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MICROBIAL DIVERSITY IN THE GULF OF TRIESTE: CHARACTERIZATION OF AEROBIC HETEROTROPHIC BACTERIAL STRAINS

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ABSTRACT

Generally the microbial ecology studies do not stress the strains taxonomic characterization, and this is the reason why microbial diversity in marine waters is poorly understood. In this paper, the results of a preliminary study on microbial diversity in the Gulf of Trieste is presented. The 61% of the isolated strains are Gram negative and, from a taxonomic point of view, are very heterogeneus. The Gram positive strains belong to Bacillus, Staphilococcus, Micrococcus, Rhodococcus, Streptomyces and Sporasarcina. Furthermore, isolation methods, with particular reference to substrata and incubation periods, are considered.

Key words: Gulf of Trieste, microbial diversity, marine microbiology
Ključne besede: Tržaški zaliv, mikrobna diverziteta, mikrobiologija morja

INTRODUCTION

Bacteria often dominate plankton biomass and are recognized as important agents of biogeochemical changes in marine ecosystems, but bacterial diversity is poorly understood yet because conventional isolation methods neglect about 90% of the micro-organisms (Pickup, 1991; Fuhrman *et al.*, 1992).

To understand the role that heterotrophic bacteria play in a certain marine environment the knowledge on the autochthonous microbial flora, not only in terms of biomass and potential activity, but also of community structure and species composition, is needed. The most frequent types of organisms must be isolated and examined for their metabolic activities and their ecological significance.

Since marine microbiologists are seldom interested in taxonomy and identification of new bacterial isolates, the compositions of bacterial communities are little known (Austin, 1988).

Microbiological researches in the Gulf of Trieste studied the influence of river outflows and sewage effluents on bacterial density and bacteriological contamination (Del Negro *et al.*, 1993). Some researches examined the dynamics in the pelagic microbial food web and the dependence of bacterial abundance on different ecological factors (temperature, depth, availability of nutrients and oxygen, etc.) (Herndl *et al.*, 1989).

The species composition of the bacterial population in the Adriatic Sea is little known (Turk, 1991): to our knowledge, the only taxonomic research in heterotrophic bacteria was related to the Central Adriatic (Šobot, 1981) and proved the presence of representatives of the genus *Pseudomonas, Vibrio, Acinetobacter, Flavobacterium* and *Micrococcus*. These results partly correspond with those from other parts of the world (e.g. Austin, 1979).

The concern of the present study was the identification and characterization of bacterial cultures isolated from the Gulf of Trieste that could contribute to the elucidation of the diversity of the predominant heterotrophic bacterial populations in this area.

MATERIALS AND METHODS

The study area

The Gulf of Trieste, the farthest north-eastern part of the Adriatic Sea, is a shallow water area characterized by a limited water exchange (Stravisi, 1983). The hydrodynamism of the whole Gulf is primarily linked to the ascending eastern current coming from the Istrian coast and carrying higher salinity waters from the Middle Adriatic into the northern basin. This current leads to anti-clockwise circulatory movements, which are also enhanced by the winds blowing from the NNE and SE (Mosetti, 1968). The seawater temperatures range from 7 to 27°C and the salinity between 12 to 36 psu, subject to the rainfall in the area (Fonda Umani, 1991).

Sampling

A superficial sea water sample was collected aseptically in May 1993 in a station 300 m off shore located near the National Marine Park "Riserva Parco Marino di Miramare" in the Gulf of Trieste (fig. 1). It was stored with ice and processed within few hours.

Enumeration of bacteria

As an indicator of water quality, coliforms were detected with Brilliant green bile (2%) broth (5 dilutions in 3 replicates), incubated at 35°C for 48 hours.

