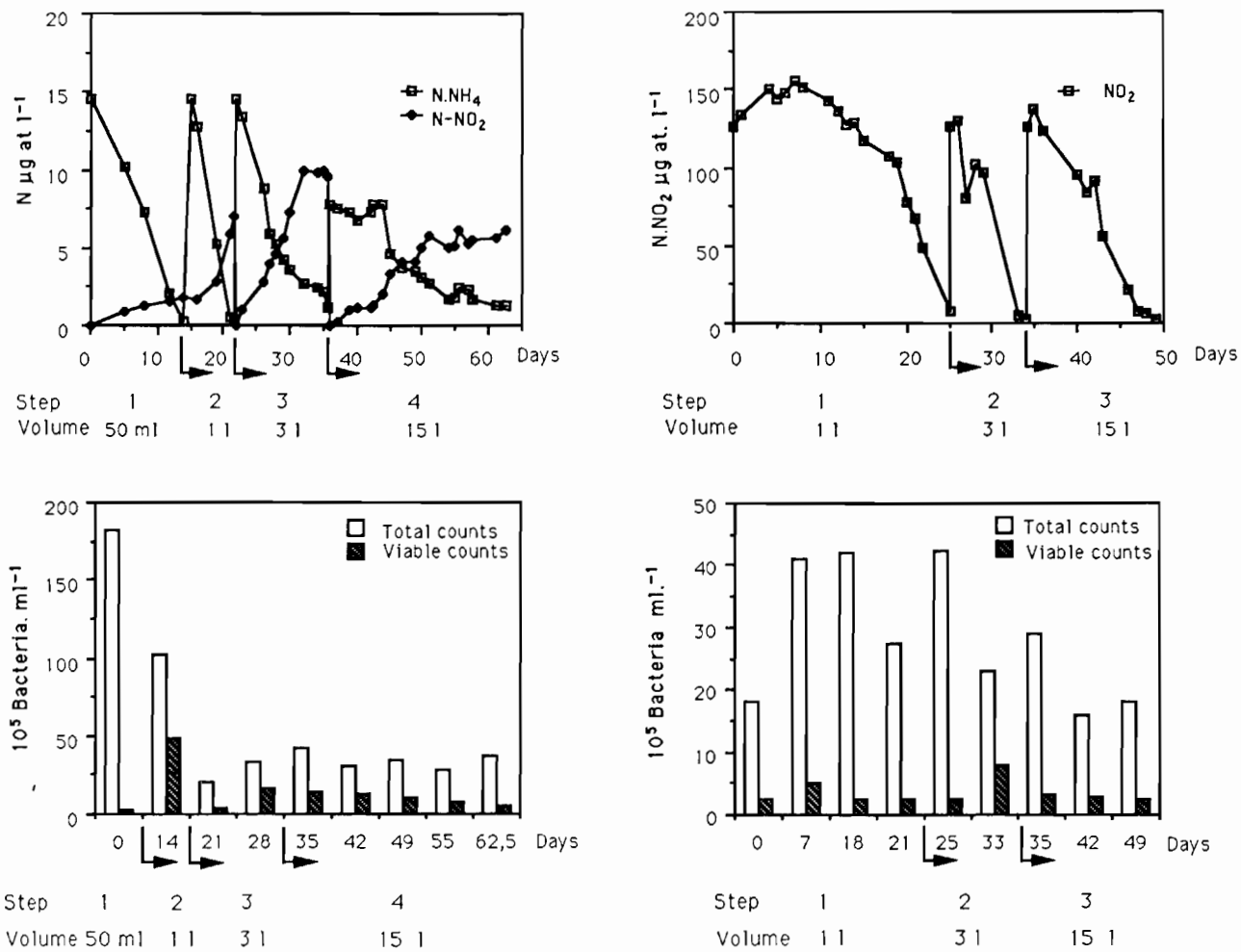

Figure 1. – Nitrogen salts and bacterial counts during the enrichment procedures. On the left: ammonia oxidizer enrichment; on the right: nitrite oxidizer enrichment.

utilization of substrates was 45%. All strains reduced nitrate to nitrite, 50% of them reduced nitrite.

The phenon 2 was composed of 9 strains associated with ammonium-oxidizers in the mineral medium. The strains exhibited an oxidative metabolism, only 50% reduced nitrate to nitrite. Depending on the Gram stain, the Gram-positive strains could be identified as *Nocardia* sp., while the Gram-negative strains were *Pseudomonas* sp. Although taxonomically different, the strains showed good physiological and nutritional homologies. They were able to hydrolyze most of the tested polymers (average exoenzyme indices: 52%), and they were not able to use amino acids as carbon and energy sources (*table* 4).

