Intraerythrocytic *Plasmodium falciparum* utilize a broad range of serum-derived fatty acids with limited modification for their growth

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SUMMARY

Plasmodium falciparum causes the most severe form of malaria. Utilization of fatty acids in serum is thought to be necessary for survival of this parasite in erythrocytes, and thus characterization of the parasite fatty acid metabolism is important in developing a new strategy for controlling malaria. Here, we examined which combinations of fatty acids present in human serum support the continuous culture of *P. falciparum* in serum-free medium. Metabolic labelling and gas chromatography analyses revealed that, despite the need for particular fatty acids for the growth of intraerythrocytic *P. falciparum*, it can metabolize a broad range of serum-derived fatty acids into the major lipid species of their membranes and lipid bodies. In addition, these analyses showed that the parasite's overall fatty acid composition reflects that of the medium, although the parasite has a limited capacity to desaturate and elongate serum-derived fatty acids. These results indicate that the *Plasmodium* parasite is distinct from most cells, which maintain their fatty acid composition by coordinating *de novo* biosynthesis, scavenging, and modification (desaturation and elongation).

Key words: malaria, desaturase, elongase, lipid metabolism, *Plasmodium falciparum*, fatty acid.

INTRODUCTION

Plasmodium falciparum causes the most severe form of malaria, afflicting people in endemic areas with a very high rate of morbidity and mortality. The clinical symptoms and pathogenesis of this disease are exclusively associated with the asexual multiplication of this parasite in erythrocytes. This parasite requires human serum for its intraerythrocytic proliferation (Trager and Jensen, 1976). Thorough investigation of the essential factors in human serum and their metabolism in the parasite is essential for identifying pathways critical for its growth and, therefore, identification of novel approaches to the treatment of malaria.

Previous studies on serum components that support the intraerythrocytic development of *P. falciparum in vitro* have identified several factors, including high and low density lipoproteins (Grellier *et al*. 1991), long-chain saturated and unsaturated fatty acids in association with BSA (Ofulla *et al*. 1993), and a mixture of lysophosphatidylcholines in association with BSA (Asahi *et al*. 2005); however, studies of fractioned human serum have not been completed. Recently, by fractionating and reducing the components in human serum, we identified palmitic ($C_{16:0}$) and oleic ($C_{18:1,\ n\text{-}9}$) acids in association with lipid-free BSA as a minimal component necessary for complete cell cycle progression and intraerythrocytic development of *P. falciparum in vitro* (Mitamura *et al*. 2000). Indeed, these essential fatty acids have been shown to be metabolized into various lipid species, such as phosphatidylcholine, phosphatidylethanolamine, diacylglycerol, and triacylglycerol, all of which are major constituents of membranes and lipid bodies in the parasite (Vial *et al*. 1982; Palacpac *et al*. 2004).

Several lines of evidence suggest that intraerythrocytic *P. falciparum* has the capacity to generate middle-chain ($C_{10:0}$, $C_{12:0}$, and $C_{14:0}$) but not long-chain fatty acids ($C_{16:0}$ and $C_{18:0}$) through a *de novo* biosynthetic pathway and that this pathway is essential for parasite growth (Surolia and Surolia, 2001). Nevertheless, intraerythrocytic proliferation largely relies on fatty acids derived from human serum; basal medium lacking these fatty acids does not support growth (Mitamura *et al*. 2000). This agrees with the idea that serum-derived long-chain saturated and unsaturated fatty acids are necessary for parasite growth and that scavenging fatty acids

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from serum is essential for survival of the intra-erythrocytic *Plasmodium* parasite (Holz, 1977; Vial and Ancelin, 1998; Mitamura and Palacpac, 2003). Moreover, a recent report shows that the intra-erythrocytic *P. falciparum* has no or little capacity to elongate or desaturate fatty acids (Krishnegowda and Gowda, 2003). These features of fatty acid metabolism in *P. falciparum* stand in contrast with those in a majority of organisms, wherein both *de novo* lipid biosynthesis and modification (elongation and desaturation) are important for homeostasis.

In this study, we screened combinations of 3 fatty acids (saturated and unsaturated) to determine which of them can sustain the long-term culture of *P. falciparum in vitro*. We also used metabolic labelling and gas chromatography (GC) to determine how serum-derived fatty acids essential for growth are metabolized by the parasite.

