Short Communication

Occurrence of penta-amines, hexa-amines and N-methylated polyamines in unicellular eukaryotic organisms belonging to the phyla Heterokontophyta and Labyrinthulomycota of the subdomain Stramenopiles

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We have analyzed cellular polyamines of various lower eukaryotic micro-algae and protists to consider the phylogenetic significance of cellular polyamine distribution profiles in the early evolution of plants and animals (Hamana, 2008; Hamana and Mastuzaki, 1982, 1985; Hamana and Niitsu, 2006; Hamana et al., 1990, 2004a, 2004b, 2006, 2013). Putrescine and spermidine were major polyamine and homospermidine, norspermidine, norspermine, spermine and/or thermospermine spread within phototrophic green algae belonging to the two divisions Chlorophyta and Charophyta containing plastids from the primary endosymbiosis of a phototrophic cyanobacterium. The phylum Glaucophyta containing putrescine and spermidine, and the phylum Rhodophyta containing putrescine, spermidine and spermine evolved independently after the primary endosymbiosis; however, norspermidine, homospermidine and norspermine were added as a major polyamine in some red algae. Putrescine, norspermidine, spermidine and norspermine spread within the phototrophic algae belonging to the phyla Cercozoa, Cryptophyta, Euglenozoa, Haptophyta, and Heterokontophyta (Heterokonta, Ochrophyta) evolved by the secondary symbiotic process of green alga or red alga. Non-photosynthetic, heterotrophs in the phyla were absent in norspermidine and norspermine. Phototrophic dinoflagellates of the phylum Dinophyta evolved by a tertiary symbiosis contain norspermidine and norspermine, and lack spermidine.

For the detection of penta-amines, hexa-amines (Hamana et al., 1991, 1992a, 1994, 1996, 1998; Mastuzaki et al., 1990b) and N-methylated polyamines (Hamana et al., 1992b, 1998; Matsuzaki et al., 1990a; Niitsu et al., 2014) found in higher-land plants, large-scale cultures of phototrophic heterokontophytes located in the subdomain Stramenopiles (Stramenopila) and a high-performance gas chromatography (HPGC) with a long capillary column were used for the present algal polyamine analyses. We detected many novel N-methylated polyamines in addition to penta-amines and hexa-amines in diatoms belonging to the class Bacillariophyceae of the phylum Heterokontophyta evolved by the secondary symbiotic process of red alga. This is the first report on the occurrence of N-methylated polyamine and hexa-amine in algae. The phylogeny of the phylum Labyrinthulomycota (also known as the class Labyrinthulomycetes or Labyrinthulea) showed the evolutionary loss of chloroplasts as well as the phyla Bicosoecacea (class Bicoecea), Oomycota (class Oomycetes) and Opalozoa (class Opalinata), and gain of ectoplasmic gliding within the subdomain Stramenopiles (Tsui et al., 2009). We first analyzed cellular polyamines of the non-phototrophic, saprotrophic labyrinthulomycetes belonging to the phylum Labyrinthulomycota, and, in them, found the major occurrence of caldopentamine.

Axenic or non-axenic strains of heterokontophytes supplied from NIES (National Institute for Environmental Studies) and NBRC (Department of Biotechnology, National Institute of Technology and Evaluation) were cultured phototrophically in light (10–14 h/24 h) at 20–25°C using 2–10 L of the liquid media designed by NIES (f/2 medium in Kasai et al., 2004) or NBRC (IMK-SW medium (Daigo’s IMK, Wako Pure Chemical, dissolved in seawater), TY-SW medium and H-SW medium). Photosynthetic liquid cultures (1–2 L) of uni-algal, non-axenic

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DBT and WDB strains were purchased from Department of Biotechnology, Institute of Environmental Biology Co. (DBT) and WDB Environmental and Biological Research Institute Co., respectively. When the non-axenic algal strains (has not been proven as an axenic strain) were cultured axenically and photosynthetically, a significant amount of contaminated organism was not detected after the washing of the algal pellets. *Labyrinthula* sp. belonging to the order *Labyrinthulales* was heterotrophically grown with a marine bacterium, *Vibrio alginolyticus* NBRC 15930 in the liquid seawater medium (TY-SW) (0.1 g Trypticase Peptone and 1 g yeast extract are dissolved in 1 L seawater) at 20–25°C. Axenic labyrinthulomycetes belonging to the order *Thraustochytriales* were grown.