The enumeration of bacterial population was performed by:

1. direct counting with epifluorescence microscopy:

5 ml of the collected sample were filtered on 0,22 µm polycarbonate black membrane (Nucleopore) stained with DAPI, and the bacteria were counted under fluorescent microscope (Porter & Feig, 1980)

2. plate counts of viable heterotrophic bacteria:

Serial dilutions with artificial sea water (ASW - g/l: $CaCl_2$ 2; KBr 0,15; KCl 0,75; $MgCl_2$ 5,71; $MgSO_4 \cdot 7H_2O$ 5,8; NaCl 23,8; $NaHCO_3$ 0,3; Na_2SO_4 3,6; $SrCl_2 \cdot 6H_2O$ 0,05) from 10^{-1} to 10^{-6} were inoculated in 6 replicates by spreading on 90 mm Petri dishes with 10 different media:

- MAR: marine agar 2216, Difco
- PCA plate count agar, Difco
- PCA prepared with sea water
- PCA prepared with ASW
- PYA: peptone yeast extract agar (Schneider & Rheinheimer, 1988)
- PYA prepared with ASW
- PYS (g/l: peptone 1; yeast extract 1; KCl 0,75; $MgSO_4 \cdot 7H_2O$ 6,98; NaCl 23,8; agar 20)
- CEL: cellulose agar (Schneider & Rheinheimer, 1988)
- CHI 1: chitin agar (Schneider & Rheinheimer, 1988)
- CHI 2: modified chitin agar - two layer plates with basal medium ($(NH_4)_2SO_4$ 2 g; Agar 15 g; ASW 1000 ml) and 3 g/l of crude chitine added to the upper layer.

The counts of CFU (colony forming units) were made after incubation at 25°C for 48 hours.

Isolation and preservation of bacterial strains

From the enumeration plates that were incubated at 25°C for 3-4 further days, 100 colonies were picked on the bases of colony morphology and streaked onto MAR plates, with serial restreaking of each culture being done until the purity of the strains was ensured. Heat-fixed smears of the cultures were Gram stained and the preparations examined under the microscope. After the purification, the cultures were inoculated onto MAR slants, incubated at 25°C and subcultured every 4-6 weeks.

For the isolation of Actinomycetes the membrane method (Hirsch & Christensen, 1983) on 3 special media (Okazaki & Okami, 1976) was used (filtered sample: 500 ml):

-MYS (maltose yeast extract agar),
-GG (glycerol glycine agar)
-SC (soluble starch casein agar).

After all the isolation and purification steps had been followed, the isolates were examined for the phenotypic characters. Those unable to grow on MAR were not considered typical marine bacteria and were eliminated from further study.

For the preservation, all the strains were freeze dried and are now preserved at the National Cultures Bank in Gorizia.

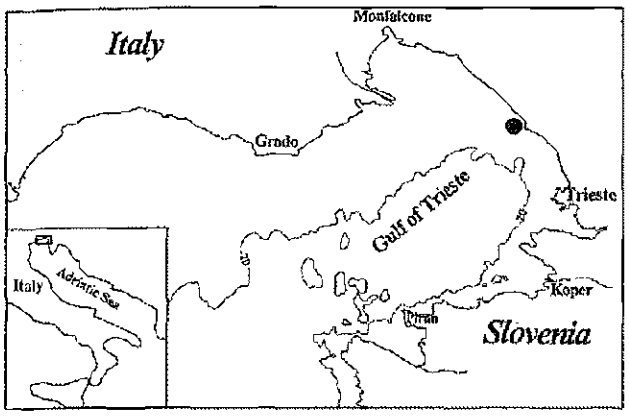

Fig. 1: Study area with the sampling station (near Miramare).
Slika 1: Obravnavano območje z vzorčevalno postajo (Miramare).

Characterization of the bacterial strains

Each strain was examined for at least 28 phenotypic characters. All test media were inoculated using cultures that had been grown on MAR for a week at 25°C. Whenever possible, ASW or MAR were used for the preparation of the media, and the incubation temperature was 25°C.