The last cluster (phenon 3) contained several clusters composed of strains of mixed origin, but mostly isolated from the seawater inoculum and the end of the nitrite-oxidizing bacteria enrichment. 6 out of the 20 strains associated with nitrite-oxidizers clustered at a low taxonomical distance. They constituted the phenon 3*a*. These strains were small, asporogenous, Gram-positive, non-fermentative rods. They showed low hydrolytic capacity (average exoenzyme index: 31%) but a very high nutritional diversity (average utilization index of organic compounds tested: 76%), being able to use nearly all substrates tested (*tables* 3 and 4). These bacteria could be identified as *Brevibacterium*, *Corynebacterium* or *Arthrobacter*, three genera of Gram positive asporogenous rods.

The eight other clusters of the group 3, containing at least three strains, were composed of strains of mixed origin. Strains of phenon 3*b* were oxidase negative, Gram-negative, aerobic, non-fermentative rods, nitrate was not reduced. They can be tentatively identified as *Pseudomonas marina*. Phenon 3*c* was composed of Gram negative aerobic rods, oxidase reaction negative, nitrate was reduced but not nitrite. These may be strains of *Alcaligenes aquamarinus*. Phenon 3*d* contained Gram-negative rods, 1/3 of the

Table 1. – Comparison of features of strains from the inoculum and from the last step of the enrichment of ammonia oxidizing bacteria.

	Strains from inoculum	Strains from day 62
Number of isolates	17	20
Characters:		
Cocci	0	0
Coccoids	53	30
Rods	59	80
Gram-positive	47	45
Gram-negative	53	55
Oxidase production	12	35
Catalase production	41	45
Glucose oxidation	65	35
Glucose fermentation	59	15
Growth at 0% NaCl	23	40
Growth at 44°C	88	40
Average Exoenzyme Index *	31	22
Gelatinase	0	50
DNAse	53	5
Phosphatase	82	30
Average Utilization Index *	34	53
A. U. Amino acids *	23	42
A. U. Carbohydrates *	54	67
A. U. Alcohols *	68	86
A. U. Aromatic compounds *	6	52
A. U. Organic acids *	36	52
A. U. Fatty acids *	25	49
NO_3^- reduction	76	65
NO_2^- reduction	41	20
Growth factor requiring strains	23	30

Results are expressed in percentage of positive responses of the 17 and 20 strains of each sample. *: see methods for calculation of indexes.

Table 2. – Comparison of features of strains from the inoculum and from the last step of the enrichment of nitrite oxidizing bacteria.

	Strains from inoculum	Strains from day 49
Number of isolates	17	20
Characters:		
Cocci	6	5
Coccoids	23	40
Rods	71	65
Gram-positive	36	70
Gram-negative	64	30
Oxidase production	12	30
Catalase production	36	55
Glucose oxidation	36	15
Glucose fermentation	6	10
Growth at 0% NaCl	23	5
Growth at 44°C	47	0
Average Exoenzyme Index *	23	34
Gelatinase	47	75
DNAse	24	10
Phosphatase	35	80
Average Utilization Index *	34	31
A. U. Amino acids *	25	18
A. U. Carbohydrates *	35	40
A. U. Alcohols *	55	68
A. U. Aromatic compounds *	17	25
A. U. Organic acids *	44	38
A. U. Fatty acids *	32	21
NO_3^- reduction	76	65
NO_2^- reduction	35	40
Growth factor requiring strains	12	40

Results are expressed in percentage of positive responses of the 17 and 20 strains of each sample. *: see methods for calculation of indexes.

strains were fermentative, nitrate was reduced. No identification was carried out. Phenon 3*e* was made up of Gram-positive non-fermentative rods. Such Gram-positive bacteria were difficult to identify, they were attributed to the group *Brevibacterium-Arthrobacter-Corynebacterium*. Phenon 3*f* contained Gram-negative short rods, their fermentative and oxidase reactions were positive. The possible identification is *Vibrio* sp. Phenon 3*g* and Phenon 3*h* were made up of non-motile aerobic oxidase negative Gram-negative aerobic rods which reduced nitrate but not nitrite; growth factors were not required. The tentative identification was: *Acinetobacter* sp. Phenon 3*i* were all Gram-positive coccoids, which gave a positive catalase reaction.

DISCUSSION

During the enrichment procedure of ammonium-oxidizing bacteria, the inoculum was diluted 10^{-6} folds in the step 4. The corresponding dilution of total number of bacteria would be from 10^7 to 10 cells per ml, but 4×10^6 bacteria were enumerated in this last step of culture (*fig.* 1), meaning that some bacteria were able to proliferate in this inorganic medium. This bacterial abundance could correspond to the growth of nitrifying cells. However, concomitantly, the heterotrophic bacteria must actively grow to compensate the dilution due to successive steps of the enrichment procedure, as the viable counts stayed around 5×10^5 bacteria per millilitre (*fig.* 1).