**Fig. 1.** HPGC analysis of polyamines of *Skeletonema costatum* DBT No. 1 (A) and No. 2 (B), *Synedra acus* DBT No. 1 (C) and No. 2 (D) and *Skeletonema japonicum* NIES-2535 (E) on a JEOL JMS-700 at the column temperature 90°C–16°C/min–280°C.

Other non-amine peaks on the GC charts of the concentrated polyamine samples were detected. Abbreviations for polyamines and N-methylated polyamines are shown in Table 1 and the text, respectively.
heterotrophically in the liquid seawater medium (H-SW) (1 g glucose, 0.2 g yeast extract and 0.5 g monosodium glutamate are dissolved in 1 L seawater) at 20–25°C.

Organisms (1–10 g wet weight) harvested in our laboratory at the early stationary phase were homogenized in 5% perchloric acid (PCA). The PCA extract was subjected to a column of a cation-exchange resin, Dowex 50WX8 (1 cm I.D. × 3 cm or 3 cm I.D. × 1 cm) to concentrate polyamines and then analyzed by high-performance (high speed) liquid chromatography (HPLC) on a Hitachi L6000 using a column of cation-exchange resin, Hitachi 2619F (4 mm I.D. × 50 mm) using post-labeled fluorometry after heating with o-phthalaldehyde (Hamana, 2002; Hamana et al., 2005). After heptafluorobutyration (HFB) of the concentrated polyamine samples, HPGC on a SHIMADZU GC-17A and HPGC-mass spectrometry (HPGC-MS) on a JEOL JMS-700, equipped with a capillary column of Inert Cap 1MS (0.32 mm I.D. × 30 m) (GL Sciences), developed from the previous standard GC with a short packed column (3 mm I.D. × 2.1 m) (Niitsu et al., 1993), were operated (Furuchi et al., 2015a, b; Niitsu et al., 2014). Some HPGC charts are shown in Figs. 1 and 2. Mass spectra of the HFB derivatives of all polyamines detected in HPGC-MS were obtained for their identification. Typical mass spectra of penta-amines, hexa-amines and methylated polyamines are shown elsewhere (Furuchi et al., 2015a, b; Niitsu et al., 2014). Molar concentrations of cellular polyamines per gram of wet weight of the starting pellets were estimated from the HPLC analysis and the two HPGC analyses using authentic polyamine standards and are shown in Table 1. The contents of methylated polyamines could not be calculated, and therefore are not included in Table 1.

1. Penta-amines and hexa-amines in Heterokontophyta

Polyamine distribution profiles of 38 heterokontophytes belonging to the classes Chrysophyceae, Phaeophyceae, Xanthophyceae, Pelagophyceae, Pongiophyceae, Schizochlaioiphyceae, Bacillariophyceae, Eustigmatophy-
Table 1. Cellular concentration of polyamines of heterokontophytes and labyrinth honeymoones.

