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Adaptational changes in cellular fatty acids of cultured bacteria as a response to trophic differences

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*The adaptational changes in cellular fatty acids and morphology of γ-*Proteobacteria *belonging to different genera* (Vibrio, Halomonas, Pseudoalteromonas) *isolated from northern Adriatic waters and cultured in four media (Marine Broth, R2 Broth and tenfold dilutions of both) were investigated. The bacterial strains with identical 16S rRNA sequences isolated from different waters used different mechanisms, or different extent of the same mechanism, to adapt their growth in equal culture media. In contrast, a similar growth strategy in each of the used cultures was employed by different strains isolated from the same water body. Divergence in nutrient quality and requirement in cultures from that presented in the bacterias' natural environment induced important changes in cellular fatty acids; (i) unsaturation, (ii) cis to trans isomerisation, (iii) chain elongation (iv) branching and/or morphology (Winnie-the-Pooh effect) or even cell division blocking. The results strongly suggest that growth and the appropriate cellular response of bacteria in culture is predetermined by nutritional conditions in their true environment and can be useful for interpretation of trophic differences between proximate environments.*

Key words: adaptation**,** cultured bacteria, cellular fatty acids, physiological response, Nothern Adriatic

INTRODUCTION

In the natural environment, just as in different culture media, heterotrophic bacteria adapt their growth rate and cell size to both the nature and concentrations of organic nutrients (EGUCHI *et al.,* 1996). Knowledge of microbial growth and substrate utilisation is important for the prediction of the fate of organic compounds in natural and engineered environments (GRADY *et al.*, 1996). Bacteria generally have distinct properties when starved for each class of essential nutrients. The related depletions require completely different physiological and regulatory responses (FERENCI, 1999, 2001). Cellular fatty acids play a role as a good biomarker of changes in the physiological status of microorganisms caused by external factors (MROZIK *et al*., 2004). Abrupt changes induce stress in a bacterial cell; adaptive response differently influences their growth and morphology (THINGSTAD *et al.,* 2005) and has a pronounced effect on the overall fatty acid profile (HEIPIEPER *et al.,* 2003; LOFFHAGEN *et al.*, 2004; HÄRTIG *et al*., 2005). The bacterial growth in culture is a reflection of the cell's history and currently existing environmental conditions from which the cells are obtained (GRADY *et al.*, 1996). Accordingly, the bacteria from different phylogenetic lineages are capable of developing similar strategies to survive nutritional changes (FERENCI, 1999, 2001).

We investigated and compared changes in the fatty acid composition and cell morphology of bacterial strains with identical 16S rRNA sequences, isolated from different water layers, after growth in cultures with different nutrient enrichments. It was hypothesised that differences in adaptation mechanisms used would allow interpretation of the trophic differences between their true environments.

MATERIAL AND METHODS

Samples were taken in July 2007 at two stations in the northern Adriatic Sea; 104 (9 m and 20 m) and 105 (10, 15 and 20 m). The chosen depths, according to CTD profiler data, correspond to water bodies above (PSU< 37.5), below (PSU> 38.3) and in the salinity gradient.

The samples were spread (100 µL) on solid media for isolation of single bacterial colonies onboard. The solid media for growth of oligotrophic bacteria (1:10 diluted R2A (Difco)) was prepared with aged seawater (after two months in the dark) filtered through 0.2 µm GSM filters (Millipore, USA) and Bacto Agar (Difco) (CHO & GIOVANNONI, 2004). The bacterial colonies were grown for 10 days at 20°C. The procedure with grown bacterial colonies was as follows: Each of the isolated strains was 16S rRNA sequenced and identical strains from different water layers as well as different strains from the same water layers were subjected to growth in different media. Identity of strains was verified by repeated 16S rRNA sequencing of bacteria from each of the media used.