MATERIALS AND METHODS

Materials

Fatty acid-free BSA (used as lipid-free BSA) (Mitamura *et al*. 2000), various fatty acid species, and $C_{20:3,\ n\text{-}9}$ and $C_{22:6,\ n\text{-}3}$ methyl esters were purchased from Sigma-Aldrich. The $C_{18:1,\ n\text{-}7}$ methyl ester was from Supelco. Other fatty acid methyl esters (FAMEs) were from Matreya. Stock solutions of fatty acids (30 mM) and FAMEs (100 mM) were dissolved in ethanol and acetone, respectively, and stored at −20 °C until use. [1-^{14}C]-oleic acid (51 mCi mmol^{-1}) was from Amersham Biosciences Corp. [1-^{14}C]-palmitic acid (60 mCi mmol^{-1}) was from NEN Life Science Products. [1-^{14}C]-myristic acid (55 mCi mmol^{-1}), [1-^{14}C]-stearic acid (55 mCi mmol^{-1}), and [1-^{14}C]-linoleic acid (50 mCi mmol^{-1}) were from American Radiolabeled Chemicals Inc. Silica gel 60 high-performance TLC and silanized silica gel 60 TLC plates were from Merck.

Parasite culture

Culture of the *P. falciparum* Honduras-1 line and the growth-promoting activity assay were performed essentially as described previously (Mitamura *et al*. 2000; Hanada *et al*. 2000) with slight modification. If the parasitaemia at 96 h was above 0·1%, the culture was diluted to 0·1% and incubated for another 96 h. The growth-promoting activities were assessed at 96 h and 192 h. The media used were as described previously (Palacpac *et al*. 2004).

Gas chromatography analysis of fatty acid

Total lipids of pooled human serum from 10 individuals were extracted by Bligh and Dyer's method (Bligh and Dyer, 1959). Fatty acids were purified from the obtained total lipids using an NH_2 Sep-Pak plus column (Waters) as described previously (Kaluzny *et al*. 1985). The fatty acids collected were transmethylated in 1 ml of 5% methanolic HCl at 80 °C for 2 h (Khunyoshyeng *et al*. 2002). The resulting FAMEs were extracted twice with 1·5 ml of *n*-hexane, dried with a Speed Vac concentrator, and stored at 4 °C until use. Prior to analysis, the FAMEs were dissolved in 140 μl of acetone, and a 2 μl sample was automatically injected into a GC353B gas chromatograph (GL sciences) equipped with a TC-FFAP capillary column (0·25 mm × 30 m; df = 0·25 μm) and a flame ionization detector (GL sciences). The column pressure was set at 1·1 kg/cm^2, the split mode was set at a ratio of 50 : 1, and the programme to control the oven temperature was as follows: 45 °C for 3 min, followed by an increase from 45 °C to 230 °C at 10 °C/min, and, finally, maintenance at 230 °C for 40 min.

Total lipids were extracted from *P. falciparum*-infected erythrocytes enriched using Percol from cultures as described previously (Palacpac *et al*. 2004). The obtained lipids were dissolved in 1·8 ml of 10% KOH in 66% methanol and saponified at 65 °C for 60 min (Matsuzaka *et al*. 2002). After acidification with 1 ml of ice-cold 6 M HCl, the fatty acids liberated were extracted by Bligh and Dyer's method (Bligh and Dyer, 1959). The collected fatty acids were transmethylated, and the resulting FAMEs were analysed as described above. To determine the background of the erythrocyte fatty acid content, the same number of uninfected erythrocytes incubated in various media used for parasite cultures were similarly treated and analysed. In all the infected erythrocytes samples, more than 90% are schizont stage.

At least 4 injections per sample were performed, and the average of the results obtained from 3 independent chromatograms was used for quantification. The concentration of each fatty acid species detected in the sample was estimated from the peak area using standard curves for various control FAMEs as determined from the analyses of 4 different concentrations of each control FAME (the linear regression coefficients of the standard curves were >0·999). Changes in the fatty acid content due to parasitic infection were determined by subtracting the results for uninfected erythrocytes from those for *P. falciparum*-infected erythrocytes. In all cases, the results for infected erythrocytes refer to the changes from uninfected erythrocytes. Also, the amounts of fatty acids in the uninfected erythrocytes were less than 4% of those in infected erythrocytes. All results were normalized by the amount of fatty acid in 10^7 infected erythrocytes as determined by measurement of the parasitaemia in Percol-enriched samples.