In the first inoculum, used for an ammonium-oxidizer enrichment, enterobacterial strains were predominant. They constituted a well separated phenon, without any strains from other origin (*fig.* 2). Such bacteria, coming from the fecal pellets of animals, have already been reported in the seawater used for shrimp rearing for several weeks (Sohier and Bianchi, 1985). Fermentative bacteria are commonly associated with marine aquaculture waters (Austin and Allen-Austin, 1985).

At the third and last step of ammonium oxidizer enrichment, meaning around 45 days of cultivation and three transfers, most of the strains exhibited a

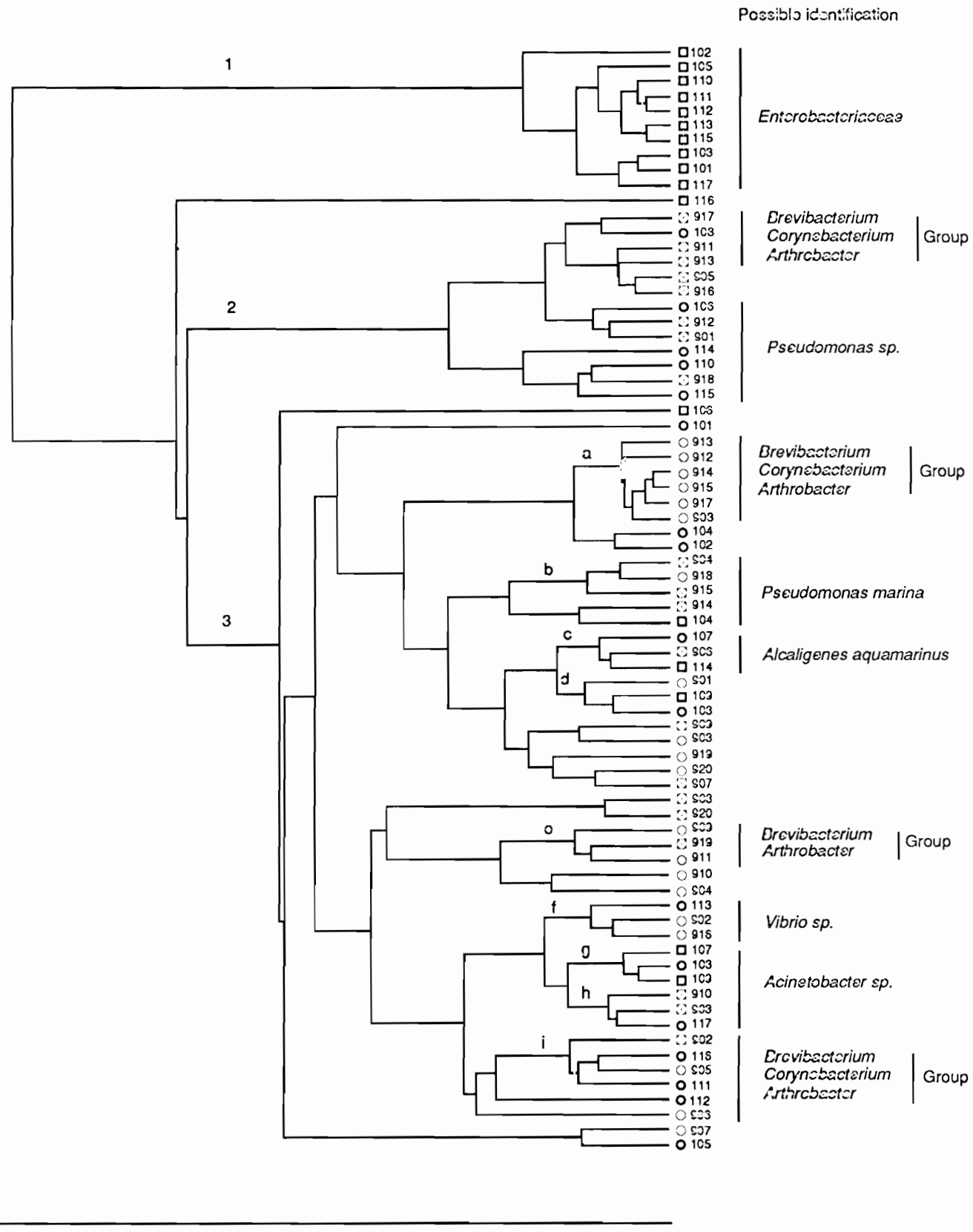

Figure 2. – Dendrogram resulting from the hierarchical classification of the 74 strains. □: strains from ammonium-oxidizers inoculum. ⊡: strains associated with ammonium-oxidizers. ○: strains from nitrite-oxidizers inoculum. ◎: strains associated with nitrite-oxidizers.

