

Perkinsus marinus, a protozoan parasite of the Eastern oyster (*Crassostrea virginica*): effects of temperature on the uptake and metabolism of fluorescent lipid analogs and lipase activities

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Abstract

The effects of temperature on the uptake and metabolism of fluorescent labeled palmitic acid (FLC16) and phosphatidylcholine (FLPC) and lipase activities in the oyster protozoan parasite, *Perkinsus marinus*, meront stage were tested at 10, 18, and 28 °C. Temperature significantly affected not only the uptake, assimilation, and metabolism of both FLC16 and FLPC in *P. marinus*, but also its triacylglycerol (TAG) lipase activities. The incorporation of both FLC16 and FLPC increased with temperature and paralleled the increase in the amount of total fatty acids in *P. marinus* meront cultures. The incorporation of FLC16 was higher than FLPC at all temperatures. The percentage of FLC16 metabolized to TAG was significantly higher at higher temperatures. Trace amounts of incorporated FLC16 were detected in monoacylglycerol (MAG) and PC at 18 and 28 °C. *P. marinus* meronts metabolized FLPC to TAG, diacylglycerol (DAG), monoacylglycerol (MAG), free fatty acids (FFA), phosphatidylethanolamine (PE), and cardiolipin (CL). The conversion of FLPC to TAG and PE was highest at 28 °C. The relative proportions of individual fatty acids and total saturated, monounsaturated and polyunsaturated fatty acids changed with temperatures. While total saturated fatty acids (SAFAs) increased with temperature, total monounsaturated fatty acids (MUFAs) decreased with temperature. Total polyunsaturated fatty acids (PUFAs) increased from 28 to 18 °C. The findings of increase of total SAFAs and decrease of total MUFAs with the increase of temperatures and upward shift of total PUFAs from 28 to 18 °C suggest that, as in other organisms, *P. marinus* is capable of adapting to changes in environmental temperatures by modifying its lipid metabolism. Generally, higher lipase activities were noted at higher cultivation temperatures. Both TAG lipase and phospholipase activities were detected in *P. marinus* cells and their extra cellular products (ECP), but phospholipase activities in both the cell pellets and ECP were very low. Also, lipase activities were much lower in ECP than in the cells. The observations of low metabolism, bioconversion of incorporated fluorescent lipid analogs and lipase activities at low temperatures are consistent with the low in vitro growth rate and low infectivity of *P. marinus* at low temperatures.

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Index Descriptors and Abbreviations: Lipid incorporation, lipid metabolism, Chromatography, Parasitic protozoan, *Perkinsus marinus*, Oyster, *Crassostrea virginica* FLPC, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanol)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; FLC16, 4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3 hexadecanoic acid; CHE, cholesteryl ester; FFA, free fatty acids; TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; CHO, cholesterol; FFH, free fatty alcohol; CER, ceramide; CL, cardiolipin; PG, phosphatidyl glycerol; PI, phosphatidyl inositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; ¹⁴C-PC, ¹⁴C-labeled dipalmitoyl-phosphatidylcholine; HPLC, high performance liquid chromatography; GLC, gas liquid chromatography; FAME, fatty acid methyl ester; psu, per salinity unit (part per thousand), HPTLC, high performance thin layer chromatography; ECP, extracellular products.

1. Introduction

Numerous studies have shown that most of parasitic protozoans require an exogenous source of essential lipids to support growth and differentiation. Incorporation of fatty acids, cholesterol, phospholipids, or lysophospholipids from the host has been reported in

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parasitic protozoans such as *Trypanosoma* (Coppens et al., 1995; Dixon et al., 1971), *Plasmodium* (Vial and Ancelin, 1998), *Giardia lamblia* (Lujan et al., 1996; Stevens et al., 1997), and *Trichomonas* spp. (Lindmark et al., 1991). Although the ability of de novo synthesizing essential fatty acids and sterols is either limited or lacking in these parasites, they can modify host lipids to some extent (Coppens et al., 1995; Dixon et al., 1971; Lindmark et al., 1991; Lujan et al., 1996; Stevens et al., 1997; Vial and Ancelin, 1998). Our recent studies on the oyster protozoan parasite, *Perkinsus marinus* (Dermo) demonstrated that unlike other parasitic protozoans, this parasite not only incorporated and modified fluorescent lipids provided in the medium (Chu et al., 2000), but synthesized a range of saturated and unsaturated fatty acids, including the essential fatty acid, arachidonic acid (Chu et al., 2002; Soudant and Chu, 2001).

Perkinsus marinus is an alveolate in the class Perkinsozoa (Levine, 1988). It is one of the two important pathogenic protozoans causing severe mortality in American (eastern) oysters (*Crassostrea virginica*) from the mid-Atlantic to the Gulf coasts since the 1950s. The disease caused by *P. marinus* is infectious (see review by Chu, 1996). Four life stages: meront (trophozoite), prezoosporangia (hypnospores), zoosporangia, and biflagellated zoospores have been identified and described (Perkins, 1966, 1988). The meront/merozoite stage is believed to be the primary agent for disease transmission (Chu, 1996; Perkins, 1988). Temperature and salinity are two important factors controlling the geographic distribution and local abundance of *P. marinus* (Burreson and Ragon-Calvo, 1996; Andrews and Ray, 1988; Andrews, 1988). Disease prevalence and intensity are maximal in the summer and increase with rising salinity during drought periods. Laboratory investigations revealed similar results; *P. marinus* prevalence and intensity in oysters are positively correlated with experimental temperature and salinity (Chu et al., 1993; Chu and La Peyre, 1993; Chu, 1996). Similarly, in vitro studies showed that *P. marinus* proliferates and develops rapidly at high temperatures between 20 and 30 °C and salinities greater than 10 psu (Chu and Greene, 1989; Perkins, 1966; Volety, 1995; Volety and Chu, 1997).