Metabolic labelling

Tightly synchronized cultures of Honduras-1 line *P. falciparum*, which have a 5 h life-span, were

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Table 1. Fatty acid content in pooled human serum and *Plasmodium falciparum*-infected erythrocytes

(The samples were prepared from the pooled human serum from 10 individuals and the culture maintained in standard medium supplemented with this serum. Data for the pooled human serum and the parasitized erythrocytes are expressed as μM and nmole per 10^7 infected erythrocytes, respectively.)

	14 : 0	16 : 0	16 : 1*n*-7	18 : 0	18 : 1*n*-9	18 : 1*n*-7	18 : 2*n*-6	18 : 3*n*-3	20 : 4*n*-6	22 : 6*n*-6
Pooled human serum	0·29	7·78	0·51	2·83	6·95	0·44	3·56	0·26	0·72	0·79
Parasitized erythrocyte	0·84	19·01	0·73	2·18	12·24	0·55	8·62	0·50	1·00	0·22

metabolically labelled either in standard or serum-free medium with ^{14}C-labelled fatty acids, and total lipids were extracted and analysed as described previously (Palacpac *et al*. 2004) except that a silica gel 60 high-performance TLC plate was used. In experiments performed in standard medium, cultures contained the pooled human serum from 10 individuals (for which the fatty acid content was determined by GC) mixed with one of the following ^{14}C-labelled fatty acids at a final specific activity of 8·3 mCi mmole^{-1}: [1-^{14}C]-myristic acid ($C_{14:0}$), [1-^{14}C]-palmitic acid ($C_{16:0}$), [1-^{14}C]-stearic acid ($C_{18:0}$), [1-^{14}C]-oleic acid ($C_{18:1,\ n\text{-}9}$), and [1-^{14}C]-linoleic acid ($C_{18:2,\ n\text{-}6}$). The purity of these radio-isotope labelled fatty acids was confirmed by TLC on a silanized plate in 70 : 50 : 35 : 1 (v/v/v/v) acetone/ methanol/ water/acetic acid prior to use. In experiments performed in serum-free medium, cultures were treated with 60 μM reconstituted lipid-associated BSA containing combinations of 3 fatty acids that supported parasite growth for at least 7 subcultures along with the appropriate ^{14}C-labelled fatty acids. In both standard and serum-free media, parasite development and appearance were monitored by microscopical observation of Giemsa-stained thin smears.

To identify the fatty acid species produced by parasite-associated elongase and desaturase activities, fatty acids were liberated from the total lipids extracted from metabolically labelled erythrocytes and then derivatized to methyl esters as described above for GC analysis. The obtained products were separated by TLC on a silanized plate in 70 : 50 : 35 : 1 (v/v/v/v) acetone/methanol/ water/acetic acid and analysed using an image analyser (Fuji Photo Film Co.) as described previously (Palacpac *et al*. 2004).

RESULTS

Comprehensive analysis of the combinations of three human serum-derived fatty acids for the intraerythrocytic proliferation of P. falciparum

To establish the baseline fatty acid composition of human serum, we used GC to analyse the average fatty acid composition of human serum pooled from 10 individuals. We identified 8 fatty acid species ($C_{16:0}$, $C_{18:1,\ n\text{-}9}$, $C_{18:2,\ n\text{-}6}$, $C_{18:0}$, $C_{22:6,\ n\text{-}6}$, $C_{20:4,\ n\text{-}6}$, $C_{16:1,\ n\text{-}7}$, and $C_{18:1,\ n\text{-}7}$) that each accounted for more than 1·5% of the total amount of fatty acid. Their estimated concentrations were 7·78, 6·95, 3·55, 2·83, 0·79, 0·72, 0·51, and 0·44 μM, respectively (Table 1).