Table 3. – Morphological and physiological features of the phena associated with the first inoculum (1); the nitrite oxidizing bacteria (3*a*) and the ammonia oxidizing bacteria (2).

	Phenon 1	Phenon 2	Phenon 3*a*
Number of strains	10	9	6
Gram +	60	67	100
Gram −	40	33	0
Oxidase +	20	50	67
Catalase +	40	25	83
Oxidative	60	12	0
Fermentative	90	0	0
Urease	0	0	0
Phosphatase	100	100	67
Esculinase	30	100	33
Hydrolysis of:			
Tween 80	30	100	50
Starch	80	67	0
Gelatine	0	100	67
DNA	70	0	17
Chitin	40	50	33
Growth at:			
4°C	10	0	0
37°C	100	100	100
44°C	100	0	67
0% (w/v) NaCl	20	0	67
10% NaCl	100	100	100
NO_3^- reduced	100	50	50
NO_2^- reduced	50	16	33

Table 4. – Nutritional features of the phena associated with the first inoculum (1); the nitrite oxidizing bacteria (3*a*) and the ammonia oxidizing bacteria (2).

	Phenon 1	Phenon 2	Phenon 3*a*
Utilization of:			
Arabinose	10	0	83
Ribose	70	0	100
Glucose	100	100	100
Lactose	0	50	100
Sucrose	90	100	83
Starch	100	100	83
Gluconate	100	0	83
Glucosamine	90	100	100
Acetate	60	12	100
Propionate	60	33	83
Butyrate	10	0	100
Caproate	0	0	25
Malonate	0	12	66
Succinate	70	12	100
Fumarate	100	12	50
Adipate	0	0	50
Lactate	90	100	100
Glycollate	0	0	33
Citrate	100	100	100
Ketoglutarate	0	12	33
Glycerol	100	75	66
Mannitol	70	100	100
Sorbitol	70	75	100
Mandelate	10	50	50
Benzoate	10	0	83
Proline	90	12	83
Tryptophane	30	0	83
Glycine	70	12	25
Alanine	90	0	67
Serine	90	0	50
Aspartate	0	0	50
Glutamate	0	0	67
Lysine	0	12	83
Arginine	30	0	83
Ornithine	0	0	83
Asparagine	0	0	67
Methionine	0	0	83
Sarcosine	0	0	50
A. E. I.	37	52	31
A. U. I.	45	35	76
A. U. Amino acids	33	4	68
A. U. Carbohydrates	70	56	91
A. U. Alcohols	80	83	88
A. U. Aromatic compounds	10	25	66
A. U. Organic acids	45	31	66
A. U. Fatty acids	32	11	77

non-fermentative metabolism. These strains were of mixed taxonomic origin: some were Gram-negative strains identified as *Pseudomonas* sp. and a Gram-positive group which included *Brevibacterium-Arthrobacter-Corynebacterium*. Both of these groups of bacteria were identified by Jones and Hood (1980) in a culture experiment of ammonium-oxidizing *Nitrosomonas* sp. If these bacteria were well equipped with exoenzymes (table 3: phenon 2) and used mostly sugars and alcohols as carbon and energy sources (*table* 4), their nutritional characteristics were marked by their inability to use amino acids as carbon and energy sources. Strains showing the same nutritional characteristics were isolated by Sohier and Bianchi (1985) during ammonium oxidation step in water, heavily loaded with organic matter, from a rearing hatchery. The presence of these pseudomonads and Gram-positive strains, detected in such habitats, suggests their close association with ammonium-oxidizers.

The inoculum used for the nitrite-oxidizer enrichment came from the same shrimp rearing tank, but was sampled several weeks later. This could explain that the strains isolated from this sample were different and did not cluster with the isolates of the ammonium-oxidizer inoculum.

They clustered with strains isolated from the fourth culture for nitrite-oxidizers, into the large phenon 2 showing many bacterial species. Such taxonomic diversity confirms the lack of nutritional selection due to presence of nitrite-oxidizers. Their specificity was to use almost all the substrates tested as carbon and energy sources (*tables* 3 and 4). The increase of growth factor requiring strains suggested that these heterotropic bacteria were able to find various organic compounds in the mineral culture medium.

In conclusion, the two types of nitrifiers (ammonium and nitrite-oxidizers) were able to induce two

different heterotrophic microflora. Half of the strains associated with ammonium-oxidizers showed restricted nutritional potential, and used mostly sugars and alcohols. In contrast, bacteria growing in association with nitrite oxidizers, demonstrated the capability to use a wide range of organic compounds, inferring that in natural habitats a large number of heterotrophs would be able to develop in the environment of nitrite-oxidizers.

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