The strains were grown in liquid media, prepared in the same composition as R2A solid media (R), diluted 1:10 with aged sea water (dR) and then inoculated in 100 µL aliquots into three different types of liquid media: Marine Broth (Difco 2216; M), diluted Marine Broth (dM) to 1:10 with aged seawater and full strength R media. For fatty acid analysis cultures were incubated in liquid media for 48 hours at 20°C. The 0.8ml aliquots of cultures were separated, added to 0.2ml of 50% glycerol and preserved at -80°C for further molecular analysis.

Fatty acid methyl esters (FAME) were isolated from bacterial pellets and analyzed by gas-liquid chromatography (GLC) on a 6890N Network GC System equipped with a 5973 Network Mass Selective Detector (MSD) with a capillary column (30m x 0.25mm x 0.25µm; cross linked 5% phenylmethylsiloxan) and ultra high purity helium as the carrier gas (BLAŽINA *et al.,* 2005).

A fragment of bacterial 16S rRNA (positions 28 to 518 of the *Escherichia coli* numbering) was PCR amplified using primers: 5′-CTC-GAGGTCGACGGTATCGGAGT TTGATC-CTGGCTCAG-3′ and 5′-CACGCTCTAGA-ACTAGTGGAT (AT) ATTACCGC GGC (GT) GCTG-3′. The 5′-terminal twenty nucleotides of two primers were added as annealing sites for sequencing primers (MITSUI *et al.,* 1997). The reaction mixture contained 1µM of each primer, 200 µM of each deoxynucleoside triphosphate, 1.5mM $MgCl_2$, 1.25U of *Taq* DNA polymerase, 1x *Taq* buffer with KCl, and as a template 1µL of each bacterial culture grown on different types of media. The mixture was layered with mineral oil and the following protocol was used: 2 min initial denaturation at 94ºC, 35 cycles of 94ºC for 1min, 53ºC for 1min, 72ºC for 1min and a final extending step at 72ºC for 5 min after the last cycle. The PCR products were electrophoresed in 1% agarose gel in 1xTAE buffer (40mM Tris-acetate, 1mM EDTA, pH 8.3). Sequencing of PCR products was carried out by Macrogen (Seoul, Korea) and conducted under BigDyeTM terminator cycling conditions. The determined sequences in this study were compared and analyzed 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/]).

Samples for bacteria enumeration by epifluorescent microscopy were fixed with 2 % formalin and stained with DAPI (PORTER & FEIG, 1980). The cell volume was calculated according to:

$$V(\mu m^3) = \left(\frac{\pi}{4}\right) \times w^2 \times \left(L - \frac{w}{3}\right),$$ and the cell area: $$A(\mu m^2) = w \times \pi \times \left(\frac{w}{2} + L\right)$$

(w is cell width (µm) and L is cell length (µm))

and biovolume was converted to carbon biomass, *m* (μgC ml^{-1}), assuming 0.220 pgC μm^{-3} (BRATBAK, 1985).

RESULTS

The accession numbers, sources (sampling location and water body type) and the closest relatives of bacterial strains used in this study are given in Table 1.

In R media isolates from both environments showed very similar C16:1trans isomer proportions and consequently cis/trans ratios. In spite of the significantly larger cell volume of *Vibrio* 1B the biomass yields of both strains were similar. The most pronounced difference in fatty acid profiles and biomass yields was observed between cells grown in dM media. While in 1A C16:1trans isomer comprised a substantial proportion, in 1B a complete shift toward C18:1cis

Table 1. Bacterial strains with taxonomic affiliation to γ- Proteobacteria, *isolated from different locations, related bacteria in GenBank with accession number and 16S rDNA sequence similarity*