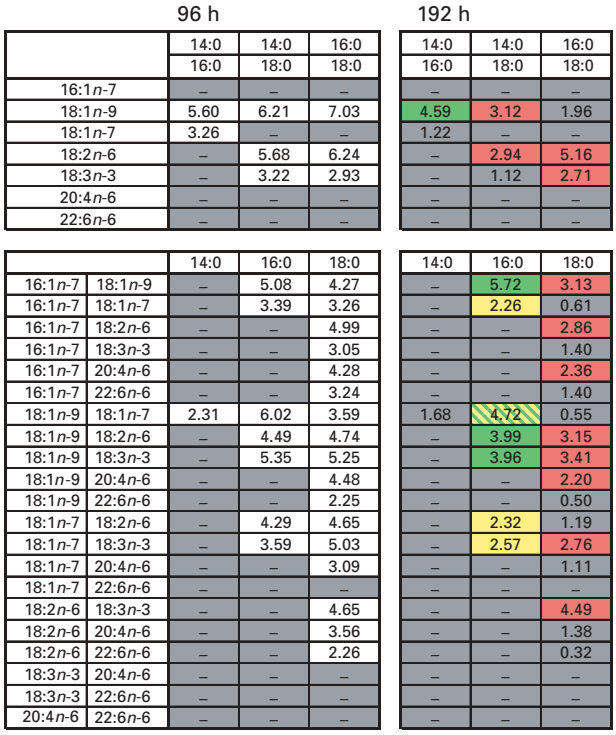

Fig. 1. Parasite growth rates in serum-free medium supplemented with reconstituted lipid-associated BSA containing a combination of saturated and unsaturated fatty acids. The combinations giving growth rates less than 2 are coloured grey; those containing $C_{18:0}$ are shown in red; those containing both $C_{16:0}$ and $C_{18:1,\ n\text{-}9}$ are in green; and those containing both $C_{16:0}$ and $C_{18:1,\ n\text{-}7}$ are in yellow. The growth rates in standard and in serum-free medium supplemented with reconstituted lipid-associated BSA containing $C_{16:0}$/$C_{18:1,\ n\text{-}9}$ were 8·14 and 6·07, respectively.

We next examined the effect of these 8 fatty acids together with the $C_{14:0}$, which is the final product of *de novo* fatty acid synthesis in *P. falciparum* (Surolia and Surolia, 2001), and $C_{18:3,\ n\text{-}3}$, an essential fatty acid species in humans, on the intraerythrocytic proliferation of *P. falciparum*. We first determined the growth rates at 96 h and 192 h in a serum-free medium supplemented with reconstituted lipid-associated BSAs containing combinations of 3 fatty acids. All of the fatty acids identified were examined

