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Characterisation and differentiation of oligotrophic waters by culturable particle-attached and free-living bacterial communities

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ABSTRACT

This study reports the development of a tool to characterise and differentiate northern Adriatic waters, particularly oligotrophic, high-salinity waters, based on the cellular fatty acids of culturable heterotrophic bacterioplankton. The growth abilities and population dominance were observed for particle-attached and free-living bacteria cultured in three types of media: Marine Broth, diluted Marine Broth (1:10) and R2 broth. Three groups of water layers were distinguished by hierarchical clustering analysis: eutrophic, oligotrophic and oligotrophic nutrient-selected. Significant differences between the resulting groups were tested by two-way ANOVA (with replication). Eutrophic layers were characterised by readily culturable particle-attached and free-living fractions of the bacterial community in all three media, all dominated by fast-growing γ-*Proteobacteria*. In contrast, oligotrophic water layers with low productivity had a much weaker culturability and a different population dominance for the free-living community, as compared to their attached or growth-arrested counterparts, for all media. The free-living bacteria from strictly oligotrophic environments demonstrated minimum culturability in Marine Broth, while those from selective oligotrophic environments were culturable and were dominated either by *Cytophaga–Flavobacter* complex, α-*Proteobacteria* or γ-*Proteobacteria*. The conclusive evidence regarding the selective and refractory nature of organic compounds in these waters demonstrates the dominant culturability of the *Cytophaga–Flavobacter* complex and α-*Proteobacteria* in free-living communities in all growth-media. The response of fatty acid dominance ratios depends significantly on the trophic state and fraction ($p < 0.05$), although the effect of the trophic state is completely different in attached and free fractions. Both fractions were tested separately, demonstrating a significant influence of the trophic state ($p < 0.05$), while the effect of the media on the fatty acid response was not significant ($p > 0.05$). An interaction between media and trophic status was present in the attached fraction ($p < 0.05$), yet this was not observed in the free fraction ($p > 0.05$), indicating that any systematic difference between trophic states was the same for each media tested. Accordingly, the free-living fraction of bacterioplankton is a more informative attribute and can be used solely as an indicator of the water layer trophic condition.

1. Introduction

The population of heterotrophic bacterioplankton can be separated into two major groups based on the uptake of organic matter. Bacterial groups that successfully colonise organic aggregates or other nutrient rich micro-niches are considered to be copiotrophs. These are opportunistic species with low nutrient affinity and a rapid growth strategy, and they depend on high concentrations of organic substances (Pinhassi et al., 2004; Palijan and Fuks, 2006). In contrast, most typical free-living marine bacteria with unique physiological characteristics are able to survive and grow under conditions of an extremely low and discontinuous supply of nutrients and are defined as oligotrophs (Cavicchioli et al., 2003). Particle-attached bacterial communities are usually larger and more active than the free-living bacteria. Although there may be an interchange of members between both communities, marked taxonomical and physiological differences between them have been found (DeLong et al., 1993; Acinas et al., 1999). Since the physical and chemical properties of the microenvironments of these communities are extremely different, it was assumed that their enzymes have adapted to work efficiently under different conditions. Accordingly, the degree of similarity between those two fractions is variable and depends on environmental conditions (LaMontagne and Holden, 2003).

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Functional differences between microbial communities have already been recognised as an indicator of local environmental conditions, and have potential applications in water quality assessment (Ahn et al., 2007; Marshall et al., 2008). In natural environments, as in different culture media, heterotrophic bacteria adapt their growth rate to both the nature and concentration of organic nutrients. Bacteria generally take on distinct properties when starved for each class of essential nutrients. The related depletions require completely different physiological and regulatory responses (Ferenci, 2001). Therefore, changes in microorganisms' taxonomic and physiological status caused by external factors can be successfully inferred from measurements of their cellular fatty acids (Blažina et al., 2005).

The aim of this study was to develop a tool based on the cellular fatty acids of the culturable fraction of heterotrophic bacterioplankton in order to better characterise and differentiate northern Adriatic water masses, particularly oligotrophic high-salinity waters. This study employs the novel approach of comparing the responses to fatty acids of the particle-attached and free-living fractions of the natural bacterioplankton communities after cultivation in three different media.

2. Materials and methods

Seawater was collected at seven stations in the northern Adriatic Sea during several cruises (June–September, 2006) aboard the Vila Velebita research vessel. The choice of sampling points was made according to salinity data obtained by CTD profile, and choices were made to correspond to water layers with different levels of salinity. Samples were transferred into sterile polycarbonate tubes and processed onboard the research vessel.

In total, 30 samples for bacteria enumeration by epifluorescence microscopy were fixed with 2% formalin and stained with DAPI (Porter and Feig, 1980). In order to determine the number of colony-forming units (CFU) and the proportion of the total number of bacteria, as well as to isolate single bacterial colonies, samples were spread (100 μL) on three diverse solid media. Bacterial colonies were grown on agar for 10 days at 20 °C.