In response to temperature changes, many organisms, especially poikilotherms which cannot regulate their own temperature, alter their membrane lipid composition (Gurr and Harwood, 1991). Temperature significantly affected the incorporation and metabolism of saturated and unsaturated fatty acids in oysters, which are poikilothermic (Chu and Greaves, 1991). *P. marinus* locates intracellularly and intercellularly in the host, therefore, environmental temperature may play a role in governing its lipid uptake/incorporation and metabolism. The increased *P. marinus* activity and virulence at high temperature may be due to the increased uptake/incorporation and bioconversion of

host lipids for membrane synthesis. Our previous study showed effective incorporation and metabolism of the fluorescent lipid analogs of palmitic acid (FLC16) and the phospholipid, phosphatidylcholine (FLPC) in this parasite (Chu et al., 2000). This paper reports the results of studies testing (1) the effects of temperature on the incorporation and interconversion of FLC16 and FLPC by *P. marinus* meronts and meront's fatty acid composition; and (2) the effect of temperature on the lipolytic enzyme activity in *P. marinus* meronts.

2. Materials and methods

2.1. Chemicals and solvents

Fluorescent labeled lipid analogs were purchased from Molecular Probes (Eugene, Oregon, USA). They included: (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diazas-indacene-3-pentanoyl) sphingosyl phosphocholine (Bodipy FLC₅-sphingomyelin, FLSM), 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diazas-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine, (β-Bodipy FLC₁₂-HPC, FLPC), 2-(4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diazas-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine, β-Bodipy FL 530/550 C₁₂-HPE, FLPE), 4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diazas-indacene-3-hexadecanoic acid (Bodipy FLC₁₆, FLC16), cholesteryl 4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diazas-indacene-3-dodecanoate (cholesteryl Bodipy FL C₁₂, FLCHE). Non-labeled lipid standards, cholesteryl ester (CHE), free fatty acids (FFA), triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), cholesterol (CHO), free fatty alcohol (FFH), ceramide (CER), cardiolipin (CL), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SM), lyso-PS (LPS), and lyso-PC (LPC) were obtained from Sigma.

Radioisotopes for the lipase assays (triolein [carboxyl ¹⁴C] and L-α-dipalmitoyl phosphatidylcholine [dipalmitoyl-1-¹⁴C]) were purchased from DuPont NEN (Boston, MA, USA). Fatty acid-free bovine serum albumin, Triton X-100, Tris-HCl, triolein, and dipalmitoyl phosphatidylcholine were obtained from Sigma (St. Louis, MO). EcoLite (+) liquid scintillation cocktail was purchased from ICN (Costa Mesa, CA, USA).

Hexane, isopropanol, water, methyl acetate, chloroform, methanol, toluene, and diethyl ether were HPLC grade (Burdick and Jackson, Muskegon, MI, USA). Carbon disulfide was ultra resi-analyzed quality (J.T. Baker, Phillipsburg, NJ, USA). The 10% (w/w) boron trifluoride in methanol (BF₃) was obtained from Supelco (Bellefonte, PA, USA).

2.2. Experiments

2.2.1. Effects of temperature on incorporation and inter-conversion of FLPC and FLC16

Perkinsus marinus meronts that had been cultured at 28 °C in modified DMEM:HAM's F-12 medium (GIBCO-BRL, Gaithersburg, Maryland; Gauthier and Vasta, 1993) prepared with 0.22 µm filtered York River water (YRW) adjusted to a salinity of 20 psu with artificial sea salts were used to test the effect of temperature on incorporation and interconversion of FLPC and FLC16. It was found previously that meronts cultivated in the above medium effectively incorporated FLPC and FLC16 and converted them to other lipids (Chu et al., 2000). The lipid in this medium was derived from the 5% (v/v) bovine fetal serum added to the medium. Lipid analysis conducted on the medium revealed a concentration of 58.9 ± 6.0 µg lipid/ml of medium. To test temperature effect, cells from a 14-day-old (stationary phase) *P. marinus* meront culture were seeded at a concentration of 2×10^6 /ml and grown at 10, 18, and 28 °C for 7 days ($n = 3$) to acclimate the culture, and then harvested. Cell pellets from all temperature treatments were washed and resuspended separately in 5.0 ml of 20 psu YRW. No cell counts were conducted on the meront cell suspension. The two fluorescent-labeled lipid analogs, 10 µl (4×10^{-4} µmol) of FLC16 and 50 µl (20×10^{-4} µmol) of FLPC dissolved in dimethylsulfoxylate (DMSO), were added to meront cell suspension and incubated at respective temperatures (10, 18, and 28 °C) in the dark for 24 h. Previous study indicated no deteriorating effects and/or mortality due to incubation of fluorescent lipid analogs dissolved in DMSO (Chu et al., 2000). Following incubation, cells were washed three times with 20 psu YRW to remove free (non-incorporated) lipid analogs. Cell pellets were freeze-dried, and stored at -20 °C for later lipid analysis.