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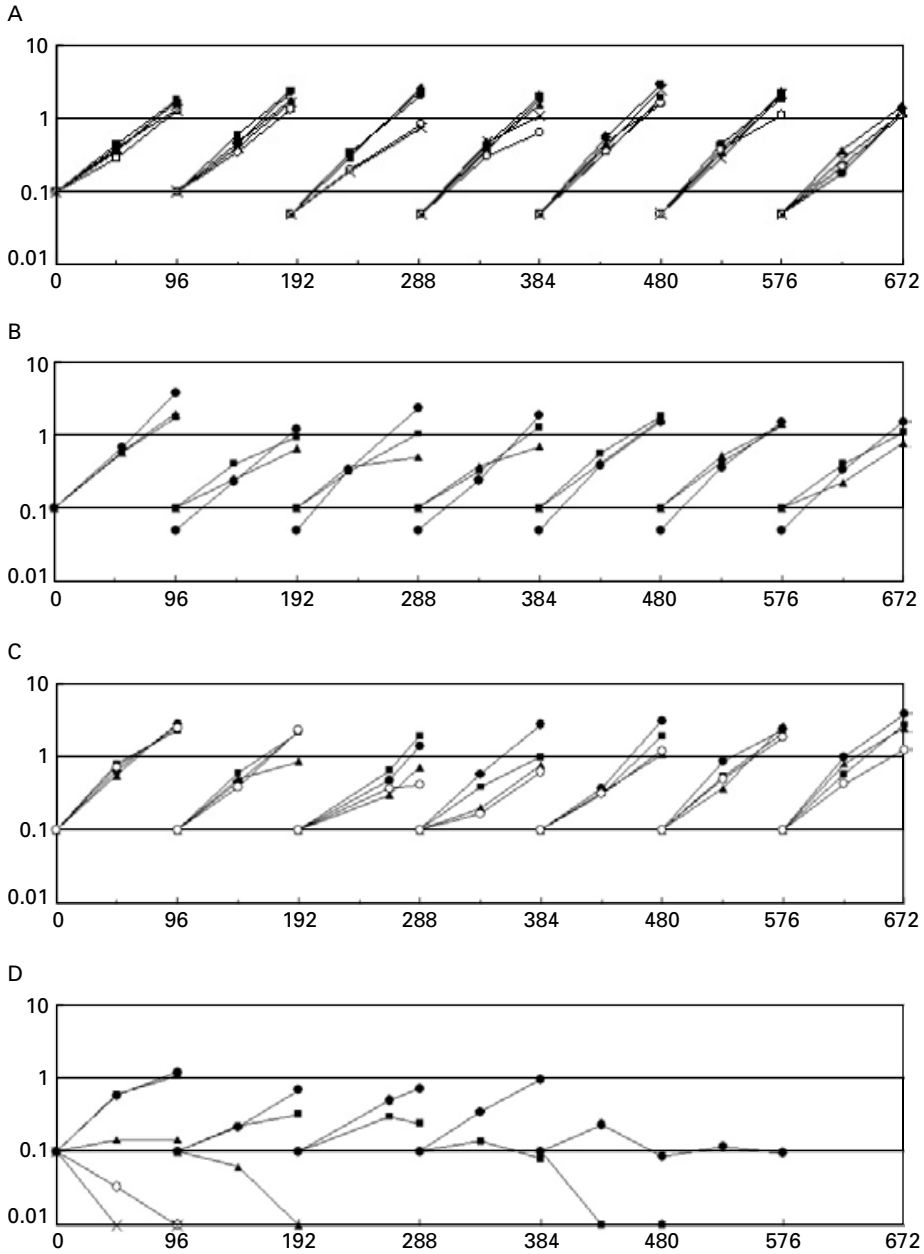

Fig. 2. The growth profile of the parasites in serum-free medium supplemented with reconstituted lipid-associated BSA containing a combination of fatty acids. (A) $C_{16:0}/C_{18:1,\ n\text{-}9}$ (●), $C_{16:0}/C_{18:1,\ n\text{-}9}/C_{14:0}$ (■), $C_{16:0}/C_{18:1,\ n\text{-}9}/C_{16:1,\ n\text{-}7}$ (▲), $C_{16:0}/C_{18:1,\ n\text{-}9}/C_{18:2,\ n\text{-}6}$ (○), and $C_{16:0}/C_{18:1,\ n\text{-}9}/C_{18:3,\ n\text{-}3}$ (×). (B) $C_{16:0}/C_{18:1,\ n\text{-}9}/C_{18:1,\ n\text{-}7}$ (●), $C_{16:0}/C_{18:1,\ n\text{-}7}/C_{18:2,\ n\text{-}6}$ (■), and $C_{16:0}/C_{18:1,\ n\text{-}7}/C_{18:3,\ n\text{-}3}$ (▲). (C) $C_{16:0}/C_{18:0}/C_{18:2,\ n\text{-}6}$ (●), $C_{14:0}/C_{18:0}/C_{18:2,\ n\text{-}6}$ (■), $C_{14:0}/C_{18:0}/C_{18:1,\ n\text{-}9}$ (▲), and $C_{18:0}/C_{18:1,\ n\text{-}9}/C_{18:2,\ n\text{-}6}$ (○). (D) $C_{18:0}/C_{16:1,\ n\text{-}7}/C_{18:1,\ n\text{-}9}$ (●), $C_{18:0}/C_{18:2,\ n\text{-}6}/C_{20:4,\ n\text{-}6}$ (■), $C_{16:0}/C_{18:1,\ n\text{-}9}/C_{20:4,\ n\text{-}6}$ (▲), $C_{14:0}/C_{16:1,\ n\text{-}7}/C_{18:2,\ n\text{-}6}$ (○), and $C_{14:0}/C_{18:2,\ n\text{-}6}/C_{20:4,\ n\text{-}6}$ (×). X- and Y-axes indicate parasitaemia (%) and period (h) of the parasite cultures, respectively.

with the exception of completely saturated or unsaturated species because they are expected to be detrimental for parasite growth (Mitamura *et al.* 2000). Of the total of 84 combinations, 20 supported parasite growth for at least 192 h. Overall, the growth rates were significantly affected by changing only 1 fatty acid (Fig. 1).

