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Correlation between thermotolerance and membrane properties in *Paramecium aurelia*

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Summary

The relationship between thermotolerance and membrane properties was studied by using a ciliated protozoan, *Paramecium aurelia*. *P. aurelia* is a complex of sibling species termed 'syngens' whose cell morphology appear similar on microscopic examination. From the comparison of tolerance to increasing temperature among 14 syngens of *P. aurelia*, we selected syngens 2 and 3 as low thermotolerant examples, and syngens 8 and 10 as high thermotolerant examples. The membrane resistance of high thermotolerant syngens measured by injection of a constant inward current was greater than that of low thermotolerant syngens. Membrane fluidity measurements of living cells using the fluorescent dye, 6-lauroyl-2-dimethylaminonaphtalene (laurdan) showed that the fluidity at the cultured temperature was decreased in high thermotolerant syngens compared to that of low thermotolerant syngens. However, when the temperature was increased to the killing temperature of each syngens, the fluidity was increased to almost the same level irrespective of syngen. Furthermore, analysis of fatty acids extracted from whole cells showed that the ratios of unsaturated to saturated fatty acids was smaller in high thermotolerant syngens than in low thermotolerant syngens. These results suggest that the thermotolerance of *P. aurelia* syngens is determined by the membrane fluidity which is related to the fatty acids composition.

Key words: thermotolerance, tonic permeability, fatty acids, membrane fluidity, laurdan fluorescence, *Paramecium aurelia.*

Introduction

Biological membranes are crucial for the cell to survive at high temperature. Environmental temperature markedly influences the physical properties of membranes, particularly in poikilothermic organisms. A rapid rise in temperature induces an increase in membrane lipid fluidity, which alters the activity of proteins embedded in the membranes. Many cells can adapt to temperature change by altering their lipid composition and restoring membrane fluidity to a constant level *via* a process termed homeoviscous adaptation (Martin et al., 1976; Anderson et al., 1981; Soicic et al., 1992; Hazel, 1995; Williams and Somero, 1996; McKinley and Hazel, 2000). Thermotolerance is established at a temperature at which the cells can restore membrane fluidity. However, the correlation between membrane fluidity and thermotolerance has yet to be proved definitively. Rather, a negative correlation has been reported in various cells (Lepock et al., 1981; Gonzalez-Mendez, 1981; Konings and Ruifrock, 1985; Swan and Watson, 1997).

Laurdan is a lipophilic fluorescent probe that shows spectral sensitivity to the lipid phase with a red shift of the emission maximum when passing from a gel to liquid crystalline phase (Parasassi et al., 1990; Parasassi et al., 1991; Chong and Wong, 1993; Sheffield et al., 1995). Based on this property, laurdan is used to estimate the fluidity of the membrane in various living cells (Chapman et al., 1995; Palleschi and Silvestroni, 1996; Yu et al., 1996; Mamdouh et al., 1998; Harris et al., 2001; Vest et al., 2004). Laurdan is, therefore, useful to test the relationship between thermotolerance and membrane fluidity.

We collected initially, a form, or 'syngen' of *Paramecium aurelia* from a mountain stream near a hot spring (Przybos et al., 2003). A test of thermotolerance showed that this syngen could survive at relatively high temperature compared to other sibling species of *P. aurelia*. We expected differences in the membrane and compared the membrane properties of *P. aurelia* sibling species. For the comparison, the membrane fluidity of living *P. aurelia* cell was measured by laurdan

fluorescence imaging. In association with membrane resistance and fatty acid composition, the membrane fluidity consistently differed between high and low thermotolerant sibling species of *P. aurelia.*

Materials and methods

Chemicals

2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate (NE-OTf) was prepared as previously described (Yasaka et al., 1990). A fatty acid mixture was purchased from LFC (Malmõ, Sweden), 6-lauroyl-2-dimethylaminonaphtalene (laurdan) from Molecular Probes (Eugene, OR, USA), cholesterol and dipalmitoilphosphatidylcholine (DPPC) from Sigma (Tokyo, Japan). All other reagents were from Wako Chem. (Osaka, Japan).

