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RESEARCH ARTICLE

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Aerobic and facultative anaerobic heterotrophic bacteria associated to Mediterranean oysters and seawater

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Summary A comparative study on the composition and seasonal fluctuations of the main heterotrophic bacterial groups and species isolated from Mediterranean oysters and their growing-seawater was carried out. For the study we used 574 strains isolated from Marine Agar (MA) and submitted to numerical analysis of phenotypic traits in previous studies, plus 323 isolates recovered on Thiosulphate Citrate Bile Sucrose (TCBS) agar from the same samples and identified in this study. Oyster samples were dominated by halophilic fermentative bacteria during most of the year with predominance of two *Vibrio* species, *V. splendidus* (at temperatures lower than 20°C), and *V. harveyi* (at higher temperatures). On the contrary, *Vibrio* spp. was not the predominant microbiota of seawater, where most isolates had remained unidentified but corresponded to α-*Proteobacteria*, as shown by rDNA hybridization with phylogenetic probes in this study. Among the strict aerobes that could be identified, none of them showed a clear dominance, and many different groups were represented in very low percentages, in contrast with the major species from oyster samples. Shannon-Weaver diversity index revealed significant differences between both types of samples. No apparent seasonality was found in the distribution of seawater species, in sharp contrast with oyster-associated bacteria.

Key words Heterotrophic bacteria · Phenotypic characterization · Phylogenetic probes · Oysters · Seawater

Introduction

The microbiota of bivalve molluscs has been examined mainly from the public health hazard, since they may concentrate pathogenic microorganisms and cause diseases in humans through their consumption. The recovery of human pathogenic bacteria from shellfish samples has been widely reported, and most of the studies have focused on fecal contamination, enteric pathogens and pathogenic species of *Vibrio* [6, 8, 9]. In addition, studies on the natural bacterial biota of different oyster species, mainly *Crassostrea gigas* and *C. virginica*, have been carried out [7, 9], although these studies have not been focused in the comparison of the seasonal bacterial content of bivalves and the growing-water.

Several early studies reported the presence of *Achromobacter*, *Pseudomonas*, *Flavobacterium/Cytophaga* and *Vibrio* in samples of the above mentioned oyster species, genus *Vibrio* being one of the most frequently detected [7, 24]. Parallel analyses of bivalves and the surrounding water and/or sediments revealed qualitative differences in the species composition [19, 22, 30]. Some authors reported a higher amount of Gram-negative fermentative bacteria in the flesh and gut samples than in the surrounding water [22, 30]. Besides, other studies found that the bacterial species found in sediment and sea water were similar to those of the bivalves, although generally the numbers were smaller [32]. In all the mentioned studies, bivalve samples yielded higher numbers of heterotrophic bacteria than seawater samples, but different results regarding specific bacterial composition were reported.

In previous papers we reported separately the detailed results of the taxonomic study by numerical analysis of phenotypic traits of aerobic and facultatively anaerobic Gram-negative bacteria recovered on Marine Agar (MA) from Mediterranean oysters and the growing water from June 1989 to May 1990 [27, 28]. Several phena, especially among the oxidative isolates, remained unidentified. In these studies, the isolates were grouped according to their metabolism (fermentative vs non-fermentative), and no attempt was made to compare between both types of samples. In the present paper we have clarified

the taxonomic adscription of representatives of these phena by hybridization with phylogenetic probes, and have identified the isolates grown on Thiosulphate Citrate Bile Sucrose (TCBS). The main aim of the study was to compare the evolution and densities of the different bacterial groups/species throughout the year in oysters and seawater.

Methods

Sampling Sampling site was an oyster hatchery of *Ostrea edulis* located 34 miles off the coast of Vinaroz, at the Mediterranean Spanish coast, in which oysters are cultivated by the long line method. Sampling was performed monthly over an annual cycle, from June 1989 to May 1990. A diver collected cultured oysters and seawater in sterile plastic containers from depths ranging from 10 to 20 m. We kept the samples at 5–10°C until processed, always within 3 h after collection. Water temperature, pH and conductivity were also determined in each sampling.

Bacteriological analysis Seven to eleven oysters per sample were cleaned, opened aseptically and shucked as recommended [15], weighted in a sterile baker, diluted to 1/5 (w/v) in filtered seawater and grinded in a blender. We made serial decimal dilutions of both types of samples on sterile seawater for determining viable counts, by spreading plates of Marine Agar 2216 (MA, Difco) and TCBS Agar (Oxoid). Both plating media were incubated at 25°C for 10 days and 48 h respectively.