We next examined whether these 20 combinations could sustain the continuous culture of *P. falciparum*. We found 11 combinations (Fig. 2A–C) that could maintain the exponential growth of the parasite during the course of continuous culture for at least 28 days (equivalent to 7 subcultures), whereas the remaining 9 combinations caused a gradual decrease in the growth rates until the parasites finally disappeared (Fig. 2D; data not shown). These results indicate that particular combinations of saturated and unsaturated fatty acids are needed to sustain long-term intraerythrocytic proliferation of *P. falciparum* and that chain length, extent of unsaturation,

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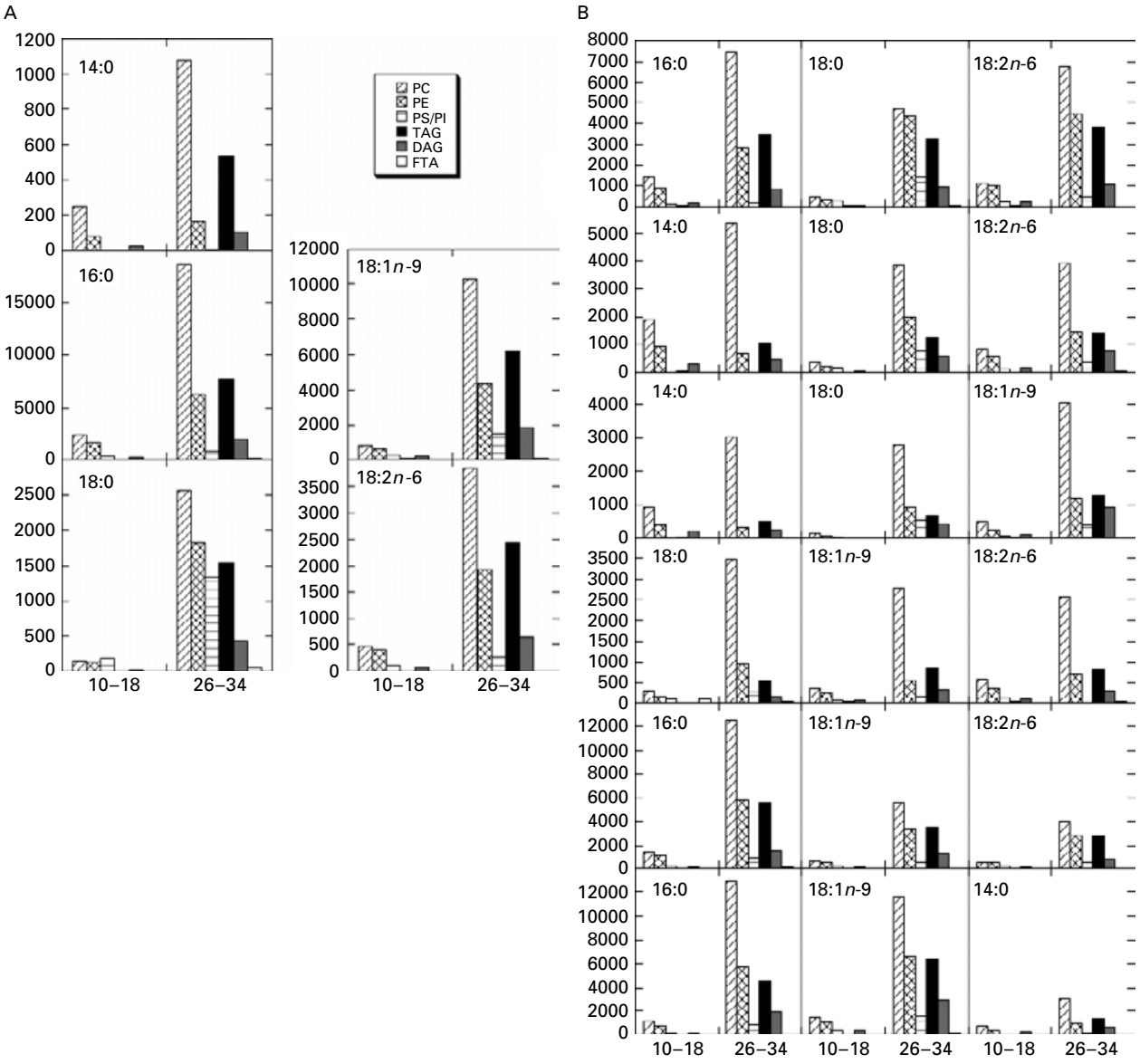