To form liposomes, DPPC and cholesterol were dissolved in chloroform and mixed at a molar ratio of 1:50 (cholesterol:DPPC). The solvent was then removed by evaporation under a nitrogen stream. The completely dried lipid was then hydrated at 50°C for 1 h in 10 mmol l^{-1} Hepes buffer (pH 7.0). The sample was mixed with a vortex mixer for several seconds every 10 min during the hydration step to form multilamellar vesicles. The total lipid concentration was 1 mmol l^{-1}.

Cell culture

Syngen 10 of *Paramecium aurelia* (Sonneborn, 1957) was isolated from a mountain stream near a hot spring in Nara Prefecture, Japan (Przybos et al., 2003). Other syngens of *P. aurelia* were stocks established at the Polish Academy of Science. *P. aurelia* cells were cultured in a hay infusion inoculated with *Klebsiella pneumoniae*. The culture temperature was kept constant by incubation in a water bath. *P. aurelia* cells in the stationary phase, 7–14 days after inoculation, were collected by low speed centrifugation and suspended in a standard solution containing (mmol l^{-1} in final concentration) 0.25 $CaCl_2$, 2 KCl, 0.5 $MgCl_2$, and 2 Tris-HCl (pH 7.2). For adaptation of the cells to temperature, the culture temperature of the water bath was set as desired for a day or more prior to examination.

Thermotolerance test

A few hundred *P. aurelia* cells cultured at 25°C were suspended in standard solution (1 ml) in a test tube, and the tube was immersed in a water bath (Neslab endocal, NH, USA). In order to know the upper limit temperature to which the cells can adapt, the temperature of the bath was initially kept at 33°C for 30 min, which was safe for all syngens, then raised stepwise by 1°C and kept constant at each temperature for about 10 min. At each temperature, swimming cells were counted using a stereomicroscope for observation. The temperature at which no cells were swimming and the cells became round was determined as the killing temperature. It was confirmed that no cells were alive after 1 day at 25°C.

Intracellular recording

The cells were deciliated by incubation in a standard solution containing 6% ethanol and gently pipetting for 0.5–1 min. They were then transferred to the standard solution without ethanol. The method of recording membrane resistance was similar to that described previously (Nakaoka et al., 1991). An electrode was filled with 0.1 mol l^{-1} KCl and the resistance was measured as 100–150 MΩ. The deciliated cells were placed in a glass vessel mounted on an inverted microscope and electrodes were inserted from above. The temperature was changed by switching the water flow beneath the vessel, and monitored with a thermistor probe placed in the vessel (Nakaoka et al., 1987).

Laurdan labeling and fluorescence imaging

P. aurelia cells cultured at 25°C were deciliated and incubated in a standard solution for 30 min at 25°C in the dark with 10 μmol l^{-1} laurdan from a 2 mmol l^{-1} stock solution in dimethylformamide. Laurdan-labeled cells were dropped on a coverslip and the coverslip was adhered to the lower side of a temperature-controlled glass vessel that was placed on the stage of an inverted epifluorescence microscope (Olympus IX-70 with IX-FLA; Tokyo Japan). It was confirmed that the deciliation procedure gave no effect on the fluorescence ratio imaging.

For fluorescence ratio imaging, excitation light was supplied from a 75 W xenon lamp. An electric shutter (Copal No. 0, Tokyo, Japan) and neutral density filter, which cut out 50% of the excitation intensity, were placed in the excitation light path. Laurdan was excited with a dichroic mirror (band pass 360–375 nm) and emitted fluorescence was divided into two light paths with double-view optics (Hamamatsu Photonics A4313, Japan). Two images, passed through bandpass filters (Omega Optical, VT, USA) of 440/20 nm and 495/20 nm, were simultaneously recorded as a single image with an EB-CCD camera (Hamamatsu Photonics C7190-20) coupled with an image intensifier (Videoscope VS4-1845, VA, USA). The image averaged with eight frames during 0.27 s was stored and assessed with an image processor system (Argus-20 and Aqua Cosmos, Hamamatsu). In order to subtract cellular autofluorescence, the cell image without laurdan was set at no fluorescence by reducing the gain of the image intensifier. The same instrumental condition was adopted for the fluorescence measurement in the presence of laurdan.