We picked 30 to 40 colonies at random from the highest dilutions of MA and TCBS plates from each sample, using a plate or combination of plates from the same dilution, to obtain reliable values of diversity indexes [5]. Colonies were purified by at least two isolation steps on MA. We cultured the strains routinely on MA or MB (Marine Broth, 2216, Difco) at 25°C, and maintained the cultures at room temperature in the dark on stab-inoculated tubes of MB supplemented with 0.3% agar (facultative anaerobes) or MA slants (aerobes). Long-term maintenance was carried out at 80°C on cultures suspended on MB adding 20% glycerol.

In previous studies [27, 28], we had identified 524 MA isolates. Following previously described procedures [2], we carried out the phenotypic identification of 323 TCBS isolates. After identification, we calculated the percentages of occurrence of the different species/phena for each sample and medium. By multiplying the viable counts by the percentage of isolates belonging to the species in each sample, we obtained an estimation of the densities of individual species/genera. Besides, we calculated the Shannon-Weaver diversity index for the taxonomic groups obtained throughout the study in both types of samples.

Hybridization with phylogenetic probes We identified the representative strains from the main unidentified phena of the mentioned previous studies [27, 28] by hybridization with phylogenetic probes, by using fluorescent in situ hibridization (FISH) and/or RNA membrane hybridization. For FISH, we used the method of Amann et al. [1]. Briefly, after fixation with 4% (w/v) paraformaldehyde, we collected the cells by centrifugation, washed them in PBS, and finally resuspended them in PBS and stored in a 1:1 mixture of PBS and 96% ethanol at 20°C. Following previously described procedures [20], we performed hybridizations on glass slides. Afterwards we spotted aliquots of fixed cells on single wells, immobilized them by drying at 46°C for 15 min and dehydrated them by passing them through an ethanol series (50, 80 and 96%, v/v) for 3 min each. Amounts of 9 µl of hybridization solution containing 50 ng probe and the specific amount of formamide [20] were applied on each well of slides and incubated for 1.5 h in a humid chamber at 46°C. We removed the probe by rinsing with 1 ml prewarmed (48°C) washing buffer followed by a 20 min incubation in washing buffer at 48°C. Washing buffer contained 20 mM Tris/HCl, pH 8.0, 0.01% SDS and 0.056 to 0.9 M NaCl, depending on the hybridization stringency. Subsequently we rinsed the slides with deionized water. After air-drying the slides, we mounted them in Citifluor.

The following oligonucleotides, provided by Pharmacia (Barcelona, Spain), were used: (i) Alf1b, complementary to a region of the 16S rRNA conserved in the alpha subclass of *Proteobacteria* and some other bacteria [20]; (ii) Bet42a, Gam42a: oligonucleotides complementary to selected regions of the 23S rRNA specific for the beta- (Bet42a) and gamma (Gam42a) subclass of *Proteobacteria* [20]; (iii) CF, complementary to a region of the 16S rRNA characteristic for cytophaga-flavobacterium subgroup of cytophaga-flavobacterium-bacteroides-phylum [21]. We labeled them with -tetrametylrhodamine-5-isothiocyanate (TRITC, Molecular Probes, Eugene, USA) or with 5(6)-carboxy-fluorescein-N-hydrosuccinimide ester (FLUOS; Boehringer Mannheim, Mannheim, Germany) as described previously [1].

Slot-blot analysis RNAs from the unidentified strains were isolated and immobilized on nylon membranes (Quiagen) by using a slot blot apparatus. We performed probing with DIG-labeled oligonucleotides as described previously [20].