Isolate	Location	Closest relatives (Accession number)	Similarity (%)
ADRIF 1 EU649682	1A - 104, 9 m (above gradient) 1B - 105, 10 m (above gradient)	*Vibrio campbellii* (ATCC 25920T), X74692.1	100
ADRIF 2 EU649683	2A - 105, 15 m (gradient) 2B - 105, 20 m (below gradient) 2C - 104, 9 m (above gradient)	*Halomonas meridiana* DSM5425, AJ306891.1 *Halomonas aquamarina* DSM30161, AJ306888.1	99 99
ADRIF 3 EU649684	104, 20 m (below gradient)	*Pseudoalteromonas sp.* strain 03/034 AJ874351.1	100
ADRIF 4 EU649685	104, 20 m (below gradient)	*Pseudoalteromonas citrea* (NICMB 1889T) X82137.1	98

The relevant fatty acid (FAME) ratios and morphological characteristics of two identical strains belonging to genus *Vibrio* (ADRIF1) isolated from two different environments (1A and 1B) after cultivation in dR, R, M and dM media are presented in Fig. 1 and Table 2. The highest C16:1 cis/trans ratio, up to 5, was achieved in 1A and 1B strains when grown in different media, dR and M, respectively. Morphology of the respective cells was similar but biomass varied inversely to the proportion of C16:1trans. fatty acid domination occurred. Both isolates had similar morphology, yet biomass yield of 1B was notably smaller than that of 1A.

The marked differences in fatty acid ratios and morphological features after cultivation in all four media were also observed for three identical strains belonging to genus *Halomonas* (ADRIF2) isolated from three different environments - 2A, 2B and 2C (Fig. 2, Table 3). The branched fatty acids were present in varying but significant proportions in the 2B profile, and

Table 2. Morphological features of identical Vibrio *strain, ADRIF1, isolated from two different water layers at stations 104, 9 m (1A) and 105, 10m (1B), after cultivation in 1:10 diluted R2 media (dR), R2 broth (R), Marine broth (M) and 1:10 diluted Marine broth (dM)*

Media	dR		R		M		dM	
Isolate	1A	1B	1A	1B	1A	1B	1A	1B
w, µm	0.5	0.4	0.7	1.0	0.7	0.7	0.6	0.5
L, µm	0.7	1.0	1.3	1.7	1.3	1.4	0.9	1.0
V, µm³	0.1	0.1	0.4	1.1	0.5	0.5	0.2	0.2
A/V	14.4	12.8	8.5	6.3	8.5	8.1	11.5	12.2
m/µgC ml^{-1}	5.7	3.1	16.3	19.6	40.1	56.6	16.0	3.9

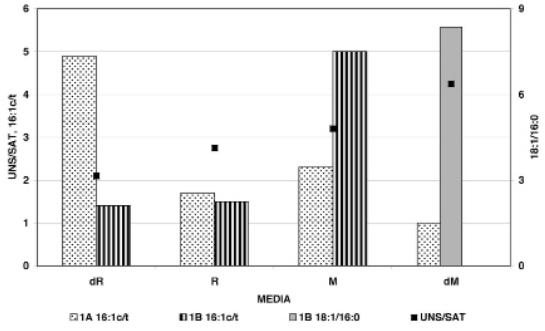

Fig. 1. C16:1 cis to trans (16:1c/t), unsaturated to saturated (UNS/SAT) and C18:1/16:0 fatty acid ratios of identical Vibrio strain, ADRIF1, isolated from two different water layers at stations 104, 9 m (1A) and 105, 10m (1B), after cultivation in 1:10 diluted R2 media (dR), R2 broth (R), Marine broth (M) and 1:10 diluted Marine broth (dM)

reaching a maximum in dM, contrary to 2A and 2C in all four media. In dR media *Halomonas* 2B showed the lowest biomass yield, different cell volume and unsaturation degree in comparison to 2A and 2C. In R media the fatty acid profiles of all three isolates were entirely different; in 2A and 2C being dominated by C16:1cis and C18:1cis, respectively. In spite of the varying cell morphology the biomasses of all three strains were similar. Though the fatty acid profiles of 2A and 2C in M media were similar, in both strains cell elongation occurred, although was substantially pronounced in 2A and accompanied by lower biomass than that measured in strain 2C (Fig. 3). While strain 2B showed no cell division in M media, in dM media growth was characterised by significant contribution of saturated branched fatty acids and cell elonga-

