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FEATURE ARTICLE

Fatty acid composition of the heterotrophic nanoflagellate *Paraphysomonas* sp.: influence of diet and de novo biosynthesis

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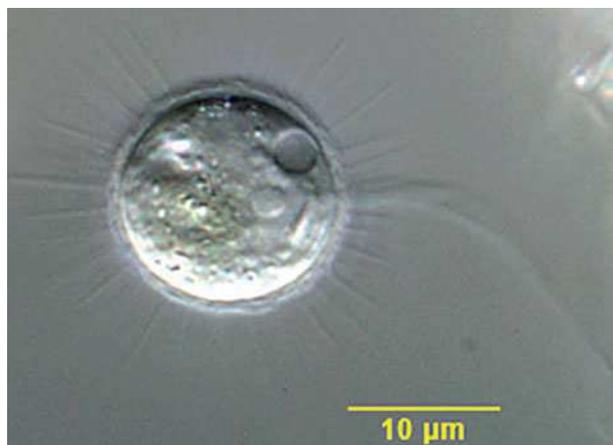
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ABSTRACT: We compared the relative importance of dietary factors versus de novo synthesis in determining the polyunsaturated fatty acid (PUFA) composition of the heterotrophic nanoflagellate *Paraphysomonas* sp. The flagellate was fed with different mutants of the picocyanobacterial strain *Synechocystis* PCC6803, which differ in their capability to synthesize specific PUFAs. The *desA*, *desB*, and *desD* genes of *Synechocystis* PCC6803 encode lipid desaturases at the $\Delta 12$, $\Delta 15$, and $\Delta 6$ positions of 18C fatty acids (FAs), respectively. Thus, the use of *desA*⁻, *desB*⁻, *desD*⁻, and *desA*⁻/*desD*⁻ mutants of *Synechocystis* PCC6803 as food sources permitted us to provide the heterotrophic flagellate with decreasing levels of unsaturated FAs. In each treatment, *Paraphysomonas* sp. exhibited the same FA composition pattern, i.e. high levels of 16:0 and 18:1, and significant amounts of 18C PUFAs and long-chain PUFAs such as 20:4(n-6), 20:5(n-3), and 22:6(n-3), which indicated that *Paraphysomonas* sp. is capable of synthesizing these PUFAs de novo. Results also showed that dietary 18C PUFAs seem to be preferentially accumulated in *Paraphysomonas* sp. lipids. This demonstrates that heterotrophic protists could play a key role in transferring essential compounds from primary producers to metazoan consumers.

KEY WORDS: Heterotrophic protist · Fatty acids · Picoplankton · Trophic upgrading

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Paraphysomonas vestita, a colourless stramenopile flagellate.

Photo: W. Bourland (see starcentral.mbl.edu/microscope)

INTRODUCTION

As heterotrophic protists (HP) are ubiquitous and abundant in all types of habitats, they are important components of food webs (Sherr & Sherr 2002, Montagnes et al. 2008). In aquatic environments, the populations of HP often are diverse, not only in species richness but also in feeding behavior and ecological roles. Indeed, HP can feed on a wide size range of prey, ranging from viruses (Bettarel et al. 2005) to fish