2.2.2. Effect of temperature on *P. marinus* meront lipase activities

To avoid potential introduction of any lipolytic enzymes that may be present in fetal bovine serum, in this experiment *P. marinus* meronts were cultivated in a medium that contained no fetal bovine serum (La Peyre et al., 1993). This medium contained 1% (v/v) lipid concentrate (100×, GIBCO-BRL, Gaithersburg, Maryland). Based on the amount of lipid added to this medium, the lipid concentration was estimated to be 14.5 µg lipid/ml. Meront cells in log growth phase (7-day-old) were used for the experiment. Ten milliliters of fresh medium (20 psu) were inoculated with 10×10^6 meronts/ml and cultivated at 10, 18 or 28 °C ($n = 6$ replicates per temperature). After 7 days, 3 replicates from each temperature were pelleted and the supernatant (extracellular product, ECP) and cell pellets were stored at -80 °C for subsequent analyses for lipase

activity. The other three replicates were harvested at 40 days. Prior to lipase activity analysis, protein content of cell pellets, and EPC were determined by the method of Lowry et al. (1951).

2.3. Lipid and fatty acid analyses

Total lipids were extracted from meront cells with chloroform-methanol-water mixture according to the procedure described by Bligh and Dyer (1959). To prevent loss of fluorescence, all extraction steps for the meronts incubated with fluorescent lipid analogs were performed without direct light and in glassware protected from light.

The incorporation and bioconversion of fluorescent lipids analogs in meronts/merozoites were analyzed according to Chu et al. (2000) and Soudant et al. (2000) with a Waters HPLC system (equipped with 717plus auto-sampler, 600E multisolvent delivery system, 996 photodiode array detector, and 474 scanning fluorescence detector), using a Lichrosorb diol column (5 µm; 250×4.6 mm ID) (Phenomenex, California). Fluorescent lipids were detected with fluorescent detector and non-fluorescent lipids were detected with a UV photodiode array detector. The response of the fluorescence detector was tested with various fluorescent lipid analogs including FLSM, FLPC, FLPE, FLC16, and FLCHE. A linear response was obtained in concentrations from 0.5 pmol to 0.5 nmol for all the tested components. Peak identification was furnished by the comparison of retention times with fluorescent labeled and non-labeled lipid standards. To further confirm the identification, components eluted from HPLC were collected and reanalyzed by high performance thin-layer chromatography (HPTLC) using a double development system as described by Olsen and Henderson (1989) and Chu et al. (2000). Incorporated FLPC and FLC16 and their metabolites were expressed as nmoles of fluorescent lipid per culture and nmoles of fluorescent lipid/mg of meront fatty acids.

Fatty acid content and composition of lipid samples were analyzed as fatty acid methyl esters (FAMES) to: (1) assess the biomass of meront cells for FLPC and FLC16 incubation temperature effect experiment since number of cells were not counted at the end of the experiment; and (2) determine the effects of temperature on meront's fatty acid profiles. Results from our previous study (Soudant and Chu, 2001), revealed that the increase of total lipids/fatty acids paralleled the increase of meront cell numbers and there was no significant change in fatty acid/lipid content per meront cell. Fluorescent and non-fluorescent lipid extracts were transesterified and FAMES analyzed according to Chu et al. (2000) and Soudant and Chu (2001). The FAME content (mg FA) in meront cells was used as a biomass reference for quantification of fluorescent lipid analog incorporation.

2.4. Analysis of lipase activities

All lipase assays were conducted at room temperature (21–22 °C) according to the procedure of Ozkizilcik et al. (1996). Triacylglycerol lipase substrate was prepared by the addition of [¹⁴C]triolein in ethanol and cold triolein to a 0.1 M Tris buffer (pH 8.0) containing 8 mM calcium chloride, 5% fatty acid-free bovine serum albumin and 0.1% Triton X-100. The final concentration of triolein in the substrate solution was 1 mM with a specific activity of 1 μ Ci/ml. The phospholipase substrate was prepared by adding ¹⁴C-labeled dipalmitoyl-phosphatidylcholine (¹⁴C-PC) and non-labeled phosphatidylcholine in ethanol to a glass vial and drying it under a stream of nitrogen. The same buffer used for the triacylglycerol lipase assays was then added to the vial containing the dry ¹⁴C-PC which was resuspended in the buffer by sonication. Final concentration of the ¹⁴C-PC in the phospholipase substrate was 1 mM with a specific activity of 1 μ Ci/ml. The phospholipase assay used in the present study measured the total phospholipase activity (phospholipase A₁ plus phospholipase A₂), but not the activity of a specific phospholipase.

To assay lipase activities, meront cell pellets were thawed on ice, resuspended in 1 ml buffer (0.1 M Tris, 8 mM calcium chloride, pH 8.0), sonicated and aliquoted into 4 fractions: 100 μ l for TAG assay, 100 μ l for PL assay, 100 μ l for protein determination, and 700 μ l stored at –80 °C for additional analyses. ECPs were thawed on ice and used directly for determination of lipolytic activities. Buffer only and fresh media were used as negative controls for cell pellets and ECPs, respectively.