| Organism | Polyamines (μmol/g wet weight) |
|----------|--------------------------------|
|          | Dap | Put | Cad | NSpd | Spd | HSpd | NSpm | Spm | TSpm | CPen | HCPen | TPen | CHeX | HChex | THeX |
| Heterokontophytes |      |     |     |      |     |      |      |     |      |      |        |      |      |       |      |
| Pylum Heterokontophyta (Heterokonta, Ochrophyta) |      |     |     |      |     |      |      |     |      |      |        |      |      |       |      |
| Class Bacillariophyceae (diatoms) |      |     |     |      |     |      |      |     |      |      |        |      |      |       |      |
| Chaetoceros calcitrans WDB strain | —   | 0.40 | —   | 0.06 | —   | 1.22 | 0.02 | 0.03 | 0.01 | —    | —     | —     | —    | —     | —    | —  |
| Chaetoceros gulaulis WDB strain | 0.06 | 0.06 | —   | 0.60 | 1.19 | 0.08 | 0.50 | 0.02 | 0.01 | 0.11 |      | —     | —    | —    | —    | —  |
| Cyclotella meneghiniana DBT strain | —   | 0.70 | —   | 0.60 | 1.18 | —    | 0.02 | —    | —    | —    | —     | —     | —    | —    | —    | —  |
| Ancalosira granulata v. angustissima DBT strain | —   | 0.85 | —   | —    | —    | 1.25 | 0.56 | —    | —    | —    | —     | —     | —    | —    | —    | —  |
| Ancalosira japonica DBT strain | —   | 0.90 | —   | —    | —    | 1.17 | 1.22 | —    | —    | —    | —     | —     | —    | —    | —    | —  |
| Asterionella formosa DBT strain | —   | 1.10 | —   | —    | —    | 1.03 | 1.20 | —    | —    | —    | —     | —     | —    | —    | —    | —  |
| Skeletonema costatum DBT strain No. 1 | 0.07 | 0.23 | —   | 0.85 | 0.83 | 0.10 | 1.20 | 0.03 | 0.01 | 0.15 |      | —     | —    | —    | —    | —  |
| Skeletonema costatum DBT strain No. 2 | 0.10 | 0.31 | —   | 1.15 | 1.05 | 0.06 | 0.11 | 0.03 | 0.03 | 0.06 |      | —     | —    | —    | —    | —  |
| Skeletonema japonicum NIES-2535 | —   | 0.01 | —   | 1.20 | 1.10 | 0.01 | 0.13 | 0.01 | 0.02 | 0.03 |      | —     | —    | —    | —    | —  |
| Synedra acus DBT strain No. 1 | —   | 0.53 | —   | —    | —    | 1.20 | 1.11 | 0.01 | 0.06 | 0.02 | 0.03 | 0.02 |      | —    | —    | —  |
| Synedra acus DBT strain No. 2 | —   | 0.30 | —   | —    | —    | 1.22 | 0.01 | 0.02 | 0.02 | 0.01 | —    | —     | —    | —    | —    | —  |
| Class Eustigmatophyceae |      |     |     |      |     |      |      |     |      |      |        |      |      |       |      |
| Namechloropsis sp. DBT strain | —   | 0.40 | —   | —    | 1.05 | 0.14 | 0.02 | —    | —    | —    | —     | —     | —    | —    | —    | —  |
| Class Raphidophyceae |      |     |     |      |     |      |      |     |      |      |        |      |      |       |      |
| Heterosigma akashiwo (H. inlandica) DBT strain | —   | 0.17 | —   | 0.85 | —    | 0.03 | 0.03 | 0.01 | 0.03 | 0.04 |      | —     | —    | —    | —    | —  |
| Heterosigma akashiwo (H. inlandica) NBRC 103001 | —   | 0.05 | —   | 0.12 | —    | 0.01 | 0.01 | —    | —    | —    | —     | —     | —    | —    | —    | —  |
| Labyrinthulomycetes |      |     |     |      |     |      |      |     |      |      |        |      |      |       |      |
| Pylum Lobalbatonulomycota (Class Labyrinthulomycetes, Labyrinthidae) |      |     |     |      |     |      |      |     |      |      |        |      |      |       |      |
| Order Labyrinthidae |      |     |     |      |     |      |      |     |      |      |        |      |      |       |      |
| Labyrinthula sp. NBRC 33215 No. 1 | 0.01 | 0.13 | 0.10 | 0.44 | —    | —    | —    | —    | 0.02 | —    | —     | —     | —    | —    | —    | —  |
| Labyrinthula sp. NBRC 33215 No. 2 | 0.10 | 0.65 | 0.52 | 1.23 | —    | 0.02 | 0.02 | —    | 0.02 | —    | —     | —     | —    | —    | —    | —  |
| Order Thraustochytriales |      |     |     |      |     |      |      |     |      |      |        |      |      |       |      |
| Aurantiochytrium sp. NBRC 102614 No. 1 | 0.02 | 0.20 | —   | 0.13 | —    | —    | —    | —    | 0.08 | —    | —     | —     | —    | —    | —    | —  |
| Aurantiochytrium sp. NBRC 102614 No. 2 | —   | 1.20 | 0.15 | 0.25 | —    | 0.01 | 0.02 | 0.01 | 0.65 | —    | —     | —     | —    | —    | —    | —  |
| Botryochytrium radiatum NBRC 104107 | 0.18 | 0.10 | 0.01 | 0.35 | —    | 0.04 | 0.02 | 0.02 | 0.57 | 0.01 | 0.02 |      | —     | —    | —    | —  |
| Oblongichytrium sp. NBRC 102618 | 0.20 | 0.07 | 0.90 | —    | 0.04 | 0.01 | 0.10 | 1.80 | 0.01 | 0.01 | —     | —     | —    | —    | —    | —  |
| Parietichytrium arkaraium NBRC 104108 | 0.12 | 0.10 | 0.93 | 0.70 | —    | 0.13 | 0.02 | 0.03 | 0.60 | —    | —     | —     | —    | —    | —    | —  |
| Schizochytrium sp. NBRC 102615 | 0.18 | 0.12 | 0.03 | 0.12 | —    | 0.04 | 0.04 | 0.12 | 0.88 | 0.01 | 0.01 | —     | —     | —    | —    | —  |
| Sicyoidichytrium minutum NBRC 102975 | 1.15 | 0.40 | 0.02 | 0.60 | —    | 0.04 | 0.01 | 0.74 | 0.63 | 0.01 |      | —     | —    | —    | —    | —  |
| Ulkenia amoeboida NBRC 104106 | 0.60 | 0.02 | 0.02 | 0.45 | —    | 0.10 | 0.04 | 0.01 | 0.65 | 0.01 | 0.01 | —     | —     | —    | —    | —  |