(Burkholder & Glasgow 1997). HP are reported to be a major source of mortality for both heterotrophic and autotrophic bacteria (Sherr & Sherr 2002). There is growing evidence that HP are the major consumers of primary production in aquatic ecosystems (Calbet & Landry 2004, Sherr & Sherr 2007). The assimilation of organic matter by HP leads to substantial losses in carbon, which has led to a debate about the quantitative significance of the transfer of organic matter to higher trophic levels via HP. However, it has been demonstrated that HP, as intermediate trophic levels, can upgrade the quality of their prey for crustacean grazers (Klein Breteler et al. 1999, Martin-Creuzburg et al. 2005, Bec et al. 2006, Martin-Creuzburg & Von Elert 2009). Of course, energy dissipates rapidly through trophic transfer processes, so trophic upgrading cannot ultimately counteract carbon losses. However, in systems where low quality food resources—such as prokaryotic organisms or refractory organic matter—cannot sustain metazoan production, HP constitute a necessary biosynthetic step and provide essential lipid compounds for metazoan growth. Indeed, trophic upgrading of food quality has been shown to be due to the lipid composition of HP (Klein Breteler et al. 1999, Martin-Creuzburg et al. 2005, Bec et al. 2006). However, few data are available on lipids in HP, this is discussed in several foodweb studies (Bec et al. 2003a, Boëchat et al. 2007, Chu et al. 2008, 2009). As the availability of polyunsaturated fatty acids (PUFAs) of the n-3 series has been suggested to potentially constrain secondary production in aquatic ecosystems (Müller-Navarra 1995, Wacker & Von Elert 2001), recent studies have investigated which factors could determine HP lipid composition and more particularly the variability of n-3 PUFAs in HP. The high n-3 content of algalivorous HP has been associated with accumulation of dietary PUFAs (Boëchat 2005, Boëchat & Adrian 2005, Veloza et al. 2006, Chu et al. 2009). Such accumulation tends to be species specific, as HP species fed the same diet exhibited biochemical differences (Boëchat & Adrian 2005). Studies analyzing HP fed 2 different diets suggested that accumulation of dietary PUFAs by HP is also compound specific (Broglia et al. 2003, Bec et al. 2003b). In some cases, an inability to extract some PUFAs from the diet has been suggested (Broglia et al. 2003). Another point is that dietary PUFAs could serve as precursors and then be converted by elongation/desaturation processes (Bec et al. 2003b). Thus bioconversion and differential accumulation of dietary PUFAs have been hypothesized to be the main factors explaining differences in fatty acid (FA) composition between HP and their diet. These studies have not excluded the importance of de novo synthesis in determining HP PUFA composition. (Chu et al. 2002, Lund et al. 2008)

In this context, we aimed to characterize more clearly the relative importance of dietary factors versus de novo synthesis in determining HP PUFA composition. The heterotrophic flagellate *Paraphysomonas* sp. was cultivated on 4 diets only differing in their PUFA contents, i.e. *desA*⁻, *desB*⁻, *desD*⁻, and *desA*⁻/*desD*⁻ mutants of the picocyanobacterial strain *Synechocystis* PCC6803. The *desA*, *desB*, and *desD* genes of *Synechocystis* PCC6803 encode lipid desaturases at the $\Delta 12$, $\Delta 15$, and $\Delta 6$ positions of 18C FAs, respectively. The mutation of these genes by insertion of an antibiotic resistance gene cartridge completely eliminated the corresponding desaturation reaction (see Tasaka et al. 1996). Thus, the use of *desA*⁻, *desB*⁻, *desD*⁻, and *desA*⁻/*desD*⁻ mutants of *Synechocystis* PCC6803 as food sources permitted us to provide decreasing levels of unsaturated FAs. At the same time, potential biases in using different diet species such as different shape, size, and motility were minimized.

MATERIALS AND METHODS

The *desA*, *desB*, and *desD* genes of *Synechocystis* PCC6803 encode lipid desaturases at the $\Delta 12$, $\Delta 15$, and $\Delta 6$ positions of 18C FAs, respectively. The *desA*⁻, *desB*⁻, *desD*⁻ and *desA*⁻/*desD*⁻ mutants of the picocyanobacterial strain *Synechocystis* PCC6803 were kindly provided by Z. Gombos (Biological Research Center, Hungarian Academy of Sciences) and were batch cultivated in Cyano medium (Jüttner et al. 1983).

Paraphysomonas sp. was obtained from the culture collection of the Limnological Institute (University of Constance, Germany). In order to obtain FA compositions comparable to those used in Bec et al. (2006), who reported PUFA compositions of the same *Paraphysomonas* strain fed with 4 other picophytoplankton species, we followed the same protocol (cultures, sampling, lipid analysis). Depending on the food supply, different *Paraphysomonas* sp. cultures were obtained: *Paraphysomonas* sp. grown on *desA*⁻, *desB*⁻, *desD*⁻, and *desA*⁻/*desD*⁻ mutants of the picocyanobacterial strain *Synechocystis* PCC6803.

At the beginning of the experiment, these *Paraphysomonas* sp. cultures were grown semi-continuously at 20°C in 50 ml flasks by replacing 10% of the culture volume with new WC medium (Guillard 1975) with vitamins and food every 12 h. To increase the culture volume, 10% of the culture volume was then replaced by 20% of new WC medium and food every 12 h over 12 d. Every other day, the *Paraphysomonas* sp. cultures were gently transferred into new clean flasks to get rid of sedimented particles. This way, we obtained 6 flasks (500 ml each) per treatment (2 flasks per replicate). Two days before the *Paraphysomonas* sp. cultures were harvested for