Assays were started by vortexing 100 μ l sample with 100 μ l substrate in 1.5 ml microcentrifuge tubes. The tubes were kept in a rack on a shaker plate at ambient temperature (20 °C) for 17 h, a time previously determined to be linear for hydrolysis rates of *P. marinus* cell pellets at similar concentrations. Reactions stopped with the addition of 750 μ l chloroform/methanol toluene (2:2.4:1, v/v/v) containing 0.3 mM stearic acid followed by 25 μ l 1 N NaOH. After vortexing the samples, tubes were centrifuged at 20,000g for 5 min producing a biphasic solution with an aqueous upper phase of 550 μ l containing the released fatty acids. A 300 μ l aliquot of the upper phase from each tube was removed for liquid scintillation counting. Results were expressed as nmole fatty acid released/mg protein/h.

2.5. Statistical analysis

All results from the experiment that tested the temperature effects on incorporation/interconversion of FLPC and FLC16 are expressed as Mean, Standard Deviation (SD). All data from this experiment were subjected to one-way analysis (dependent vari-

able = temperature) of variance (ANOVA) and multiple comparisons were determined by Neuman–Keuls test. Within each temperature treatment, there was no significant difference in FA contents between the control and those incubated with fluorescent lipid analogs. Therefore, FA content data at each temperature were combined ($n = 9$) for statistical analysis. Percentage data were converted by Arcsin (\sqrt{x}) prior statistical analysis. Significant differences ($P < 0.05$) between treatments were noted with superscripted letters. Analyses were performed using Statistica computer package. Lipase activity data were compared by 2-way ANOVA (dependent variables were culture age and temperature). When results were significant at the $p < 0.05$ level, individual comparisons were conducted using Tukey's test.

3. Results

3.1. Total fatty acid content and uptake/incorporation of fluorescent lipid analogs in meronts cultured at different temperatures

The content of fatty acids (FAs) in *P. marinus* meronts differed among temperature treatments, although cultures were started with similar inoculated cell number (2.0×10^6 cells/ml culture). The total FA contents of meronts cultivated at 10, 18, and 28 °C for 7 days and incubated one day with FLC16 or FLPC at respective temperatures were statistically different, and increased with temperature (Table 1). Temperature significantly affected the incorporation and metabolism of both FLC16 and FLPC. The incorporation of both FLC16 and FLPC paralleled to the amounts of FAMES in meronts, increased with increased temperature (Table 1). FLC16 was incorporated in higher amounts compared to FLPC at all temperature treatments (Figs. 1A and B; Table 1). Most of the incorporated FLPC remained as PC and only a small fraction of incorporated FLPC was metabolized to other lipid classes. Interestingly, meronts cultivated at 18 °C had the highest FLC16 incorporation, but lowest FLPC incorporation when results were expressed as nmoles of FL lipids/mg FA (Table 1; Figs. 1A and B). FLC16 metabolized to TAG and DAG at all three temperatures (Fig. 1A). The percentage of incorporated FLC16 found in TAG was significantly higher at higher treatment temperatures: 13.5, 84.8, and 90%, respectively, at 10, 18, and 28 °C. But, the incorporated amount, expressed as nmoles of fluorescent lipids/mg FA, was highest at 18 °C and this temperature had the highest FLC16 deposited in TAG (Fig. 1A). Trace amounts (<0.5 nmol of fluorescent lipids/mg FA) of incorporated FLC16 were detected in MAG and PC at 18 and 28 °C and in PI/PS at 18 °C. Meronts metabolized FLPC to TAG, DAG, MAG, FFA, PE, and CL

Table 1

Total fatty acids (FAs, mg) per *P. marinus* culture ($n = 9$) and total FLC16 ($n = 3$) and FLPC ($n = 3$) incorporations in *P. marinus* cultures

	Temperatures					
	10 °C		18 °C		28 °C	
	Mean	SD	Mean	SD	Mean	SD
Total mg FAs/culture ($n = 9$)	0.022 ^a	0.002	0.194 ^b	0.023	0.803 ^c	0.175
Total incorporated FLC16 (nmole fluorescent lipids/culture; $n = 3$)	0.17 ^a	0.02	2.1 ^b	0.16	4.87 ^c	0.38
Total incorporated FLC16 (nmole fluorescent lipids/mg FA; $n = 3$)	7.8 ^a	0.8	11.0 ^b	1.4	5.9 ^a	1.0
Total incorporated FLPC (nmole fluorescent lipids/culture; $n = 3$)	0.09 ^a	0.00	0.44 ^a	0.03	2.49 ^b	0.27
Total incorporated FLPC (nmole fluorescent lipids/mg FA; $n = 3$)	4.0 ^a	0.1	2.1 ^b	0.1	3.7 ^a	0.7

Results are presented as means and standard deviation (SD). Different letters denote significant differences at the $p < 0.05$ level.

FLC16, 4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-*s*-indacene-3-hexadecanoic acid (Bodipy FLC₁₆); FLPC, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (β -Bodipy FLC₁₂-HPC).