Marine Agar (MA) and Marine Broth (MB) (Difco 2216) were used as representative of nutrient-rich media. Two different types of media were used to represent oligotrophic conditions, prepared by the following procedure. Marine Broth and Agar were diluted (dM) to 1:10 (Palijan and Fuks, 2006) with aged seawater (after 2 months in the dark) filtered through 0.22 μm GSM filters (Millipore, USA). The second media for growth of oligotrophic bacteria, R2A (Difco), was also prepared with aged seawater. R2 liquid media (R2) was prepared by addition of glucose, starch, sodium pyruvate and free casamino acids in the same quantities as used in original R2A solid media.

Additionally, 100 mL of each water sample was filtered on 0.7 μm GF/F filters (Whatman, USA) to retain particle-attached bacteria. Then, the filtrate was filtered on 0.22 μm filters (Millipore, USA) to retain free-living bacteria. Each filter was cut into 3 equal parts with a sterile blade, inoculated in 100 mL of three different liquid media, and incubated for 48 h at 20 °C to obtain the culturable part of particle-attached and free-living communities, respectively, for Fatty Acid Methyl Ester (FAME) analyses. Before further processing, 0.8 mL aliquots of bacterial cultures were separated, added to 0.2 mL of 50% glycerol and preserved at −80 °C for further molecular analysis. Liquid cultures of bacterial isolates and communities were centrifuged at 4000 × *g* for 20 min and washed twice with deionised water. The bacterial pellets (100–400 mg fresh weight), were saponified, methylated and analysed (Blažina et al., 2005). The samples were added to 1.2 M NaOH in 50% aqueous methanol solution. The tubes were placed in a boiling bath for 30 min. After cooling, the saponificate was acidified with 6 M HCl (pH < 2). Next, 12% BF_3 in methanol was added, and the samples were heated for 10 min in a near boiling water bath. After cooling, the FAME were extracted in dichloromethane.

The FAME were analysed by gas-liquid chromatography on a 6890N Network GC System equipped with a 5973 Network Mass Selective Detector with a capillary column (30 m × 0.25 mm × 0.25 μm; cross linked 5% phenylmethylsiloxan), using ultra-high purity helium as the carrier gas. The GLC settings were as follows: the programmed column temperature was 145 °C, increasing at 4 °C/min up to 270 °C, and a constant column pressure of 15 psi was used. The retention time and peak areas were recorded using Chemstation Software. Bacterial FAME were identified by mass spectral data, and by a family plot of equivalent chain length data obtained by GC standards (Blažina et al., 2005).

For each fraction (attached and free-living), 3 dominance ratios were calculated for each of three culture media (M, dM and R2): DRgamma = C16:1/(C18:1 + BRtot), DRalpha = C18:1/(C16:1 + BRtot) and DRCF = BRtot/(C18:1 + C16:1). A multivariate classification procedure (hierarchical clustering method), performed over dominance ratios, was used to detect natural sample groupings. These ratios emphasise the dominance of bacterial populations in the culturable community (DRgamma of γ-*Proteobacteria*; DRalpha of α-*Proteobacteria*; DRCF of *Cytophaga–Flavobacter* complex), are the highest for the dominating population (>1), and do not overlap with ratios of the other populations (Blažina et al., 2005). Significant differences between groups obtained by hierarchical clustering were tested by two-way ANOVA with replication with statistical significance set at the conventional 5% level ($p > 0.05$). The statistical data analyses were performed on a PC Systat 10.

A fragment of bacterial 16S rRNA (positions 28–518 of the *Escherichia coli* numbering) was PCR amplified using primers: 5′-CTCGAGGTCGACGGTATCGGAG TTTGATCCTGGCTCAG-3′ and 5′-CACGCTCTAGA-ACTAGTGGAT(AT)ATTACC GCGGC(GT)GCTG-3′. The 5′-terminal twenty nucleotides of the two primers were added as annealing sites for sequencing primers (Mitsui et al., 1997). The reaction mixture contained 1 μM of each primer, 200 μM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase, 1× *Taq* buffer with KCl, and 1 μL of each bacterial culture grown on different types of media as templates. The mixture was layered with mineral oil, and the following protocol was used: a 2 min initial denaturation at 94 °C followed by 35 cycles of 94 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min, with a final extension step at 72 °C for 5 min after the last cycle. The PCR products then underwent electrophoresis in a 1% agarose gel in 1×TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.3). Sequencing of PCR products was carried out by Macrogen (Seoul, Korea) and conducted under BigDyeTM terminator cycling conditions. The sequences determined in this study were compared and analysed for similarity to other known sequences in public-domain databases (NCBI GeneBank [http://www.ncbi.nlm.nih.gov/] and the Ribosomal Database Project [http://rdp.cme.msu.edu/]).

3. Results

The data set for each water layer consisted of 18 variables: 2 fractions × 3 growth media × 3 FAME dominance ratios. Multivariate hierarchical clustering procedures performed on more than 30 different water layers indicated 3 major groupings: GI, GII and GIII, each consisting of 10 water layers. The dendrogram is presented in Fig. 1. The environmental conditions met in the water layers joined to groups GI–GIII are summarised in Table 1. Water layers in GI mostly had higher chlorophyll *a* concentrations, a higher total number of bacteria, and lower salinity levels. CFUs on all three solid media were highly similar. In contrast, the water