lipid analyses, the exchange of medium was stopped. Microscopic observations revealed that *Paraphysomonas* sp. cell concentrations subsequently increased, and consequently, the concentration of picocyanobacteria decreased. However, at the end of this 2 d period, picocyanobacterial cells were still observed as free cells in the culture medium and in the *Paraphysomonas* food vacuoles. Heterotrophic nanoflagellate cells were separated from their food source by repeated centrifugations ($2000 \times g$, 4 min) and subsequent resuspension in fresh medium. The resulting concentrated supernatant was then screened with a 5 μm mesh-size filter. The retained particles were resuspended in WC medium without vitamins, and subsequently filtered on precombusted GF/F filters (Whatman, 25 mm diameter). Particulate organic carbon (POC) was measured using a NCS-2500 analyzer (Carlo Erba Instruments). Microscopic counting of subsamples following the protocol of Porter & Feig (1980) indicated that carbon contribution of heterotrophic bacteria and cyanobacteria never exceeded 6%. The contamination with autotrophic particles ranged from 2.8% (*Paraphysomonas* fed *desA*⁻) to 4.1% (*Paraphysomonas* fed *desD*⁻) of total carbon. The contamination with heterotrophic bacteria ranged from 0.6% (*Paraphysomonas* fed *desA*⁻/*desD*⁻) to 1.8% (*Paraphysomonas* fed *desD*⁻) of total carbon.

The total lipids were extracted with dichloromethane/methanol (2:1, v/v) and evaporated to dryness with nitrogen. The lipid extracts were transesterified with 3 mol l⁻¹ methanolic HCl (60°C, 15 min) for the analysis of FAs. Subsequently, FA methyl esters (FAMES) were extracted 3 times with 2 ml of iso-hexane. The iso-hexane lipid fractions were evaporated to dryness under nitrogen and redissolved in 10 to 20 μl of iso-hexane. FAs were analyzed by gas chromatography on an HP 6890 gas chromatograph equipped with a flame ionization detector and a DB-225 (J&W Scientific) capillary column. FAs were quantified by comparison to internal standards (C17:0 and C23:0 methyl esters) and identified by their retention times and their mass spectra, which were recorded with a GC-MS (Finnigan MAT GCQ) equipped with a fused-silica capillary column (DB-225MS, J&W). The limit of detection was 20 ng of FA.

Effects of food treatments on FA content of *Paraphysomonas* sp. were analyzed by 1-way ANOVAs (1 for each FA). Percent data were divided by 100 (proportions) and arcsine of the square-root transformed as described by Underwood (1997). ANOVAs were carried out using the general linear model module of Statistica 6.0 (StatSoft). Raw data met the assumption of homogeneity of variance; treatment effects were tested by Unequal N HSD post hoc tests. Bonferroni adjustment of significance levels of multiple tests according to Rice (1989) did not affect the detection of statistical differences between treatments.

RESULTS AND DISCUSSION

The 4 *desA*⁻, *desB*⁻, *desD*⁻, and *desA*⁻/*desD*⁻ mutants of the picocyanobacterial strain *Synechocystis* PCC-6803 exhibited different PUFA compositions (Fig. 1). The *desA*⁻/*desD*⁻ mutant contained no PUFA, as desaturation reactions at the $\Delta 12$ and $\Delta 6$ positions have been eliminated by mutagenesis (Tasaka et al. 1996). Similarly, *desA*⁻ cells exhibited no PUFA except 18:2(n-9). Inactivation of the *desB* gene resulted in complete elimination of n-3 PUFA. However, *desB*⁻ cells contained n-6 18C PUFAs such as 18:2(n-6) and 18:3(n-6). The *desD*⁻ cells did not contain any FA with double bonds at the $\Delta 6$ positions (namely 18:3(n-6) or 18:2(n-9)) but could synthesize 18:2(n-6) and 18:3(n-3) PUFAs. These differences are clearly reflected in *Paraphysomonas* lipids indicating a dietary influence on its PUFA composition (Fig. 1).

Saturated and monounsaturated FAs are the major FAs found in *Paraphysomonas* lipids. When feeding on *Synechocystis* that was lacking n-3 and n-6 PUFAs (*desA*⁻/*desD*⁻, *desA*⁻), the flagellate exhibited higher relative amounts of 18C monounsaturated FA (18:(1n-7)/18:(1n-9); Unequal N HSD, $p < 0.05$ following ANOVA, $F_{3,7} = 883.24$, $p < 0.001$), which suggests that monounsaturated FAs could compensate to a certain degree the unavailability of dietary PUFA. Treatments in which no dietary PUFAs were provided also showed that the flagellate can de novo synthesize 18C PUFAs and highly unsaturated FAs (HUFAs) such as 20:4(n-6), 20:5(n-3), and 22:6(n-3).