Results

We performed 11 samplings for one year, from June 1989 to May 1990. Tables 1 and 2 show that temperature was the only variable with marked differences over the year, with a maximum around 27°C and a minimum of 12°C. Salinity ranged from 34 to 39‰, which is typical of full strength marine water, undiluted by continental flows. The values of pH showed small fluctuations from 8.1 to 8.4. Oyster colony counts on MA and TCBS rose in parallel with temperature, attaining a maximum of 3.7×10^6 CFU/g on MA in July, and a minimum of 3.2×10^4 CFU/g in January (Table 2). Seawater

Table 1 Variables of seawater samples

Month	VI	VII	VIII	IX	X	XI	I	II	III	IV	V
Temperature (°C)	19.5	25.6	27.2	23.7	20.0	18.0	12.1	13.6	14.8	16.2	20.8
CFU[a]/ml on MA	370	180	5400	390	4500	1300	630	840	310	87	190
CFU/ml on TCBS	<100	6	8	110	10	9	5	2	1	5	1
Vibrio/Photobacterium	33[b]	28	15	40	—	5	—	—	—	27	12
Alter./Pseudoalt./Shew.	22	31	5	20	4	—	25	—	8	—	—
Halomonadaceae	—	6	40	—	—	35	4	—	4	—	—
Pseudomonadaceae	11	3	—	—	—	—	12	8	15	—	—
Flavob.Cytophaga	—	—	—	—	4	—	—	27	30	—	—
Gram-positives	—	6	30	12	26	—	37	23	19	23	25
Unidentified Gram-neg.	34	26	10	28	66	60	22	42	24	50	63
No. spp.-phena / No. colonies	8/9	17/32	12/20	16/25	17/23	16/20	17/24	18/26	15/26	20/22	29/32
Diversity (H)[c]	2.95	3.78	3.16	3.84	3.92	3.79	3.84	4.01	3.57	4.25	4.30

[a]CFU: colony-forming units.
[b]percentage of each group/total MA isolates.
[c]Shanon-Weaver index.

Table 2 Variables of oyster samples

Month	VI	VII	VIII	IX	X	XI	I	II	III	IV	V
Temperature (°C)	19.5	25.6	27.2	23.7	20.0	18.0	12.1	13.6	14.8	16.2	20.8
CFU[a]/g MA ($\times 10^4$)	13	370	170	120	58	8.2	3.2	5.4	49	48	63
CFU/g TCBS ($\times 10^2$)	<	1400	1000	6500	840	5.5	6.3	7.8	830	730	140
Vibrio/Photobacterium	74[b]	91	100	90	81	31	46	40	93	90	63
Alteromonas/Pseudoalt.	3	9	—	—	6	—	7	—	—	—	—
Shewanella	—	—	—	3	3	4	4	—	—	—	—
Halomonadaceae	3	—	—	—	—	8	—	8	—	—	—
Pseudomonadaceae	—	—	—	—	—	—	—	4	—	—	—
Flavobact.Cytophaga	—	—	—	—	—	—	—	4	*3*	—	—
Gram-positives	6	—	—	—	—	15	11	4	—	—	—
Unidentified Gram-neg.	14	—	—	7	10	42	32	40	4	10	37
No. spp.-phena / No. colonies	13/31	10/34	9/31	12/30	16/31	20/26	14/28	15/25	7/30	8/30	5/30
Diversity (H)[c]	2.71	2.72	2.37	2.74	3.66	4.19	3.16	3.42	1.99	1.74	1.56

[a]CFU, colony-forming units.
[b]percentage of each group/total MA isolates.
[c]Shanon-Weaver index.

heterotrophs able to grow on MA were less abundant, their counts ranging from 5.4×10^3 CFU/ml in August to 8.7×10^1 CFU/ml in April (Table 1). TCBS counts, considered to evaluate culturable *Vibrio* spp. densities, were always 1 to 2 orders of magnitude lower than MA counts in both samples (Tables 1 and 2).

Tables 1 and 2 also show the evolution of the percentages of the main bacterial groups in both types of samples related to the total number of bacteria on MA along the year, as well as the Shannon-Weaver diversity index for each group. It can be observed that the *Vibrio*/*Photobacterium* group was dominant in oyster samples from March to October, and in seawater samples only by highest temperatures (from July to September). This group increased during the warm period in both types of samples and decreased at cold temperature, especially in seawater samples, it becoming undetectable in several samplings. Seawater samples were dominated by unidentified Gram-negative bacteria, especially during the cold months. In these samples, the *Alteromonas/Pseudoalteromonas/Shewanella* group was also more abundant at higher temperatures. Other groups were very scarce. A relatively high percentage of unidentified Gram-negative isolates were

found in oyster samples from low water temperatures. Gram-positive bacteria were poorly represented in oyster samples but more abundant in seawater samples (Tables 1 and 2).

Shannon-Weaver diversity index revealed significant differences between both kinds of samples. For seawater samples, this value showed less fluctuations and remained always above 3, reaching a highest value of 4.3 in May. Oyster samples showed always lower values and marked differences along the year, reaching the lowest value in May (1.56), and the highest in November (4.19) (Tables 1 and 2).