The characters observed were:

1. Colonial morphology and staining reactions:

- presence of pigment on MAR (after 7 days);
- motility and cell shape were determined microscopically from wet mounts of 24 hours cultures;
- Gram-staining reaction (Difco staining solutions);
- the presence of spores was examined on wet preparations under a phase contrast microscope and on Schaeffer-Fulton stained smears (Doetsch, 1981);

2. Biochemical tests:

- oxidase reaction was determined with the application of 3-day cultures and Oxoid oxidase sticks;
- catalase activity was tested with transferring growth from MAR to a drop of 3% H_2O_2 (Kaneko *et al.*, 1979)
- fermentative or oxidative metabolism of glucose and 5 other carbohydrates (lactose, maltose, mannitol, mannose and sucrose) was determined following the modified Leifson's method (Smibert & Krieg, 1981);
- indole production was detected with the standard procedure (3 days at 25°C) (Smibert & Krieg, 1981);
- nitrate and nitrite reduction was recorded after 7 days (Smibert & Krieg, 1981);
- Voges-Proskauer and methyl red tests were carried out in MR-VP Difco broth at 30°C in 48 hours and in 5 days (Smibert & Krieg, 1981).

3. Degradative capabilities:

- Casein and chitin degradations were tested with a modified Smibert and Krieg (1981) method; the tests were carried out with two layer plates (the bottom one of MAR and the upper one of casein or chitine agar) and the results were recorded after 2 weeks of incubation;
- The degradation of agar, Tween 20 and DNA were tested with MAR, MAR supplemented with 0,1 g $CaCl_2$ and 10 ml Tween 20 (autoclaved separately) and DNAse test agar DIFCO prepared with ASW; the results were registered after 1 week of incubation (Kaneko *et al.*, 1979);
- after 4 days the degradation of urea (Oxoid broth base, prepared with half strength ASW) was detected.

4. Physiology tests:

- growth at 37°C (MAR, 5 days);
- growth on 0% and 4% of NaCl (7 days) (Kaneko *et al.*, 1979).

5. Resistance to:

Penicillin (10 U.I.) and to O-129 was determined on MAR plates after 24 hours by the agar diffusion technique using commercial discs

The isolated Streptomycetes were identified with numerical techniques (Langham *et al.*, 1989) comprising characterization by 50 different tests.

RESULTS

Enumeration

The Brilliant green bile (2%) broth test for the detection of the coli-aerogenes group showed no presence of this kind of bacteria.

Using epifluorescence technique 5,5 x 10^6 cells/ml were detected.

The average counts obtained on the 10 media employed are presented in Table 1:

media	MAR	PCA (0% NaCl)	PCA (sea water)	PCA (ASW)	PYA (sea water)	PYA (ASW)	PYS	CEL	CHI 1	CHI 2
CFU/ml	200	300	25	50	10	20	20	10	1000	100

Tab. 1: Bacteria enumeration on 10 different media.
Tabela 1: Število bakterij, zraslih na 10 različnih medijih.

Isolation

Among the cultures isolated many were not able to

grow on MAR and some lost viability, so a total of 84 pure cultures were obtained. The quantity of strains isolated from every media is presented in the table 2:

Isolation media	No. of strains
MAR	24
PCA (0% NaCl)	13
PCA (sea water)	13
PCA (ASW)	4
PYA (sea water)	12
A (ASW)	3
PYS	6
CEL	5
CHI 1	2
CHI 2	2

Tab. 2: Number of strains isolated from each medium.
Tabela 2: Število sojev bakterijskih vrst, izoliranih iz vsakega medija.

The special media and the technique used for the isolation of Actinomycetes proved to be inefficient, since no bacterial colony, with a myceliar type of growth could be found. But 4 Streptomyces and 2 Rhodococcus strains were isolated by the less selective method on 4 media: MAR, CHI 1, CHI 2 and PCA.

Description of the isolates

The isolates showed to be very diverse, belonging to many different bacterial groups. Some of them were identified to the genus level.

51 isolates (61%) were Gram negative and 33 (39%) Gram positive. More than half of the cultures were colourless (46) and motility was observed only by 27. Most of the isolates are able to grow also without salt (76%) or with 4% of NaCl (85%). The majority was also able to grow at 37°C (85%) and was sensitive to penicillin (82%) and O-129 (70%).