Dap (3) (abbreviations for the number of methylene chain unit between N), diaminopropane; Put (4), putrescine; Cad (5), cadaverine; NSpd (33), norspermidine; Spd (34), spermidine; HSpd (44), homospermidine; NSpm (333), norspermine; Spm (343), spermine; TSpm (334), thermospermine; CPen (3333), cadopentamine; HCPen (3334), homocadopentamine; TPen (3343), thermopentamine; CHeX (33333), cadohexamine; HChex (33334), homocadohexamine; THeX (33343), thermohexamine; DBT, Department of Biotechnology, Institute of Environmental Biology Co., LTD, Yaeru, Shizuoka, Japan; NIES, National Institute for Environmental Studies, Tsukuba, Japan; NBRC, Biological Resource Center, National Institute of Technology and Evaluation, Kisarazu, Japan; WDB, WDB Environmental and Biological Research Institute Co. Ltd., Tokushima, Japan; —, not detected (<0.005 μmol/g wet weight). Methylated polyamines found in Skeletonema costatum, Skeletonema japonicum and Synedra acus are excluded in Table 1.
ceae or Raphidophyceae have been demonstrated by HPLC analyses (Hamana, 2008; Hamana and Matsuzaki, 1982, 1985; Hamana et al., 2004b). Although it has been known by HPLC analysis (Hamana, 2008; Nishibori and Nishijima, 2004; Nishibori et al., 2006) that Chattonella, Heterosigma, Fibrocapsa, Gonyostomum and Olisthodiscus species belonging to Raphidophyceae contain spermidine as the major polyamine. Nishibori et al. (2009) first found caldopentamine (3333) and homocaldopentamine (3334) in Chattonella marina var. antiqua NIES-1 and Heterosigma akashiwo NIES-6 by HPGC analysis. In the present study, we tried additional polyamine analyses of a large culture of phototrophic algae belonging to Bacillariophyceae, Eustigmatophyceae and Raphidophyceae using HPLC and HPGC. In Bacillariophyceae, two Chaetoceras species and two Skeletonema species contained caldopentamine (Fig. 1 and Table 1). In addition to the penta-amine, homocaldopentamine, homocaldohexahexaeamine (33334) and thermohexamine (33343) were detected as a minor polyamine in Synedra acus (Fig. 1 and Table 1). Thermopentamine (33343) was not found; however, methylated derivatives of thermopentamine were detected in S. acus (Fig. 1), indicating the occurrence of the penta-amine in the alga. In Raphidophyceae, DBT and NBRC strains of Heterosigma akashiwo cultured in the present study contained caldopentamine and/or homocaldopentamine (Table 1). Penta-amines and hexa-amines were not detected in the large-scale cultures of Cyclotella, Aulacoseira and Asterionella species belonging to Bacillariophyceae and Nannochloropsis sp. belonging to Eustigmatophyceae.