Species composition in oysters and seawater The comparison between species composition in both types of samples is shown in Table 3. *Vibrio* spp. were clearly dominant in oyster samples, representing around 65% of the total MA isolates, and dominating the oyster community during most of the year. Only in the cold season they represented less than 50% of the culturable bacteria. Out of them, *Vibrio splendidus* accounted for 40% of total isolates. The second most abundant species was *V. harveyi*, which represented 14% of total MA isolates. Other identified species or unidentified groups never reached, individually, more than 4% of the total MA-culturable community (Table 3).

Some *Vibrio* species appeared only in warm and late-warm season (*V. harveyi*, *V. pelagius*, *V. mediterranei*, and *V. tubiashii*), whereas *V. splendidus* dominance was restricted to winter and spring samples (Fig. 1). The pathogen *V. vulnificus* was not detected, not even among the TCBS isolates (see below), in contrast to oysters harvested in other geographical areas. *V. parahaemolyticus*, another pathogen reported to be transmitted by bivalve consumption, was not detected among MA isolates, but two strains were identified from TCBS plates in July. Seasonality was not evident among strict aerobic species, except for *Pseudoalteromonas luteoviolacea*, that occurred in low numbers from July to November. The hybridization of representative strains from unidentified phena containing oyster isolates revealed that most of them (phena 19, 21, 22, 23 and 33, of the study by Ortigosa et al. [27]) hybridized with the specific probe for α-*Proteobacteria*. A few of them corresponded to γ-*Proteobacteria* (phenon 16), and none of them to *Cytophaga-Flavobacterium*.

Almost all oyster isolates from TCBS plates corresponded, as expected, to fermentative strains, with the sole exception of 2 isolates of *Shewanella putrefaciens*, that grew as black colonies on plates from September and October samples. Roughly, we obtained the same picture for *Vibrio* species incidence on TCBS than in MA plates. A χ^2 test demonstrated no significant differences between relative frequencies of the major *Vibrio* species in both media; some minoritary species were detected, however, only on TCBS (*V. parahaemolyticus*). Nevertheless, differences were very remarkable when considering *Vibrio* spp. densities calculated from TCBS counts versus *Vibrio* spp. densities estimated from MA counts. Apparently, TCBS underestimated *Vibrio* spp. densities, giving sometimes figures as low as 4% of the *Vibrio* CFU on MA. This underestimation was especially noteworthy on cold months.