The generalized polarization (*GP*) for each pixel was calculated using $GP=(I_{440}-I_{495})/(I_{440}+I_{495})$, where I_{440} and I_{495} are the fluorescence intensities measured at the emission maximum of laurdan that are characterized by the gel and the liquid-crystalline phases, respectively (Parasassi et al., 1990; Parasassi et al., 1991). *GP* distributions were obtained from the histograms of *GP* images, and the *GP* value was determined from the mean of the distributions. To correct the *GP* value of the present imaging system, fluorescent images of laurdan-labeled liposomes were recorded at various temperatures (20°C–55°C), and the system was set to give the *GP* values of 0.62 and –0.20, at 20°C and 55°C, respectively, as determined previously (Ohba, 1998; Harris et al., 2002).

Fatty acid analysis

Extraction and analysis of fatty acids were done using standard techniques, as follows. About 200 ml cultures of stationary phase cells were collected by low speed centrifugation and washed twice with a standard solution. The concentrated pellet was dropped on a glass slide that had been placed in an oven at 95°C, and left for 10–15 min. The dried pellet was extracted with acetonitrile. To 0.5 ml of the extracted solution in a reaction tube, 0.1 ml of 18-crown-6 (1 mmol l^{-1}) in acetonitrile and ~5 mg of anhydrous potassium fluoride were added. After a brief vortex mixing, 0.1 ml of NE-OTf (1 mmol l^{-1}) in acetonitrile was added for fluorescent labeling of carboxylic acids as previously reported (Yasaka et al., 1990). The mixture was vortex mixed for 10 min at room temperature. The resulting solution was stood for 30 s and an aliquot (10 μl) of the supernatant was analyzed in a high-performance liquid chromatography (HPLC) system consisting of a pump (Tosoh CCPS, Tokyo, Japan), injector valve (Rheodyne, Model 7125, Rohnert Park, CA, USA), spectrofluorometer (Hitachi F-1050, Tokyo, Japan) operating at 394 nm emission and 259 nm excitation and an analytical column (Kaseisorb LCODS Super, Tokyo, Japan). The elution solvent was methanol:water (9:1) delivered at a flow rate of 1.0 ml min^{-1}. Fluorescently labeled fatty acids were identified from their retention times relative to standards. The fatty acid composition was determined by integration of emission peaks.

Results

Comparison of thermotolerance

Survival at increasing temperature was compared between 14 syngens (sibling species) of *Paramecium aurelia* (Fig. 1). At the killing temperature of each syngen, all of the cells tested stopped swimming and became rounded. Based on such observation, we selected syngens 2 (39°C) and 3 (41°C) as low thermotolerant types, syngens 8 (43°C) and 10 (44°C) as high thermotolerant types. These killing temperatures changed little, even when the syngens were adapted beforehand to a sublethal temperature (5°C below the killing temperature of each syngen) for a day. Therefore, the killing temperature is close to the upper limit after adaptation to the high temperature.

Membrane resistance

In order to compare the ionic permeabilities of cell membranes between high and low thermotolerant syngens of *P. aurelia*, intracellular recordings were made by inserting two microelectrodes, one for recording potential and another for current injection. At the resting potential, membrane resistance was determined from the potential shift induced by injection of a constant inward current (10^{-10} A). The membrane resistances of syngen 8 and 10 cells were larger than those of syngen 2 and 3 cells (P<0.01; Fig. 2). When the cells were adapted to 35°C, the mean value of membrane resistance was slightly increased from that of the cells adapted to 25°C, although the values were variable.

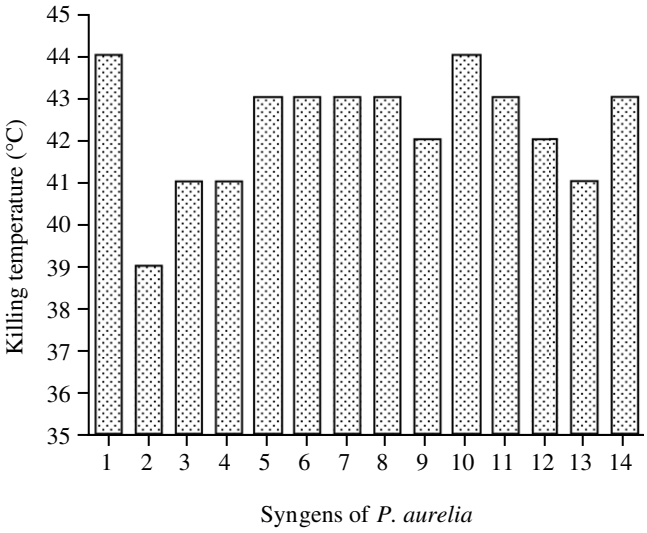

Fig. 1. Comparison of thermotolerance between *P. aurelia* syngens. Each syngen, cultured at 25°C, was incubated at 33°C for 30 min, and then the incubation temperature was raised stepwise by 1°C and kept constant for about 10 min. At each temperature, swimming cells were counted and the temperature at which no cells were swimming was determined as the killing temperature. Experiments were conducted three times.