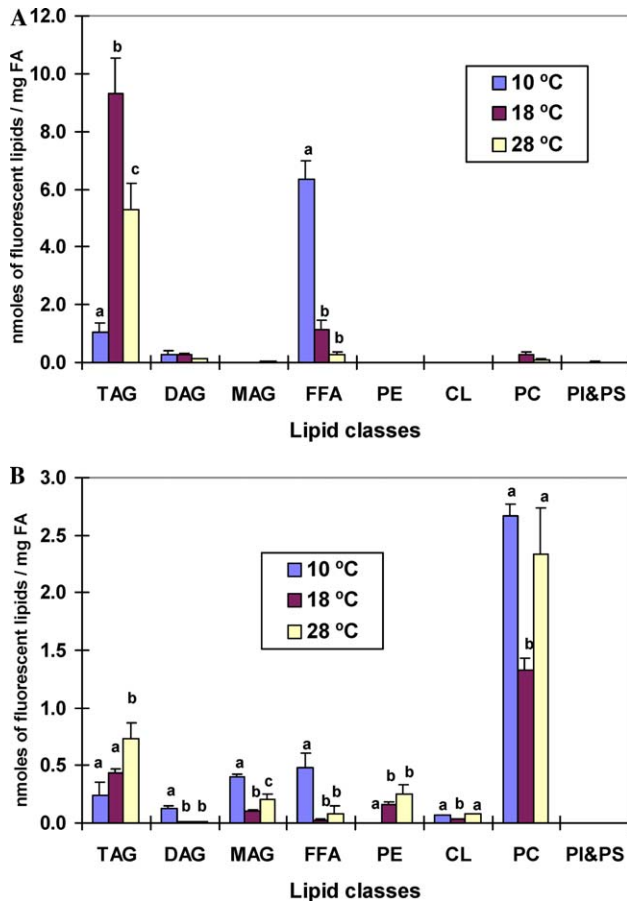


Fig. 1. Incorporation and conversion of FLC16 (A) and FLPC (B) in vitro cultured *P. marinus* cells (meronts/merozoites) at 10, 18, and 28 °C after 24 h incubation. Results are expressed as nmole of fluorescent lipid/mg of fatty acids (Means, SD; $n = 3$). FFA, free fatty acids; TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; CL, cardiolipin; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; and PC, phosphatidylcholine. Different letters denote significant differences at the $p < 0.05$ level (one-way ANOVA).

(Fig. 1B). The conversion of FLPC to TAG and PE was highest at 28 °C. At this temperature, TAG and PE represented 19.7 and 6.8%, respectively, of the total incorporated fluorescence. Though less was incorporated, the percentages of FLPC converted to TAG and PE at 18 °C were similar to those at 28 °C, 20.8 and 7.8%, respectively. Significantly higher amounts of incorporated FLPC were converted to DAG, MAG, and FFA in meronts at 10 °C than at 18 and 28 °C.

3.2. Meront fatty acid composition at different temperatures

Temperature affected fatty acid composition of meronts. The relative proportions of individual fatty acids (Table 2) and as well as total saturated fatty acids (SAFAs), mono-unsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) changed with temperature (Fig. 2). The weight % of major saturated fatty acids, 14:0 and 16:0 were significantly higher in meronts cultivated at 28 °C than at 10 and 18 °C and 18:0 augmented with temperatures. Thus, total SAFA proportion increased significantly with temperature. While the weight % of the fatty acids, 16:1($n = 7$), 18:1($n = 9$), 18:1($n = 7$), and 20:1($n = 9$), were relatively higher at higher temperatures (i.e., 18 and 28 °C), the predominant 22:1($n = 9$) decreased dramatically with temperature (Table 2). This fatty acid largely accounted for the decline of the total MUFAs with temperatures (Fig. 2). Meronts at 10 °C had the lower weight % of 20:2($n = 6$), 20:4($n = 6$), and 20:2($n = 9$) and other minor PUFAs, with the exception of 18:2($n = 6$), than meronts at 18 and 28 °C (Table 2). Generally, meronts at 18 °C had the highest weight percentage of these fatty acids and thus had the highest proportion of total PUFAs.

Table 2
Fatty acid composition of in vitro cultured *P. marinus* meronts at 10, 18 or 28 °C

% of total FA	Temperatures					
	10 °C		18 °C		28 °C	
	Mean	SD	Mean	SD	Mean	SD
<i>SAFAs</i>						
14:0	3.9 ^a	0.5	5.6 ^a	0.6	15.6 ^b	1.8
15:0	0.8 ^a	0.1	0.2 ^b	0.1	0.1 ^b	0.0
16:0	14.9 ^a	2.5	14.1 ^a	0.6	23.0 ^b	1.1
17:0	0.0 ^a	0.0	0.5 ^b	0.1	0.1 ^c	0.0
18:0	3.9 ^a	0.6	5.8 ^b	0.2	8.0 ^c	0.3
20:0	0.8 ^a	0.4	1.8 ^b	0.1	1.5 ^b	0.1
22:0	0.4 ^a	0.1	1.0 ^b	0.1	0.6 ^c	0.0
24:0	0.5 ^a	0.1	0.7 ^b	0.1	0.8 ^b	0.1
<i>MUFAs</i>						
16:1(<i>n</i> – 9)	2.1 ^a	0.5	0.9 ^b	0.1	0.7 ^b	0.1
16:1(<i>n</i> – 7)	1.3 ^a	0.2	2.1 ^b	0.2	2.8 ^c	0.3
18:1(<i>n</i> – 9)	11.1 ^a	1.0	23.7 ^b	0.4	18.4 ^c	0.8
18:1(<i>n</i> – 7)	1.7 ^a	0.2	2.9 ^b	0.3	2.3 ^c	0.4
20:1(<i>n</i> – 9)	3.6 ^a	0.3	6.3 ^b	0.3	9.4 ^c	0.8
20:1(<i>n</i> – 7)	0.1	0.2	0.0	0.1	0.0	0.0
22:1(<i>n</i> – 9)	46.2 ^a	5.7	3.9 ^b	1.6	1.3 ^c	0.3
<i>PUFAs</i>						
18:2(<i>n</i> – 6)	3.6 ^a	1.5	7.6 ^b	0.4	2.9 ^c	0.2
20:2(<i>n</i> – 9)	0.6 ^a	0.2	1.5 ^b	0.4	1.1 ^c	0.2
20:2(<i>n</i> – 6)	1.1 ^a	0.2	1.9 ^b	0.1	1.6 ^c	0.4
20:3(<i>n</i> – 6)	0.0 ^a	0.1	0.9 ^b	0.1	0.5 ^c	0.0
20:4(<i>n</i> – 6)	3.3 ^a	0.7	17.2 ^b	1.2	8.8 ^c	0.9
20:5(<i>n</i> – 3)	0.0 ^a	0.0	0.4 ^b	0.1	0.1 ^c	0.0
22:5(<i>n</i> – 3)	0.0 ^a	0.0	0.4 ^b	0.0	0.1 ^c	0.0
22:6(<i>n</i> – 3)	0.0 ^a	0.0	0.3 ^b	0.0	0.1 ^c	0.0