In our previous study we had identified only a very small

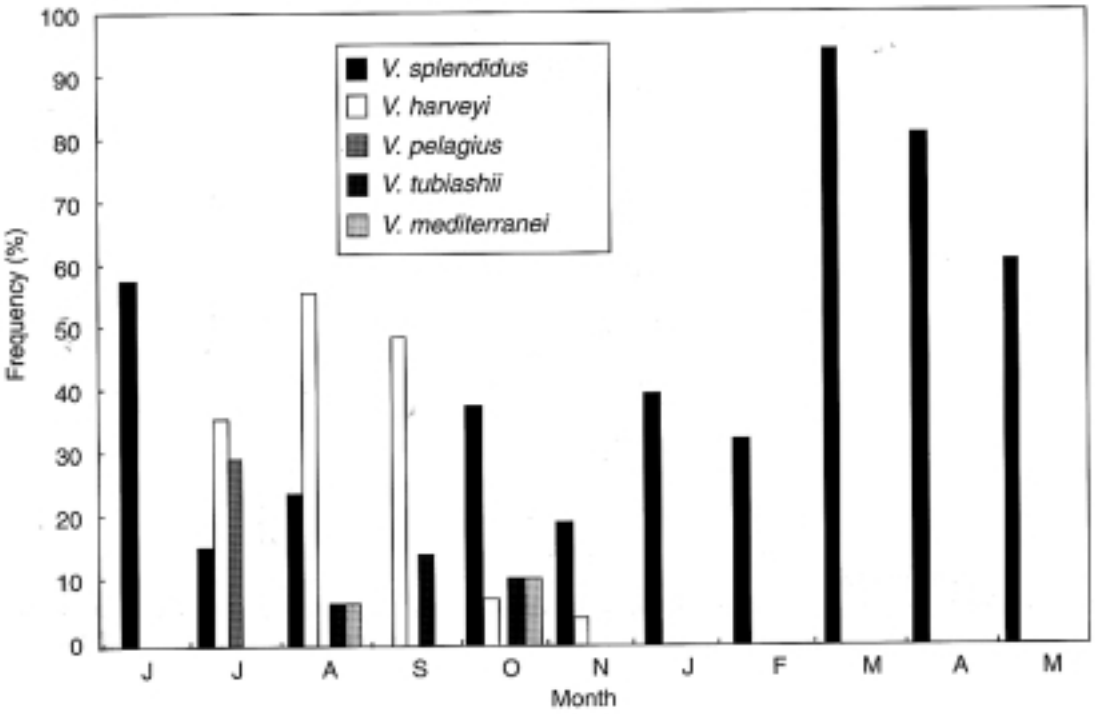

Fig. 1 Percentage of MA isolates of the five most abundant *Vibrio* species found in oyster samples over the year

Table 3 Bacterial species composition of oyster and seawater samples[a, b]

Oyster (319 strains)		Seawater (255 strains)	
Vibrio splendidus	40.0%(127)	*Halomonas cupida*	4.3%(11)
Vibrio harveyi	14.0%(44)	***Alteromonas macleodii***	4.0%(10)
Vibrio pelagius	3.5%(11)	*P. haloplanktis/undina*	3.0% (8)
Vibrio tubiashii	3.0%(9)	***Flavobacterium salegens***	2.7% (7)
α-proteobacteria, O22	2.5%(8)	***Bacillus marinus***	"
α-proteobacteria, O23	2.0%(6)	***Pseudomonas* sp.**	2.3% (6)
Vibrio mediterranei	1.5%(5)	***Halomonas aquamarina***	"
Pseudoalteromonas luteoviolacea	"	**α-proteobacteria, O45**	"
Shewanella putrefaciens	"	**G+, G7**	"
α-proteobacteria, O19	"	*Vibrio harveyi*	2.0%(5)
P. haloplanktis/undina	1.2%(4)	***Photobacterium angustum***	"
***Vibrio/Photob.* sp. F24**	0.9%(3)	**G+, G3**	"
Halomonas cupida (013)	″	α-proteobacteria, O33	1.6%(4)
G+, G1	"	*Cytophaga–Flavobacterium*, O40	"
Vibrio alginolyticus	0.6%(2)	*Marinomonas sp.* O16	1.2%(3)
Photobacterium damselae	"	α-proteobacteria, O20	"
Vibrio fisheri	"	γ-proteobacteria, O24	"
***Vibrio/Phot.* F6**	"	α-proteobacteria, O26	"
***Vibrio/Phot.* F17**	"	**G–, O30**	"
***Vibrio/Phot.* F20**	"	**G–, O39**	"
***Pseudoalteromonas* sp. O4**	"	**γ-proteobacteria, O41**	"
Marinomonas sp. O16	"	**α-proteobacteria, O43**	"
G–, O18	"	**α-proteobacteria, O44**	"
α-proteobacteria, O21		G+, G1	"
***Alteromonas* sp., O29**	"	*Vibrio splendidus*	0.8%(2)
***Bacillus* sp. G5**	"	*Vibrio orientalis*	″
Vibrio orientalis	0.3%(1)	***Vibrio/Phot.* F26**	"
Alteromonas espejiana	"	***Vibrio/Phot.* F27**	"
***Bacillus* sp.**	"	***Pseudoalteromonas* sp. *O7***	″
G–, O25	"	***Marinobacter hydrocarbonoclasticus***	"
α-proteobacteria, O26	"	***Oceanospirillum linum***	"
G–, O27	"	***Micrococcus* sp.**	"
G–, O28	"	α-proteobacteria, O21	"
α-proteobacteria, O33	"	α-proteobacteria, O23	"
Cytophaga sp. O37	"	**G–, O32**	"
Cytophaga/Flavobacterium, O40	"	**α-proteobacteria, O34**	"
		G–, O35	"
+1 × 22 G–, fermentative		**G–, O42**	"
1 × 19 G–, aerobes		**G–, O46**	"
1 × 6 Gram-positive isolates		**G+, G2**	"
		G+, G6	"
		Vibrio tubiashii	0.4%(1)
		Vibrio campbellii	"
		Vibrio mediterranei	"
		***Vibrio/Phot.* F12**	"
		P. espejiana	"
		P. luteoviolacea	"
		Shewanella putrefaciens	"
		Pseudomonas fluorescens	"
		G–, O25	"
		G–, O27	"
		Cytophaga sp. O37	"
		+ 1 × 15 G–, fermentative	
		1 × 49 G–, aerobes	
		1 × 26 Gram-positive isolates	