Fig. 3. Comparison of the profiles of the lipid species generated by metabolism of different fatty acids in standard and in a serum-free medium supplemented with reconstituted lipid-associated BSA containing the combinations of 3 fatty acids. (A) Standard medium. (B) Serum-free medium. Each row represents a combination of fatty acids added to the serum-free medium. The specific ^{14}C-labelled fatty acids used for metabolic labelling are indicated in each graph. The amount of each lipid is quantified by densitometry analysis using an image analyser (Fuji Photo Film Co.) and is expressed as arbitrary units.

and position of double bonds are critical parameters regulating the growth.

Comparison of the profiles of the lipid species metabolized from different fatty acids in standard and serum-free media

Six combinations of fatty acids ($C_{14:0}/C_{16:0}/C_{18:1,\ n\text{-}9}$, $C_{14:0}/C_{18:0}/C_{18:1,\ n\text{-}9}$, $C_{14:0}/C_{18:0}/C_{18:2,\ n\text{-}6}$, $C_{16:0}/C_{18:1,\ n\text{-}9}/C_{18:2,\ n\text{-}6}$, $C_{16:0}/C_{18:0}/C_{18:2,\ n\text{-}6}$, and $C_{18:0}/C_{18:1,\ n\text{-}9}/C_{18:2,\ n\text{-}6}$) were able to maintain the *P. falciparum* subculture for over 6 months (data not shown), suggesting that, like human serum, these combinations can support parasite culture indefinitely. We next used metabolic labelling with ^{14}C-fatty acids to examine the lipid profiles generated from these 5 fatty acids in standard and a serum-free media. All of the fatty acids tested could be metabolized into phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, diacylglycerol, and triacylglycerol. Further, the level of each lipid species and the lipid profiles were similar at early (ring to early trophozoite; 10–18 h) and mature stages (mature trophozoite to schizont; 26–34 h) (Figs 3A, B and 4A–G). These results indicate that, despite the requirement of particular fatty acids for growth, intraerythrocytic *P. falciparum* can metabolize a broad range of serum-derived fatty acids.