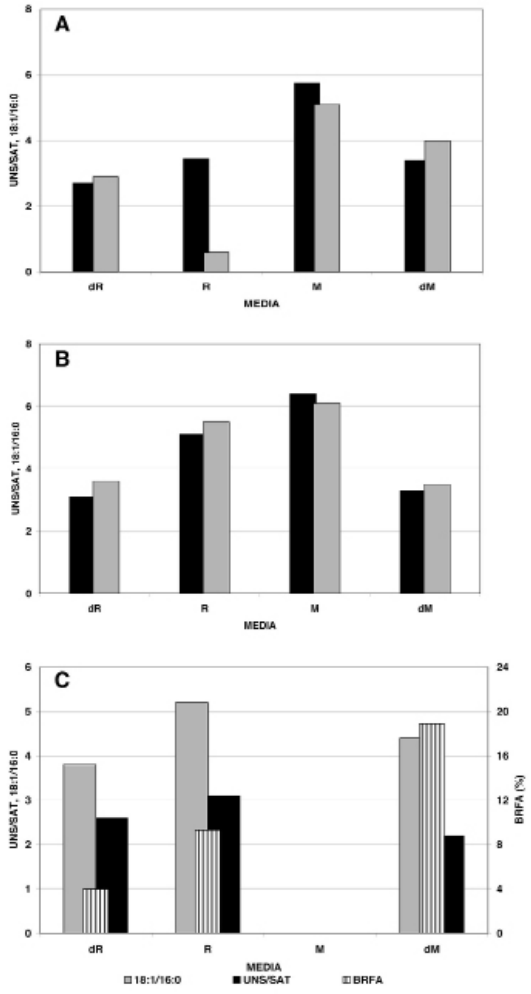

Fig. 2. C18:1/16:0 and unsaturated to saturated (UNS/SAT) fatty acid ratios and total branched fatty acid proportion (BRFA) of identical Halomonas *strain, ADRIF2, isolated from three different water layers at stations 105, 15 m (2A) and 20m (2B) and 104, 9m (2C), after cultivation in 1:10 diluted R2 media (dR), R2 broth (R), Marine broth (M) and 1:10 diluted Marine broth (dM)*