Among Gram positive strains, on the basis of typical colonies forming substrate and aerial mycelium, 4 were recognized as *Streptomyces* and 2 as nocardiforms, probably *Rhodococcus,* showing elementary branching and a marked rod-coccus growth cycle. The Streptomycetes were identified as *Streptomyces anulatus* (2 strains) and *S. exfoliatus* (1 strain); due to the high value of the standard error of taxonomic distance, the fourth *Streptomyces* strain is tentatively assigned to *S. anulatus.*

Among 22 Gram positive rods, 12 were spore forming, catalase positive with fermentative metabolism of glucose and were identified as *Bacillus.*

Only 5 Gram positive cocci were isolated and only 1 was oxidative, probably a *Micrococcus.* 1 formed specific cubical packets of 8 cells, producing endospores, and was by some specific additional tests (Holt *et al.*, 1994) confirmed as *Sporosarcina ureae.* 3 of Gram positive, catalase positive, oxidase negative strains, with fermentative metabolism of glucose, were able to grow on 10% NaCl agar and were identified as *Staphylococcus* (Baird-Parker, 1963).

Among Gram negative isolates, 3 had a helical form and were fermentative, MR-VP, indole and urease negative. The 5 Gram negative vibrios, 15 cocci and 28 rods were very heterogeneous and a lot of additional work should be done in order to identify them.

DISCUSSION

The results of many numerical taxonomy studies show the predominant presence of Gram negative rods along the water column with higher proportion of Gram positive organisms occurring in the sediments (Kaneko *et al.*, 1979; Austin, 1982). In our study more than half of the isolates (61%) were Gram negative, although not predominant, probably due to the limited depth of the monitored station.

The highest counts were obtained using the standard media for the enumeration of heterotrophic aerobic bacteria (PCA and MAR) and, exceptionally, with two chitin agar media, which gave rise to thousands of Actinomycetes colonies belonging to the genus *Streptomyces.* Anyway, the high number of streptomycetes colonies developed is not indicative of the effective dominance of this kind of bacteria (as demonstrated by the lacking of results in the specific tests); the fortuitous inclusion of few spore chains in the inoculated sample could explain the observed situation: the substantial taxonomic homogeneity of the strains seems to confirm this hypothesis.

Although the discrepancy between viable and direct counts may reflect the known presence of unculturable micro-organisms, the difference observed in this study is greater than previously reported for the area considered (Dolzani *et al.*, 1989). However, a longer incubation period (5-6 days) allowed the development of several colonies on the higher dilution plates, belonging mainly to the genus *Rhodococcus* and to an unidentified spirillum. This indicates the importance to prolong the incubation time for determining the total viable counts. As general observation, the isolation methods allowing long incubation periods (high dilution and/or poor and/or difficult media) brought a higher bacteria recovery.

As many authors report, the majority of the bacteria in marine environment are free living or loosely attached to particles, that is why mostly motile bacteria often dominate the isolated strains. In our study motility was observed only by 34% of the isolates. This may be due to the method used (wet mounts from solid media grown cultures), or of the shallow Gulf of Trieste typical bacterial population, where sediment bacteria are

mixed with the free living ones.

Not all of the bacteria characterized in this study required NaCl for their growth. A higher number of colonies developed in standard PCA without NaCl and more than half of them were unable to grow on MAR. MacLeod (1986) suggested that the Na-dependency of the transport process permits faster growth and gives organisms possessing it a competitive advantage in a saline environment. It is possible that the Na-indipendent bacteria in the marine environment grow best when nutrients are not limiting, as in the considered period. The presence of bacteria not affected by NaCl might also be due to a terrigen input but the absence of coliforms does not support this hypothesis.

This study has shown that the aerobic bacteria with and without dependency are part of the plankton community of the Gulf of Trieste, characterized by low diversity and high biomass (Fonda Umani *et al.*, 1990).

POVZETEK

Mikrobna diverziteta morskih voda je slabo raziskana, saj se mikrobne ekološke raziskave ne ozirajo na taksonomsko pripadnost bakterijskih sojev. V tem članku podajamo preliminarne rezultate raziskave mikrobne diverzitete v Tržaškem zalivu. 61% izoliranih bakterijskih sojev je bilo Gram negativnih in iz taksonomskega vidika zelo heterogenih. Gram pozitivni soji so pripadali rodovom Bacillus, Staphilococcus, Micrococcus, Rhodococcus, Streptomyces in Sporosarcina. Poleg tega smo obravnavali še izolacijske tehnike s posebnim ozirom na substrat in čas inkubacije.

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