[a]Boldface indicates species/phenon exclusive of this kind of sample.
[b]Unidentified groups are indicated by a letter (F for fermentative, O for strict aerobes and G for Gram-positives) followed by the number of their phenon in their respective studies.

percentage of the seawater isolates grown on MA (around 32%) by phenotypic traits [28]. Table 3 shows that the water samples were dominated neither by any of the species nor by unidentified groups, as were the oyster samples, and a seasonality was not obvious. The most abundant identified species was *Halomonas (Deleya) cupida*, but it only accounted for 4.3% of the isolates, closely followed by *Alteromonas macleodii*, several other aerobic Gram-negatives, and one Gram-positive, *Bacillus marinus* (Table 3). Among the fermentatives, the more commonly recovered species were *V. harveyi* and *Photobacterium angustum* (2% each). Other *Vibrio* spp. accounted for less than 1% of the isolates. We had identified also *Oceanospirillum linum, Pseudomonas nautica* (now reclassified as *Marinobacter hydrocarbonoclasticus), Pseudoalteromonas espejiana, Pseudoalteromonas luteoviolacea, Shewanella putrefaciens, Pseudomonas fluorescens* and *Cytophaga* sp., but the remaining 60% of isolates could not be identified.

The unidentified seawater strains belonged mainly to α-Proteobacteria as well (phena 20, 21, 23, 26, 33, 34, 43, 44 and 45, of the study by Ortigosa et al., [28]), two phena (24 and 41) were γ-proteobacteria, and phenon 40 contained *Cytophaga/Flavobacterium* strains. TCBS isolates from seawater samples allowed us to detect *V. harveyi* and *V. splendidus*, which were undetectable on parallel MA plates, and revealed the occasional presence of *Photobacterium damselae* and *V. alginolyticus*. Only two out of the 70 strains isolated from seawater on this medium were not vibrios, one *S. putrefaciens* strain and one unidentified Gram-positive.

Discussion

Comparisons of generic composition between the natural bacterial microbiota of bivalve molluscs and their surrounding environments, including their physiological profiles, are relatively scarce due to the methodological burdens imposed by the phenotypic characterization of many strains, which frequently needs numerical analysis. Among the few available studies, Martin [22] carried out a single analysis of both the sea water collected over a mussel bed and the gut of mussels from the same area. In that study, the gut isolates were mainly assigned to the *Vibrionaceae*, whereas these bacteria were hardly detected in the samples from sea water. Kueh and Chan [16] compared the microbiota of several species of bivalve molluscs, including oysters (*Crassostrea gigas*), to that of the surrounding sea water, and found differences in both numbers and generic composition. The most frequently detected microorganisms from shellfish were mainly *Pseudomonas*, but also *Vibrio, Acinetobacter* and *Aeromonas*, whereas coliform and coryneform bacteria were the predominant microorganisms from seawater. Prieur et al. [30] analyzed the microbiota of several bivalve molluscs in an experimental hatchery, including oysters (*C. gigas*), at different stages of culture. Gram-negative fermentative bacteria corresponding to *Photobacterium* and *Vibrio* were more abundant in the ground whole flesh than in the surrounding water. These authors suggested a relationship between the microbiota associated with the mollusc and that of the surrounding sea water. In addition, the bacteria isolated from molluscs were also characterized by a significant proportion of proteolytic and lipolytic strains.

The results of the present study agree to some extent with the mentioned studies. In fact, the culturable aerobic heterotrophic bacterial population associated with Mediterranean oysters (*O. edulis*) is dominated, during the warmer months (March through October), by facultative anaerobic halophilic bacteria (i.e. *Vibrionaceae*) which were the most abundant group during this period of the year also in water samples. These bacteria were not only able to thrive on anaerobic conditions by fermenting carbohydrates, but they could also use nitrate as alternative electron acceptor of their oxidative metabolism, being thus better adapted to anaerobic microniches which may be common in the oyster gut. During the cold season (November to February), Gram-negative oxidative bacterial groups corresponding to yet undescribed α-Proteobacteria dominated in sea water and oyster samples.