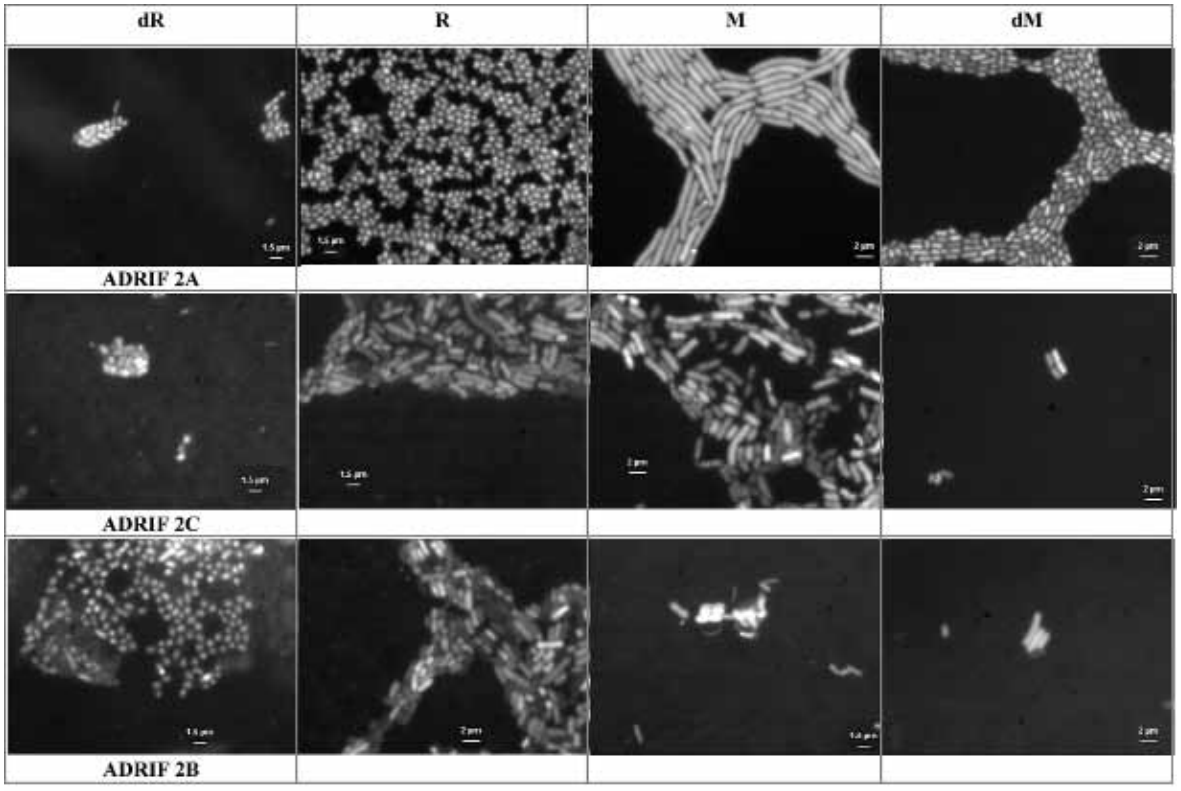

Fig.3. Growth of Halomonas *ADRIF 2 isolated from A) salinity gradient, B) below gradient and C) above gradient, on four different growth media. All photos were taken by epifluorescence microscope at 1000x magnification. Images were recorded with a Hitachi KP-M1A camera and analysed with image analysis software*

tion. The fatty acid profiles of strains 2A and 2C were similar, but larger cell volume and biomass yield were measured in 2C.

Two different *Pseudoalteromonas* strains (ADRIF3 and ADRIF4) isolated from the same environment did not show any cell division in both M and dM media (Table 4, Fig. 4). FAME profiles of both strains in dR media were characterised by high proportions of the C16:1trans isomer. In R media the C16:1cis isomer of ADRIF3 was completely, while C17:1cis and C18:1cis were only partially, transformed into the corresponding trans isomers. In contrast, the ADRIF4 strain showed a much lower contribution of C16:1trans fatty acid. With respect to their dR cultures, both strains significantly

Table 4. Morphological features of two different Pseudoalteromonas strains, ADRIF 3 and ADRIF 4, isolated from the same water layer at station 104, 20m, after cultivation in 1:10 diluted R2 media (dR), R2 broth (R), Marine broth (M) and 1:10 diluted Marine broth (dM)

Media	dR		R		M		dM	
Isolate	3	4	3	4	3	4	3	4
w, µm	0.7	0.8	0.9	0.8	0.7	1.0	0.8	0.9
L, µm	0.8	1.2	1.3	1.1	1.2	1.5	1.1	1.3
V, µm³	0.2	0.5	0.7	0.5	0.4	0.9	0.4	0.7
A/V	8.4	8.8	7.7	9.5	8.7	7.3	9.1	8.3
m/µgC ml^{-1}	19.8	4.1	116.9	43.2	0.7	0.3	0.3	0.1

increased their biomasses and in ADRIF3 cell volume was significantly enlarged, while in ADRIF4 no change occurred.