Results were expressed as weight percentage of the total fatty acids (Means and standard deviation, SD; *n* = 9). Different letters denote significant differences at the *p* < 0.05 level.

SAFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; and PUFAs, polyunsaturated fatty acids.

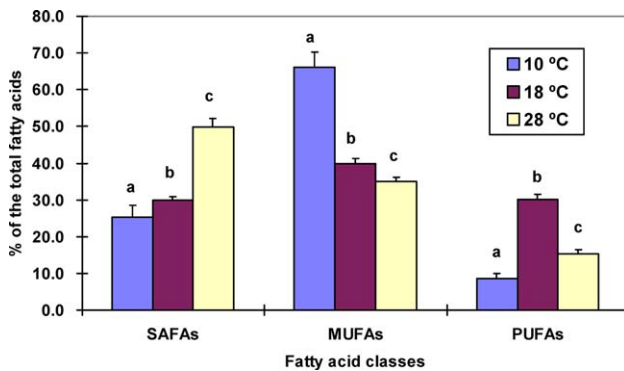


Fig. 2. Saturated, monosaturated, and polyunsaturated fatty acid proportions of in vitro cultured *P. marinus* cells (meronts/merozoites) after 8 days of growth at 10, 18, and 28 °C. Results are expressed as percentage of the total fatty acids (Means, SD; *n* = 9). Different letters denote significant differences at the *p* < 0.05 level (one-way ANOVA). SAFAs, saturated fatty acids; MUFAs, monounsaturated fatty acid; and PUFAs, polyunsaturated fatty acids.

3.3. Effect of temperature on lipase activities

Both triacylglycerol lipase and phospholipase activities were detected in cell pellets and extracellular prod-

ucts (ECPs) from *P. marinus* cultures. TAG lipase activities of cell pellets ranged from a low of 0.75 ± 0.12 nmol FA released/mg protein/h in the 7 day 18 °C treatment to a high of 3.06 ± 0.34 nmol FA released/mg protein/h in the 40 day at 28 °C (Fig. 3A). Generally, TAG lipase activities in *P. marinus* cells increased non-linearly with culture temperatures (Fig. 3A). TAG lipase activities in *P. marinus* cells differed significantly between 10 and 28 °C treatments in both 7 day and 40-day-old cultures (*p* < 0.001). The TAG lipase activities in 40-day-old cells were similar between 18 and 28 °C and significantly higher than the activity at 10 °C. There was no difference in TAG activity between 10 and 18 °C in 7-day-old cells. TAG lipase activities of the ECPs also exhibited treatment effects (*p* < 0.001). As in the cell pellets, generally TAG lipase activities in ECPs increased with increasing culture temperature (Fig. 3B). The TAG activities in 7 day ECP were non-detectable, except in the 28 °C treatment. No TAG lipase activity was detected in the 7 day ECPs at 18 °C treatment, yet the same temperature treatment had significant activities after 40 days in culture (2.28 ± 0.14 nmol FA released/mg protein/h).

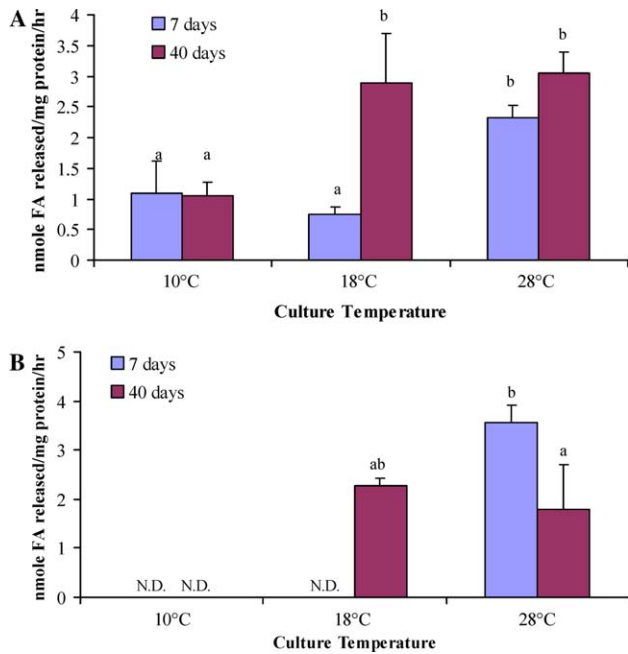


Fig. 3. TAG lipase activities in *P. marinus* cell pellets (A) and ECP (B) grown at 10, 18, and 28 °C for 7 and 40 days (Means, SD; $n = 3$). Different letters denote significant differences at the $p < 0.05$ level (two-way ANOVA). ND, non-detectable; ECP, extracellular products; and FA, fatty acid.