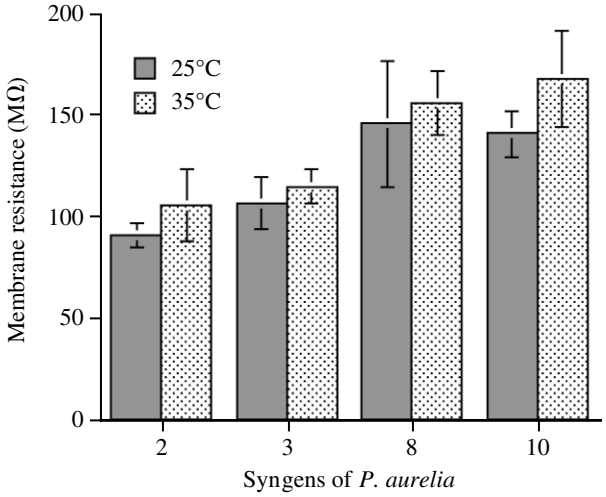

Fig. 2. Comparison of membrane resistance between *P. aurelia* syngens. Cells cultured at either 25°C or 35°C were deciliated 30 min before the measurement and suspended in a standard solution at each culture temperature. The resting potentials of syngens were in the range, –24 mV to –29 mV. Membrane resistance was measured at 25°C by application of a constant inward current (10^{-10} A). Key indicates culture temperature. Each value for membrane resistance is the mean ± s.d. (N=5–10 cells).

Membrane fluidity

Incubation of *P. aurelia* cells with laurdan labeled not only the cell membrane but also the intracellular membranes (Fig. 3A). Among the intracellular membranes, food vacuoles

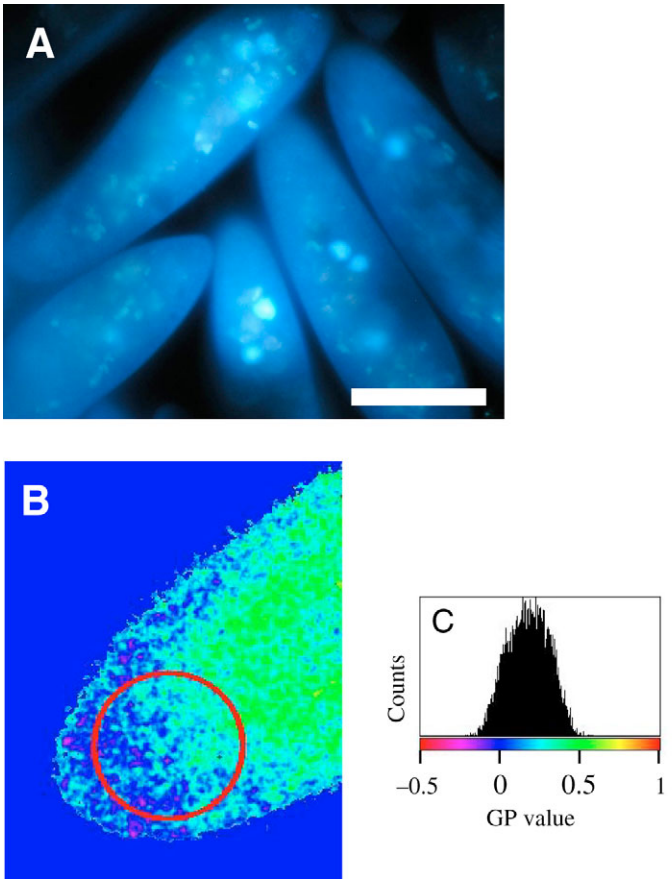

Fig. 3. Typical examples of laurdan-labeled *P. aurelia* and *GP* image. (A) Fluorescence image of laurdan-labeled *P. aurelia*. Syngen 10 cells were labeled with laurdan and observed at 25°C. The photograph was taken with a 4 s exposure using a digital camera. Scale bar, 50 μm. (B) *GP* map image calculated from fluorescence images of syngen 2 cell at 25°C. The *GP* value of each pixel was measured within the circle (diameter, 20 μm). (C) Colour bar and histogram of *GP* values obtained from (B). Total counts: 8730, bin width: 0.006. *GP* value was determined from the mean of the histogram.