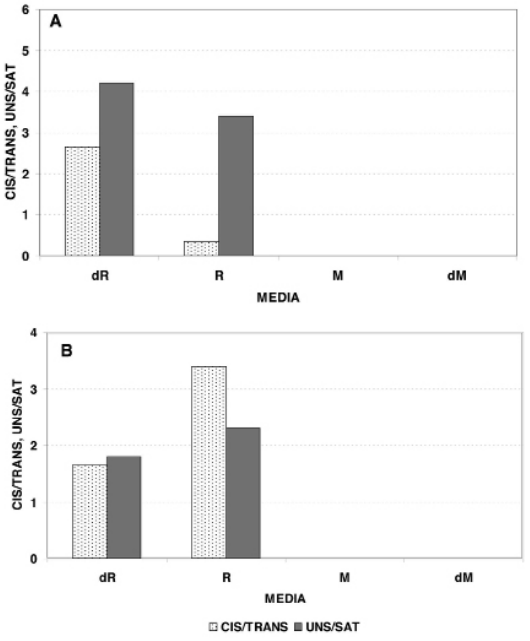

Fig. 4. Total cis to trans (CIS/TRANS) and unsaturated to saturated (UNS/SAT) fatty acid ratios of two different Pseudoalteromonas *strains A) ADRIF 3 and B) ADRIF 4, isolated from the same water layer at station 104, 20m, after cultivation in 1:10 diluted R2 media (dR), R2 broth (R), Marine broth (M) and 1:10 diluted Marine broth (dM)*

DISCUSSION

The strains with identical 16S rRNA sequences isolated from different water layers showed marked differences concerning cellular fatty acids and/or morphological features after cultivation in four media of different quality or enrichment. In contrast, a similar growth strategy in each of the used cultures was employed by different strains isolated from the same water body. These results agree with the concept of separate hunger state (FERENCI, 1999), i.e. hunger for each of the essential nutrients requires a different regulation mode and adaptation of the outer membrane permeability. The strains were cultured in Marine and R2 Broths, media that offer equally rich nutrient environments of different complex substances. Bacteria isolated from the environment where their growth was adapted to a similar quality of nutrients as offered in one of the culture media successfully grew with minor adjustments. In tenfold media dilution bacteria responded with further changes in membrane permeability to the changes of required substances concentration. Otherwise, morphological changes, i.e. volume enhancement due to use of assimilable but not required for growth substrates, with minor changes in cellular fatty acids were observed. Thus the bacteria adapt their growth in culture differently as a function of the nutritional state of bacterial isolation environment.

The different modes of a *Vibrio*-ADRIF1 adaptation were reflected in various proportions of trans isomers and consequently cis/trans ratios. Such conversion, a very efficient mechanism enabling bacteria to adapt to environmental stress, changes the permeability of the membrane (HEIPIEPER *et al.,* 2003; MROZIK *et al.*, 2004). Different adaptation expressed in dR already implied a difference in the two strains' origins with respect to availability and removal of carbon and nitrogen compounds. This observation was strengthened in tenfold richer media of the same quality (R). Non-limiting glucose addition provoked changes to the membrane of strain 1A by switching trans isomerisation in order to reduce transport through the membrane, while strain 1B used it to increase its size without any change in membrane permeability. The latter strategy, known as the Winnie-the-Pooh effect, is also implemented by bacteria in nature where there is access to a pool of assimilable organic C in excess of that required for growth (THINGSTAD *et al.*, 2005). In M media both strains grew comparably, but differences in FAME profiles revealed more a pronounced adaptation need in strain 1A. This also suggests differences in the availability of complex carbon and nitrogen sources in their true environments, particularly considering the entirely different adaptation modes of the related strains in dM. Low cell volume, enhanced surface-to-volume ratio and the highest unsaturation in both strains indicated enhanced transport of nutrients. However, the change of concentration and apparently the relation between nutrients in diluted media stimulated *trans* isomerisation in 1A and

more complex adaptation, C2- fatty acid chain-elongation, in 1B. Different responses suggested different nutrient demands in their original environments. The C2-chain-elongation requires a switch in utilisation of acyl-chain primers from the substrate (NICHOLS & RUSSELL, 1996) to provide maximum unsaturation and consequently increased membrane permeability. Such membrane fluidity regulation is directed toward assimilation of very complex high molecular weight carbon sources such as cellulose (MOON & ANDERSON, 2001). Selective intake of substrate and hunger for complex, large molecules indicated more select quality of the environmental substrate of 1B in comparison to 1A. In addition, the high energy cost of fatty acid chain elongation in comparison to *trans* isomerisation resulted in significantly lower biomass in 1B.