Phospholipase activities of both the cell pellets and ECPs were at the threshold of detection limit and exhibited high variability. Hydrolysis rates in the cell pellets ranged from 0.02 ± 0.33 to 0.33 ± 0.05 nmol FA/mg protein/h. The ECP rates for phospholipase activity ranged from 0.53 ± 0.25 to 1.30 ± 1.58 nmol FA/mg protein/h. There were no significant treatment effects for culture temperature or culture age in either the cell pellets or ECPs.

4. Discussion

Our previous study showed effective incorporation and metabolism of the fluorescent lipid analogs FLC16 and FLPC and indicated that FLC16 was incorporated to a greater degree than FLPC (Chu et al., 2000). Similarly, the present study showed that FLC16 was incorporated in higher amounts than FLPC for all the temperature treatments. Free fatty acids (e.g., FLC16) may be more readily integrated into other lipid classes, such as TAG than FLPC, because a hydrolysis step is not required.

The present study demonstrated the significant temperature effect on the incorporation and metabolism of both FLC16 and FLPC in *P. marinus* meronts. Temperature is the dominant factor in controlling the progression of *P. marinus* infections in oysters (Andrews and Ray, 1988; Burreson and Ragone-Calvo, 1996; Chu

and Volety, 1997). Infection-associated mortality usually begins in early summer (June) when water temperature increases and peaks between August and September when ambient temperatures reach up to 28 °C. During in vitro culture, warm temperatures (21–28 °C) also favor *P. marinus* development (Chu and Greene, 1989; Perkins, 1966) and meront proliferation (Volety, 1995). Our previous study (Soudant and Chu, 2001) noted that the increase of total lipids/fatty acids paralleled the increase of meront cell numbers. To facilitate further development and increased proliferation at high temperatures, increased lipid incorporation from an exogenous (e.g., host) source may be necessary. The parallel increases of total FA content (an indicator of cell mass) and total incorporated FLC16 and FLPC with temperatures support this argument (Table 1). However, the content of incorporated fluorescent lipids per unit mass (i.e., nmoles fluorescent lipid/mg FA) did not increase with temperature, except FLC16 which increased from 10 to 18 °C. This implies that the distribution of incorporated lipids in *P. marinus* cells was relatively uniform, due to the concomitant increases of cell number (cell mass) and fluorescent lipid incorporation at higher temperature.

The highest FLC16 incorporation and lowest FLPC incorporation occurred at the same temperature 18 °C. This is interesting, but difficult to explain. The rationale behind the observed phenomenon is unknown. However, as noted in previous (Chu et al., 2000) and present studies, the FLC16 incorporation was always high at all temperatures. Low temperature such as 10 °C reduced significantly the lipid metabolic activity in *P. marinus*. At this temperature, the metabolism of FLC16 and FLPC to other lipids (e.g., deposition of FLC16 into TAG and incorporation/conversion of FLPC into TAG and other phospholipids) was much lower compared to other temperature treatments. It is possible that at low temperature, *P. marinus* catabolizes exogenous lipids for energy and general maintenance. The presence of significant amounts of intermediate by-products such as DAG, MAG, and FFA in culture incubated with FLPC supports this hypothesis. On the other hand, at higher temperatures (i.e., 18 and 28 °C) efficient accumulation of FLC16 in TAG as energy storage for ongoing proliferation may be important. But, the usage of this metabolic pathway was greatly reduced at 10 °C. Thus, it suggests that metabolism of incorporated FLC16 is more temperature dependent than is the uptake of the substrate.

To cope with environmental changes in temperature, most organisms including parasitic and non-parasitic protozoans and bacteria are capable of modifying/altering their biochemistry and physiology accordingly. One of the responses to temperature change is to re-tailor a series of molecular species of the membrane lipids and/or alter the composition of membrane lipids

(Gurr and Harwood, 1991). This response is referred to as “homeoviscous adaptation” (Hochachka and Somero, 1984), which allows the organism to maintain membrane biophysical properties at optimal values for normal function. Although change in the ratio of fatty acyl unsaturation to fatty acyl saturation is commonly seen during changes of environmental temperatures, alteration of lipid classes and their ratios have also been noted (Florin-Christensen et al., 1997; Gurr and Harwood, 1991; Jones et al., 1993; Roy et al., 1991).

A recent study by Florin-Christensen et al. (1997) showed that the parasitic protozoan *Trypanosoma cruzi* increased the ratios of free sterols to phospholipids and saturated to unsaturated fatty acids in phospholipids when transferred from 28 to 37 °C. They believed that the alteration in the ratios of saturated to unsaturated fatty acids was due to changes in desaturase activities. They found a significant decrease in fatty acyl $\Delta 9$ and $\Delta 12$ desaturation at 37 °C. The free living amoeba, *Acanthamoeba castellanii* also showed increase in lipid unsaturation over a period of 24 h, when the growth temperature was shifted downwards from 30 to 15 °C (Jones et al., 1991). Chilling of late-exponential and stationary phase cultures from 30 to 15 °C resulted in a rapid and marked increase in the synthesis of PUFA (Avery et al., 1994; Jones et al., 1991). Fatty acyl $\Delta 12$ desaturation was also induced when culture temperatures were stepped down from 30 to 15 °C (Jones et al., 1993).