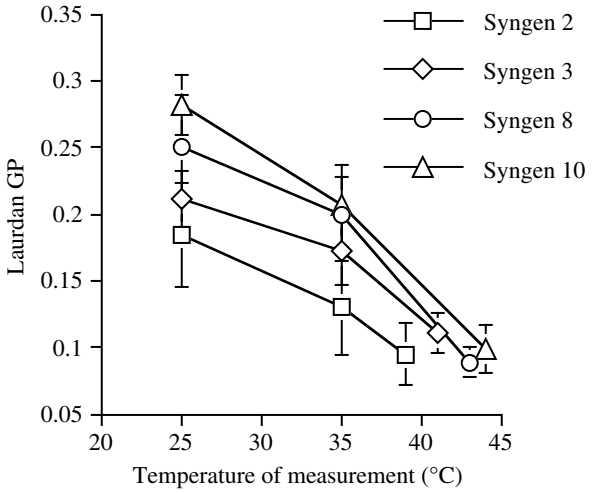

Fig. 4. Temperature dependence of *GP* value. *P. aurelia* syngens, 2, 3, 8 and 10 cells were cultured at 25°C. Fluorescence image of laurdan-labeled cells was initially taken at 25°C, and then taken 3 min after the temperature shift to either 35°C or the killing temperature of each syngen. Images were taken before the cell death. *GP* values are the means ± s.d. (*N*=5–8 cells).

were most strongly labeled. In order to avoid the strong fluorescence of food vacuoles, *GP* values at the anterior portion of the cell, where few food vacuoles are found, were measured (Fig. 3B,C). The *GP* values at 25°C were higher in the high thermotolerant syngens 8 and 10, than the low thermotolerant syngens 2 and 3 (Fig. 4). These *GP* values decreased gradually with the increase of measuring temperature. When the temperature was increased to the killing temperature of each syngen, the *GP* value decreased to approximately 0.1, irrespective of the syngens.

Fatty acid composition

Generally, as the ratio of unsaturated to saturated fatty acids in the membrane increases, the membrane fluidity will increase. In order to examine whether such a relationship holds in *P. aurelia* syngens, the fatty acid fraction was compared among syngens.

Fatty acids of whole cells were extracted from four syngens of *P. aurelia* cultured at either 20°C, 30°C and analyzed by HPLC (Fig. 5). The extracted fatty acids were mainly palmitic acid (C16:0), oleic acid (C18:1) and stearic acid (C18:0). Compared with low thermotolerant syngens, 2 and 3 (Fig. 5A,B), high thermotolerant syngens, 8 and 10 (Fig. 5C,D), contained 7–10% more palmitic acid (C16:0), and 6–10% less oleic acid (C18:1). A consistent change of fatty acid composition was not found with a change in culture temperature. Furthermore, all of the fatty acids extracted were divided into unsaturated and saturated groups, and the ratio of unsaturated to saturated fatty acids was compared between syngens (Fig. 6). The ratio of unsaturated to saturated fatty acids was lower in syngens 8 and 10 than syngens 2 and 3 ($P<0.05$). Therefore, high thermotolerant syngens, 8 and 10, have less unsaturated fatty acids than low thermotolerant syngens, 2 and 3.

Discussion

Hyperthermic killing temperature was compared among 14 syngens of *P. aurelia*, and two syngens of high thermotolerance and two syngens of low thermotolerance were selected for convenience. We assumed that disruption of the membrane structure due to heating is the main cause of cellular damage. Comparisons between high and low thermotolerant syngens showed a consistent relationship between the thermotolerance and the physical properties of the membrane.