Halomonas 2C demonstrated similar morphological characteristics and membrane adjustment to *Vibrio* 1A. With varying medium quality and concentrations the *Halomonas* kept a constantly high unsaturation degree, changing proportions of C18:1 and C16:0, while *Vibrio* achieved the same effect by cis/trans isomerisation. Both strains have used the same adaptation type in all four media in order to maximize nutrient transport ability with minimum energy consumption. *Halomonas* 2A displayed similar changes in both diluted media, likewise recognition of complex and simple substrate quality, although underwent severe biochemical and morphology changes in related concentrated media, respectively. However, in R media, unaltered and nearly coccoidal structures with saturated membranes indicated that bacteria remained starved in spite of unlimited glucose, an apparently hostile substrate. In contrast, a typical "sausage-form" morphology and high unsaturation in M point to an increase in removal of assimilable complex organic compounds in excess, a more suitable process for its original environment as storage for "a rainy day". Such a strategy was described for bacteria thriving in fluctuating environments (THINGSTAD *et al.,* 2005), e.g. gradient layers.

The growth characteristics in cultures of *Pseudoalteromonas* strains ADRIF3 and ADRIF4, as well as *Halomonas* 2B strain, pointed to the severe nutritional scarcity of their original environments of isolation. All three strains reacted extremely to the nutritional enrichment either by complete trans isomerisation, branch-fatty acid synthesis as an energy-consuming mechanism of adaptation or cell division blocking as the most serious bacterial response to nutritional stress. However, growth absence might also indicate that the related strains were not in an active state, starving for energy to overcome the present divergence between enriched media and the nutrient-scarce environment they originated from.

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Prilagodbene promjene u staničnim masnim kiselinama bakterija izdvojenim iz sjevernog Jadrana – odgovor na trofičke razlike

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SAŽETAK

Istražene su prilagodbene promjene u staničnim masnim kiselinama i morfologiji *γ-Proteobacteria* koje pripadaju različitim svojtama; *Vibrio*, *Halomonas*, *Pseudoalteromonas*; izoliranih iz sjevernog Jadrana i uzgojenim na četiri hranjive podloge (Marine Broth, R2 Broth i deseterostruko razrjeđenje od oba). Sojevi s jednakim 16S rRNK sekvencama izolirani iz različitih vodenih masa koristili su različite mehanizme ili jedan mehanizam različitog intenziteta kako bi prilagodili rast u istom mediju uzgoja. Nasuprot tome, sličnu strategiju rasta su koristili različiti sojevi izolirani iz iste vodene mase. Odstupanje u kvaliteti hranjiva i potreba u kulturi od onih prisutnih u prirodnom okolišu izaziva važne promjene u staničnim masnim kiselinama (i) nezasićenost, (ii) cis/trans izomerizacija, (iii) produljenje acilnih lanaca, (iv) razgranavanje acilnih lanaca ili/i promjene morfologije stanica (Winnie-The-Pooh učinak), te zaustavljanje dijeljenja stanica. Rezultati upućuju da su rast i odgovarajući stanični odgovor bakterija u kulturi unaprijed određeni stanjem hranjiva u prirodnom okolišu, te mogu poslužiti za interpretaciju trofičkih razlika između bliskih okoliša.

Ključne riječi: prilagodba, uzgojene bakterije, stanične masne kiseline, fiziološki odgovor, Sjeverni Jadran