Except for *Paraphysomonas* fed *desA*⁻, the other treatments also showed that the flagellate exhibited significant amounts of 18:2(n-6). The picocyanobacterial strain *desA*⁻ contained no 18:2(n-6) but provided *Paraphysomonas* with 18:2(n-9). As this compound is accumulated in the flagellate lipids, we hypothesized that 18:2(n-9) could replace 18:2(n-6). Other dietary lipids have been shown to functionally replace other lipids (e.g. cholesterol versus tetrahymanol in ciliate lipids, see Conner et al. 1982, Martin-Creuzburg et al. 2006). The *desB*⁻ and *desD*⁻ treatments revealed that dietary n-6 and n-3 18C PUFAs were also accumulated in *Paraphysomonas* lipids. *Paraphysomonas* fed on *desB*⁻ exhibited high percentages of 18:2(n-6) and 18:3(n-6) (Unequal N HSD, $p < 0.05$ following ANOVAs, $F_{3,7} = 558.76$, $p < 0.001$ and $F_{3,7} = 675.75$, $p < 0.001$, respectively), whereas *Paraphysomonas* fed on *desD*⁻ contained high levels of 18:2(n-6) and 18:3(n-3) (Unequal N HSD, $p < 0.05$ following ANOVAs, $F_{3,7} = 558.76$, $p < 0.001$ and $F_{2,5} = 464.15$, $p < 0.001$, respectively). These 18C PUFAs are known to be precursors of long-chain HUFAs such as 20:4(n-6), 20:5(n-3), and 22:6(n-3) (Von Elert 2002). Surprisingly, the HUFA content of *Paraphysomonas* fed on 18C PUFA-