Membrane resistance, measured by injecting current across the cell membrane, differed between high and low thermotolerant syngens, the former having large resistances (Fig. 2). Since membrane resistance is the reciprocal of the ionic permeability through the cell membrane, the ionic

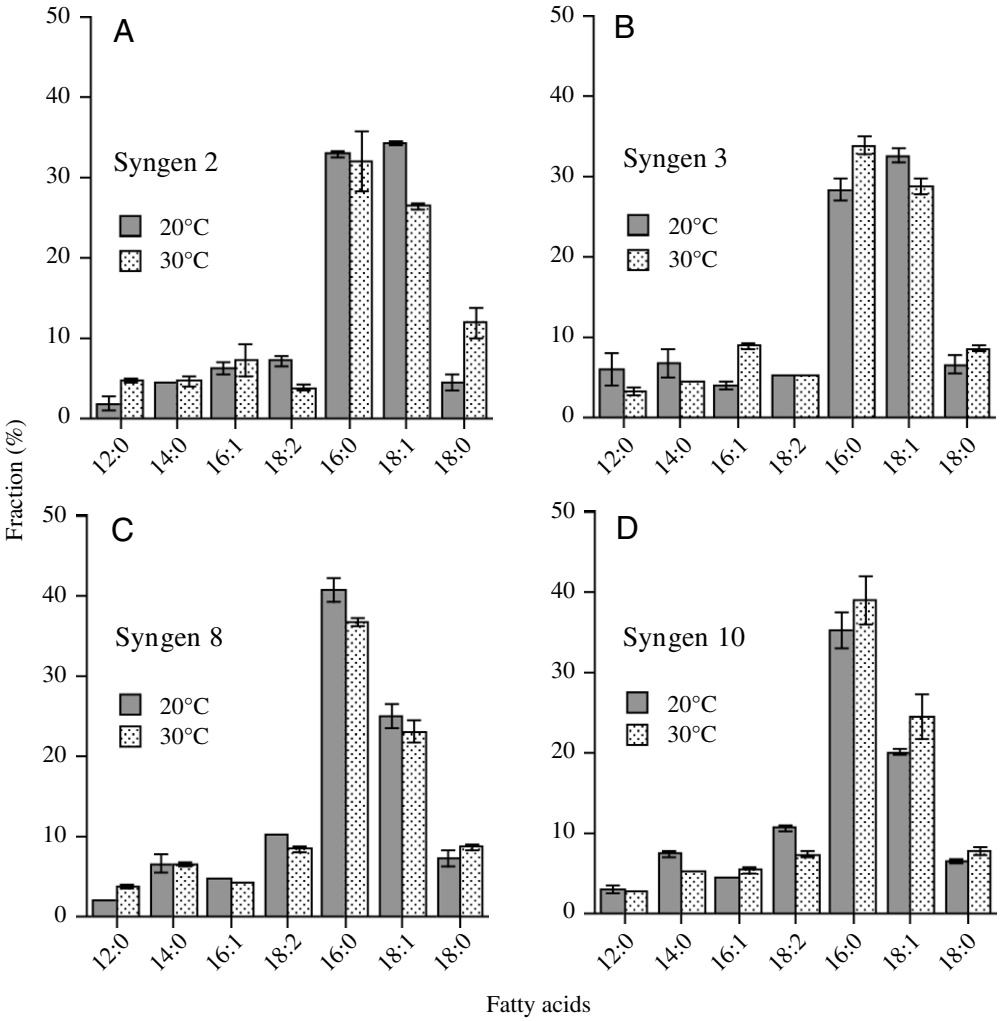

Fig. 5. Fatty acid composition of various syngens. *P. aurelia* syngens, 2, 3, 8 and 10 were cultured at either 20°C or 30°C. Fatty acids extracted from whole cells were analyzed by HPLC as described in Materials and methods. The fatty acids are denoted by the convention C with the ratio of the number of carbon atoms to the number of unsaturated linkages. (A) Syngen 2. (B) Syngen 3. (C) Syngen 8. (D) Syngen 10. Key indicates culture temperature. Values are the mean ± s.d. (*N*=3–6 experiments).

permeability of high thermotolerant syngens is lower than that of low thermotolerant syngens. As the ionic permeability of phospholipid vesicles has been related to the membrane fluidity (Rossignol et al., 1985; Lande et al., 1995), the low ionic permeability seems to correspond to a low membrane fluidity in high thermotolerant syngens.