The two major *Vibrio* species in oysters identified over the study were *V. splendidus* and *V. harveyi*. Both species showed an opposite behavior, the former being the dominant species throughout the cold season, whereas the latter dominated over the warm months. These two species shared several features which may account for their alternative dominance in this location: they are *Vibrio* species with high salinity requirements for optimal growth, quite above the typical requirements of estuarine vibrios, such as *V. parahaemolyticus* or *V. fluvialis* [4]. They are also similar in their hydrolytic capabilities, and have in common even the ability to produce an extracellular alginase, and their spectra of sole carbon sources are the most similar among *Vibrio* spp. In fact, *V. splendidus* and *V. harveyi* usually arise as closest phena in numerical taxonomic studies [3, 27]. Nevertheless, they behave in opposite ways in relation to temperature preferences: *V. splendidus* is a typical psychrotroph, able to grow at 4°C whereas *V. harveyi* is a mesophile unable to grow at 4°C. In addition, only half of the *V. splendidus* strains isolated in our study were able to grow at 35°C and none grew at 40°C [27], whereas all *V. harveyi* strains could develop at 35°C and some could even thrive at 40°C. The similarities in nutritional and salinity preferences, together with differences in temperature preferences may explain the observed alternation between these two species throughout the year in oyster samples.

The production of hydrolytic exoenzymes shown by most *Vibrio* species is undoubtedly an advantage for the development of dense populations inside the oysters, as both the bacteria and the animal benefit from the breakdown of the ingested/filtered macromolecules. This could explain why *Vibrio* spp. reach high densities in the oysters, even when the water receives inputs from the oyster-associated species through

fecal debris. It seems therefore that the wide number of other halophilic, strict aerobic bacterial species present in sea water and reaching the oyster cannot compete successfully with vibrios when they are in high densities.

When comparing the results obtained in the present study with others obtained on mussel samples from the same area, some differences are obvious: the number of *Vibrio* species detected increases notably by using directly inoculated MA instead of TCBS plates seeded with APW-enriched samples [26]. Thus, besides TCBS being selective for some strains of common *Vibrio* species, a previous enrichment in APW limitates the range of species detected to the few that can compete successfully in the first hours of incubation. This might account for the common detection of *V. alginolyticus* when enrichments are used and its scarcity on directly plated MA, even in the most favorable season. In a study on several types of cultured bivalves (including *O. edulis*) from the Ebro delta (Tarragona, Spain), Montilla et al. [23] used direct streaking of the homogenates onto TCBS and found *V. fluvialis* as the dominant species, whereas *V. alginolyticus* was minoritary. The dominance of the former species can be explained by the lower salinity values described as optimal for this species in comparison with the usual salinity values registered for open Mediterranean waters. The authors of that study also remarked the absence of *V. vulnificus* in the bivalves analyzed, and the occasional presence of *V. parahaemolyticus*. The absence of the former species in all studies performed on oysters from the Mediterranean Sea [27; this study], even in waters with lower salinity such as the Ebro delta,is remarkable in comparison with the abundance of this species in oysters from other geographic areas [12, 14, 25, 36]. Seawater samples were much more diverse that oyster samples, and allowed the detection of large numbers of readily cultured heterotrophic bacteria representing new taxa. Preliminary data on 16S rDNA sequencing of some representatives of unidentified groups indicate that some of them correspond to undescribed new species of already known genera (*Alteromonas*, *Marinomonas*, *Roseobacter*) or to undescribed genera belonging to α-3 Proteobacteria or to the *Flavobacterium-Cytophaga* branch. New marine genera, such as *Sagittula*, *Sulfitobacter*, *Octadecabacter*, *Marinosulfonomonas*, *Ruegeria*, *Arhernsia* and *Stappia* [10, 11, 13, 31, 35], which belong to α-3 Proteobacteria, have been recently described, as well as the non marine halophiles *Antarctobacter*, *Silicibacter*, *Roseivivax*, *Roseovarius* and *Rubrimonas* [17, 18, 29, 33, 34]. More research is necessary to definitively clarify the taxonomic adscription of the unidentified strains. These data stress the fact that there are still many culturable marine bacteria from non-extreme environments that remain unidentified, and, therefore, they also stress the need of accurate and extended studies on these bacteria to complete our databases on bacterial diversity before estimating the extent of uncultured vs cultured marine bacterial taxa.

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