2. Methyldiamines in Heterokontophyta

Terminal-N-methyldiamines, both terminal-N-methyldiamines, internal-N- plus terminal-N-methyldiamines and internal-N- plus both terminal-N-methyldiamines were found in three diatoms, Skeletonema costatum, Skeletonema japonicum and Synedra acus within the 10 diatom strains (10 species) of Bacillariophyceae analyzed here by HPGC. Terminal-N-methyldiamines were delayed and internal-N-methyldiamines were fastened in the retention time of HPGC. Methyldiaminopropane (M3), dimethyldiaminopropane (M3M), dimethylnorspermidine (M33), dimethylspermidine (M333), dimethylhomospermidine (M333M), 4-methyl-dimethyldiaminopropane (M3(M)3M) and N4-methyl-dimethylspermidine (M3(M)3M) were detected in the culture No. 1 and/or No. 2 of Synedra acus (Fig. 1). Methylation of polyamines occurred preferentially in aminopropyl-N, but scarcely in aminobutyl-N in the algal polyamines.

Many novel methyldiamines found in the algae were identified by HPGC-MS analysis of authentic M33, M333M, N4-methylnorspermidine (3(M)3), M34, N8-methylspermidine (34M) and N5-methylhomospermidine (4(M)4) newly chemically synthesized in the present study and our previous MS data of authentic M4, dimethylputrescine (M4M), methylhomospermidine (M44), dimethylhomospermidine (M44M), N4-methylspermidine (3(M)4) and N5-methylthermospermine (3(M)34) (Hamana et al., 1992b, 1998; Niitsu et al., 2014).

3. Penta-amines in Labyrinthulomycota

Labyrinthula sp. grown with Vibrio alginolyticus contained putrescine, cadaverine, norspermidine and spermidine as the major polyamines (Fig. 2 and Table 1). Since it has been known that Vibrio species contain putrescine and norspermidine, parts of putrescine and norspermidine found in Labyrinthula sp. might be derived from V. alginolyticus. TY-SW medium contained putrescine and spermidine. Norspermine, spermine and caldopentamine were found as the minor polyamines of Labyrinthula sp. Caldopentamine was detected as a major polyamine in addition to putrescine, cadaverine, norspermidine, spermidine, norspermine, spermine, thermopentamine in Aurantiocystium sp., and six other species of the order Thraustochytriales when they were grown in H-SW medium containing putrescine and spermidine (Fig. 2 and Table 1). Relative contents of diaminopropane, norspermidine, norspermine and caldopentamine as a series of aminopropyl derivatives were shifted in the 10 polyamine-samples (Table 1). Homocaldopentamine and/or thermopentamine were detected as a minor polyamine in the five species of Thraustochytriales (Fig. 2 and Table 1). When polyamine-free 199-SW medium was used, similar cellular polyamine profiles were obtained. These data showed the wide distribution of penta-amines and cadaverine and the absence of homospermidine as the common polyamine profile of Labyrinthulomycota. Methyldiamines were not detected in the labyrinthulomycetes. The major occurrence of caldopentamine in the Thraustochytriales is a very unique polyamine profile within eukaryotes. A cellular polyamine analysis of other non-photosynthetic bicoelomates, oomycetes and opalineans of the Stramenopiles is currently being planned.

Penta-amines and hexa-amines were found preferentially in thermophilic bacteria in which the occurrence of long polyamines are correlated to their thermophily. N-Methyldiamines seem to be involved in plant cell proliferation and differentiation in multicellular higher land plants. The biological significance of the occurrence of long polyamines and N-methyldiamines in mesophilic unicellular eukaryotic organisms is interesting.

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