In order to compare the membrane fluidity between high and low thermotolerant *P. aurelia* syngens, membranes of living cells were labeled with laurdan. Labeling occurred not only on the cell membrane but also on the intracellular membranes. The intracellular membranes probably include endosomes, because the fluorescent images in this study were of similar appearance to images of fluorescently labeled endosomes transported from the cell surface (Wiejak et al., 2004; Iwamoto and Allen, 2004). Therefore, *GP* values obtained from cell imaging are averages for the cell surface membrane and partially the intracellular membranes. The *GP* values measured at 25°C, which is the same as the culture temperature, are different between syngens, ranging from 0.18 to 0.28 (Fig. 4). *GP* values of high thermotolerant syngens, 8 and 10, are larger than those of low thermotolerant syngens, 2 and 3. Such a difference of *GP* values suggests that the membrane fluidity of high

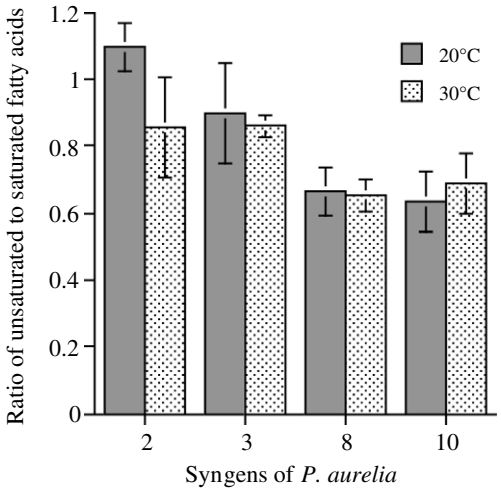

Fig. 6. Ratio of unsaturated to saturated fatty acids. Fatty acid compositions shown in Fig. 5 were divided into unsaturated and saturated fatty acids, and expressed as a ratio. Key indicates culture temperature. Values are the means ± s.d. (*N*=3–6 experiments).

thermotolerant syngens is less than that of low thermotolerant syngens at the cultured temperature. As the temperature rises these *GP* values decrease gradually and reach ~0.1 at the killing temperature of each syngen. These results are interpreted as the membrane fluidity at the killing temperature increases to almost the same level, at which point there is breakdown of the membrane functions. Because high thermotolerant syngens have membranes of lower fluidity at the culture temperature, the killing temperature, at which the membrane functions are damaged, will be greater concomitantly. Whereas the low thermotolerant syngens, having increased membrane fluidity, will be damaged at relatively low temperatures. Since the ionic permeability of membrane increases with the increase in membrane fluidity, the temperature rise up to the killing temperature will increase the ionic permeability to the critical level where the cell cannot regulate the ionic balance of the intracellular space.

Although the present analysis of fatty acids extracted from whole cells is a conventional one compared to a previous report (Hennessey and Nelson, 1983), fatty acid contents are different between high and low thermotolerant syngens. The high thermotolerant syngens, 8 and 10, have decreased ratios of unsaturated to saturated fatty acids compared with the low thermotolerant syngens, 2 and 3 (Fig. 6). These differences in the ratio of unsaturated to saturated fatty acids are consistent with differences in the membrane fluidities between high and low thermotolerant syngens. Increase in the fraction of unsaturated fatty acids is generally related to the increase of membrane fluidity. Differences in the ratio of unsaturated to saturated fatty acids between high and low thermotolerant syngens, is mainly due to differences in the fractions of palmitic acids (C16:0) and oleic acids (C18:1). Low thermotolerant syngens, 2 and 3, contain almost the same amounts of these fatty acids (Fig. 5A,B), while high thermotolerant syngens, 8 and 10 have 40–50% less oleic acid (C18:1) than palmitic acid (C16:0) (Fig. 5C,D). Such a large variation in the fatty acid content seems to be caused by the syngen-specific variation in fatty acid metabolism.

In conclusion, a comparison of high and low thermotolerant syngens of *P. aurelia* shows a consistent difference in membrane resistance, membrane fluidity and fatty acid composition. The high thermotolerant syngens exhibit less fluidity than the low thermotolerant syngens. At the hyperthermic killing temperature, the membrane fluidity increases to almost a constant level, irrespective of the killing temperature. The membrane fluidity is a crucial factor in the thermotolerance of *P. aurelia.*

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