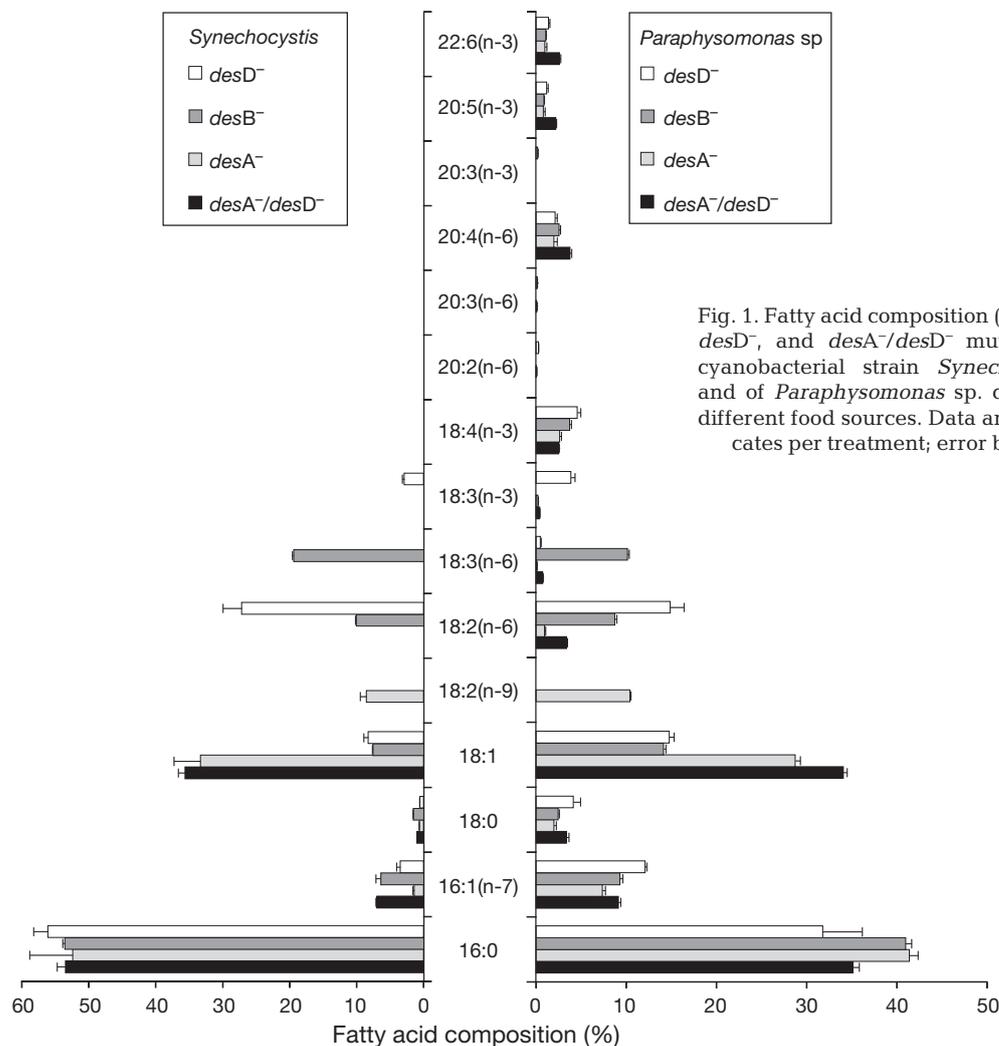


Fig. 1. Fatty acid composition (%) of *desA*⁻, *desB*⁻, *desD*⁻, and *desA*⁻/*desD*⁻ mutants of the picocyanobacterial strain *Synechocystis* PCC6803 and of *Paraphysomonas* sp. cultivated on these different food sources. Data are means of 3 replicates per treatment; error bars indicate SD

containing food sources was similar or even tended to be lower than for *Paraphysomonas* grown on a source lacking PUFA. Indeed, *Paraphysomonas* fed *desA*⁻/*desD*⁻ exhibited the highest contents in 20:4(n-6), 20:5(n-3), and 22:6(n-3) (Unequal N HSD, $p < 0.05$ following ANOVAs, $F_{3,7} = 27.07$, $p < 0.001$ for 20:4(n-6), $F_{3,7} = 68.39$, $p < 0.001$ for 20:5(n-3), and $F_{3,7} = 51.59$, $p < 0.001$ for 22:6(n-3), respectively). This indicates that the dietary influence is not the main factor determining the PUFA composition of this flagellate. This point could be linked to previous studies that have analyzed the FA composition of HP fed 2 different diets. Bec et al. (2003b) observed similar ratios between some major PUFAs, which tended to be maintained by active metabolic processes. Broglio et al. (2003) suggested that 'individual HP species may tend to maintain a certain stoichiometry in the FA composition regardless of diet'.

Our results corroborate those obtained by Bec et al. (2006) and show that *Paraphysomonas* fed 8 different

picophytoplankton strains exhibited the same FA composition pattern, i.e. high levels of 16:0 and 18:1 and significant amounts of 18C PUFAs and of long-chain HUFAs (20:4(n-6), 20:5(n-3) and 22:6(n-3)). Our results also show that the chrysoomonad *Paraphysomonas* sp. can exhibit its specific PUFA pattern exclusively by de novo synthesis. Pigmented chrysoomonads are known to produce long-chain PUFAs in high quantities (Ahlgren et al. 1992), which suggests that the capacity of *Paraphysomonas* sp. to synthesize PUFAs de novo could be attributed to its phylogenetic origin (see Desvilettes & Bec 2009 for further details). Interestingly, Véra et al. (2001) reported that *P. vestita* cultivated on heterotrophic bacteria exhibited only very low amounts of n-3 series PUFA. Nevertheless, Zhukova & Kharlamenko (1999) showed that the flagellate *Bodo* sp. exhibited great variations in its n-3 PUFA content when fed on rice- or starch-grown bacteria. Environmental factors (such as temperature, O₂, salinity, auto-, mixo-, or heterotrophic nutrition) have

also been shown to profoundly alter the FA composition of protists (Avery et al. 1994, 1996, Lund et al. 2004, Poerschmann et al. 2004, Boëchat et al. 2007). Indeed, it has been shown that HP must rapidly adjust the PUFA composition of their membranes in response to environmental temperature changes (Nozawa & Thompson 1979). As the degree of desaturation has also been linked to membrane functioning and phagotrophic activity, adjusting the membrane lipid composition is a necessary HP adaptation to keep on growing and feeding in a changing environment (Avery et al. 1995). We therefore suggest that the specific FA pattern of our cultivated flagellate fed different picophytoplanktonic diets is mainly due to environmental parameters (here culture conditions and/or food type). However, this specific pattern can significantly be altered by dietary inputs. Here, dietary 18C PUFAs seem to be preferentially accumulated in *Paraphysomonas* lipids. 18C PUFAs are considered as essential compounds for crustacean grazers (Von Elert 2002). This indicates that HP can transfer essential compounds from primary producers to metazoan consumers. However, the accumulation efficiency of dietary PUFAs by HP should be investigated more thoroughly.

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