The fatty acid composition of *P. marinus* is quite similar to *A. castellanii*. In responding to the increase of cultivation temperature, as expected, *P. marinus* meronts increased their total relative SAFAs and decreased their total MUFAs, respectively. The increases in saturated fatty acids, 14:0, 16:0, and 18:0 contributed to the shift in total SAFAs from low to high temperatures. The decline of total MUFAs with temperature is more complicated involving increase and decrease of individual MUFAs in opposite directions. The MUFAs, 16:1($n-7$), 18:1($n-9$), 18:1($n-7$), and 20:1($n-9$) were higher at higher temperatures (Table 2). The increases of 16:1($n-7$) and 20:1($n-9$) were relatively linear with increased temperatures. On the other hand, the fatty acids, 22:1($n-9$) along with 16:1($n-9$), were the two fatty acids actually decreasing with temperatures (Table 2). Surprisingly, increase of PUFAs only occurred from 28 to 18 °C and the lowest total PUFAs was at 10 °C. The striking augmentations of the fatty acids, 18:2($n-6$) and 20:4($n-6$), from 28 to 18 °C were responsible for the increase of PUFAs. It is speculated that *P. marinus* at 10 °C is more or less in dormancy. *P. marinus* activity, proliferation and development is reduced dramatically at low temperatures (Chu and Greene, 1989; Volety, 1995; Volety and Chu, 1997). Desaturation activity was not measured in the present study. It is unknown whether the low PUFAs at 10 °C

and decrease of MUFAs from 10 to 28 °C were due to a reduction in desaturation activity.

Lipases, both intracellular and extracellular, are utilized by organisms for a range of metabolic functions including assimilation and reorganization of dietary lipids and mobilization of stored lipids (Gurr and Harwood, 1991). Parasites potentially have additional uses for lipases such as acquisition of host lipids and penetration of host tissues (Furlong, 1991; Saffer et al., 1989).

Phospholipids are important structural components of the bilayer cell membrane (Gurr and Harwood, 1991). Therefore, generally, in addition to protease hydrolysis, the virulence associated with cell lysis and tissue penetration of the host are attributed to phospholipase activity rather than TAG lipase activity (McKerrow et al., 1993; Schulte and Scholze, 1989; Szamel and Resch, 1981; Vial et al., 1982; Zidovetzski et al., 1993). In the present study, phospholipase activities detected in all treatments, intracellularly and extracellularly, were low relative to TAG lipase activity. This implies that the lipolytic activity of *P. marinus* may be directed towards the acquisition and metabolism of lipids rather than virulence towards the host. *P. marinus* does not need to force entry to the host oyster. In nature, *P. marinus* infective cells are released from the infected host. The water born infective cells such as meronts enter the oyster as it feeds and filters water. After they enter the host, they are phagocytosed by the host epithelium-associated blood cells, the hemocytes (Ford and Tripp, 1996). Those that survive the host defense would then be carried to other tissues via circulation. Also, *P. marinus* is capable not only of making its own phospholipids (Lund and Chu, 2002) from scratch, but also of synthesizing a range of saturated and polyunsaturated fatty acids (Chu et al., 2002; Soudant and Chu, 2001). It is possible that the usage of phospholipases to cleave fatty acids from host phospholipids may be of minimal importance for membrane synthesis in this parasite.

The relatively low ECP and cell pellet TAG lipase activities at the lowest temperature (10 °C) are notable in that these results differ from what has been reported in higher poikilotherms. Neas and Hazel (1985) found that rainbow trout acclimated to 5 °C increased the titer of phospholipase A₂ (PLA₂) in liver microsomes relative to trout acclimated to 20 °C, when activities were measured at the same temperature. But, their lypolysis rates were nearly identical to those acclimated to 20 °C, when the PLA₂ activities were measured at the original acclimation temperatures. In contrast, cold acclimated (10 °C) *P. marinus* meronts apparently did not increase TAG lipase titer relative to cells acclimated to higher temperatures. Cell pellets and ECPs of 7-day-old culture from the 10 °C treatment actually had lower TAG lipase rates than those acclimated to 28 °C. This suggests that *P. marinus* cells may lack the ability to compensate

metabolically for low environmental temperatures. Low extracellular lipase activity was also found in the bacterium, *Acinetobacter* sp. when the culture temperatures were reduced from 25 to 5 °C (Barbaro et al., 2001).

In summary, temperature significantly affected not only the uptake, assimilation and metabolism of lipids from exogenous source in *P. marinus*, but also its TAG and phospholipid lipase activities. Generally, higher uptake, incorporation and lipase activities were noted at higher cultivation temperatures. Similarly, catabolism and bio-conversion of incorporated lipids was lower at low than at high temperatures. These observations are consistent with the low in vitro growth rate and low infectivity of *P. marinus* at low temperatures. The findings of increase of total SAFAs and decrease of total MUFAs with the increase of temperatures and upward shift of total PUFAs from 28 to 18 °C suggest that *P. marinus* is capable of adapting to changes in environmental temperatures by modifying its fatty acid composition.

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