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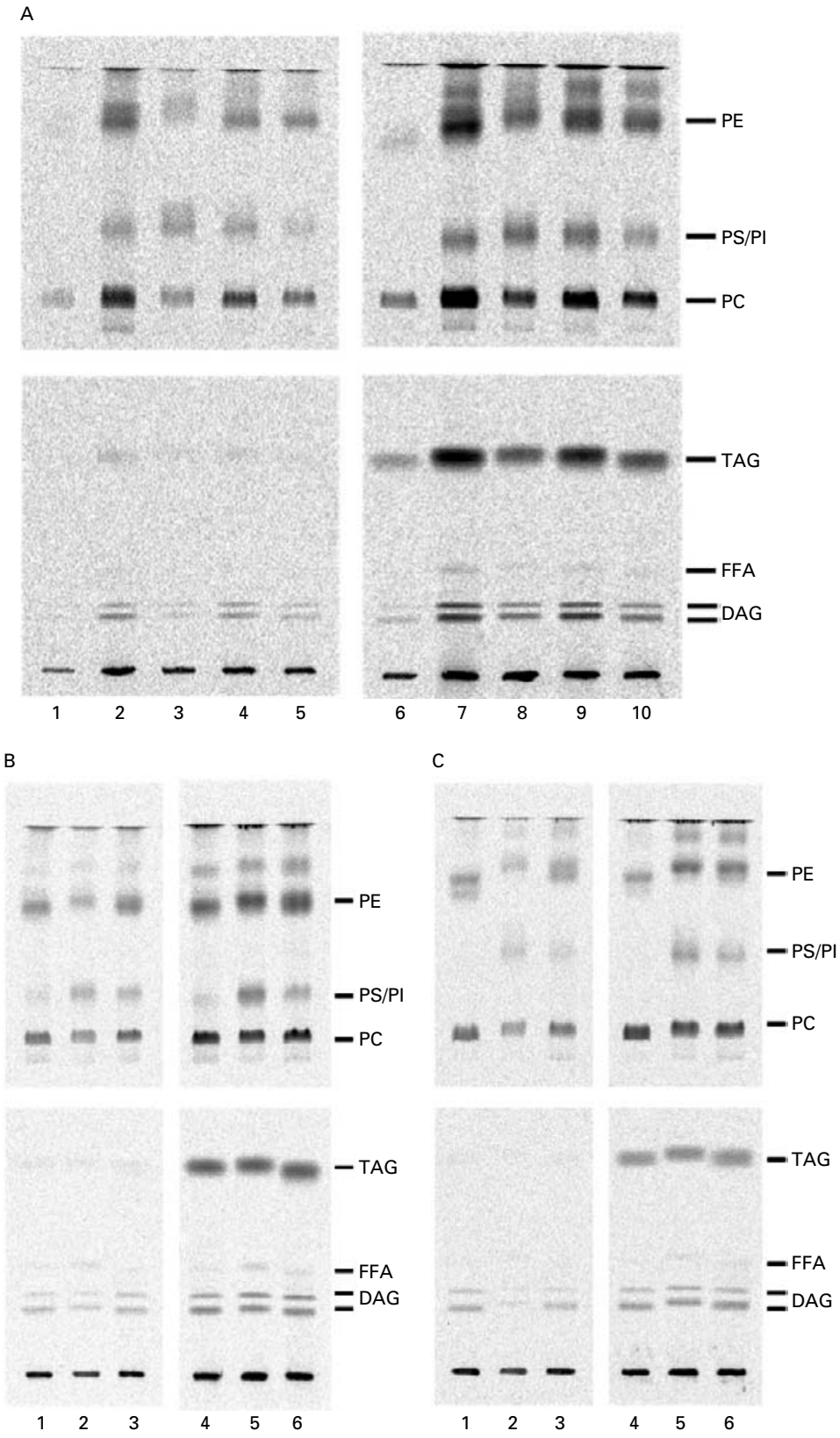

Fig. 4. Analysis of lipid species generated by metabolism from different fatty acids in standard medium or serum-free medium supplemented with reconstituted lipid-associated BSA containing combination of fatty acids. (A) Standard medium. High-performance TLC of the samples metabolically labelled from 10 to 18 h (lanes 1–5) and from 26 to 34 h (lanes 6–10) during intraerythrocytic development. The following ^{14}C-fatty acids were used for metabolic labelling: ^{14}C-$C_{14:0}$ (lanes 1 and 6), ^{14}C-$C_{16:0}$ (lanes 2 and 7), ^{14}C-$C_{18:0}$ (lanes 3 and 8), ^{14}C-$C_{18:1,\ n\text{-}9}$ (lanes 4 and 9), and ^{14}C-$C_{18:2,\ n\text{-}6}$ (lanes 5 and 10). (B-G) Serum-free media. The following combinations were added to the medium: $C_{16:0}$/$C_{18:0}$/$C_{18:2,\ n\text{-}6}$ (B), $C_{14:0}$/$C_{18:0}$/$C_{18:2,\ n\text{-}6}$ (C), $C_{14:0}$/$C_{18:0}$/$C_{18:1,\ n\text{-}9}$ (D), $C_{18:0}$/$C_{18:1,\ n\text{-}9}$/$C_{18:2,\ n\text{-}6}$ (E), $C_{16:0}$/$C_{18:1,\ n\text{-}9}$/$C_{18:2,\ n\text{-}6}$ (F), and $C_{16:0}$/$C_{18:1,\ n\text{-}9}$/$C_{14:0}$ (G). High-performance TLC of the samples metabolically labelled from

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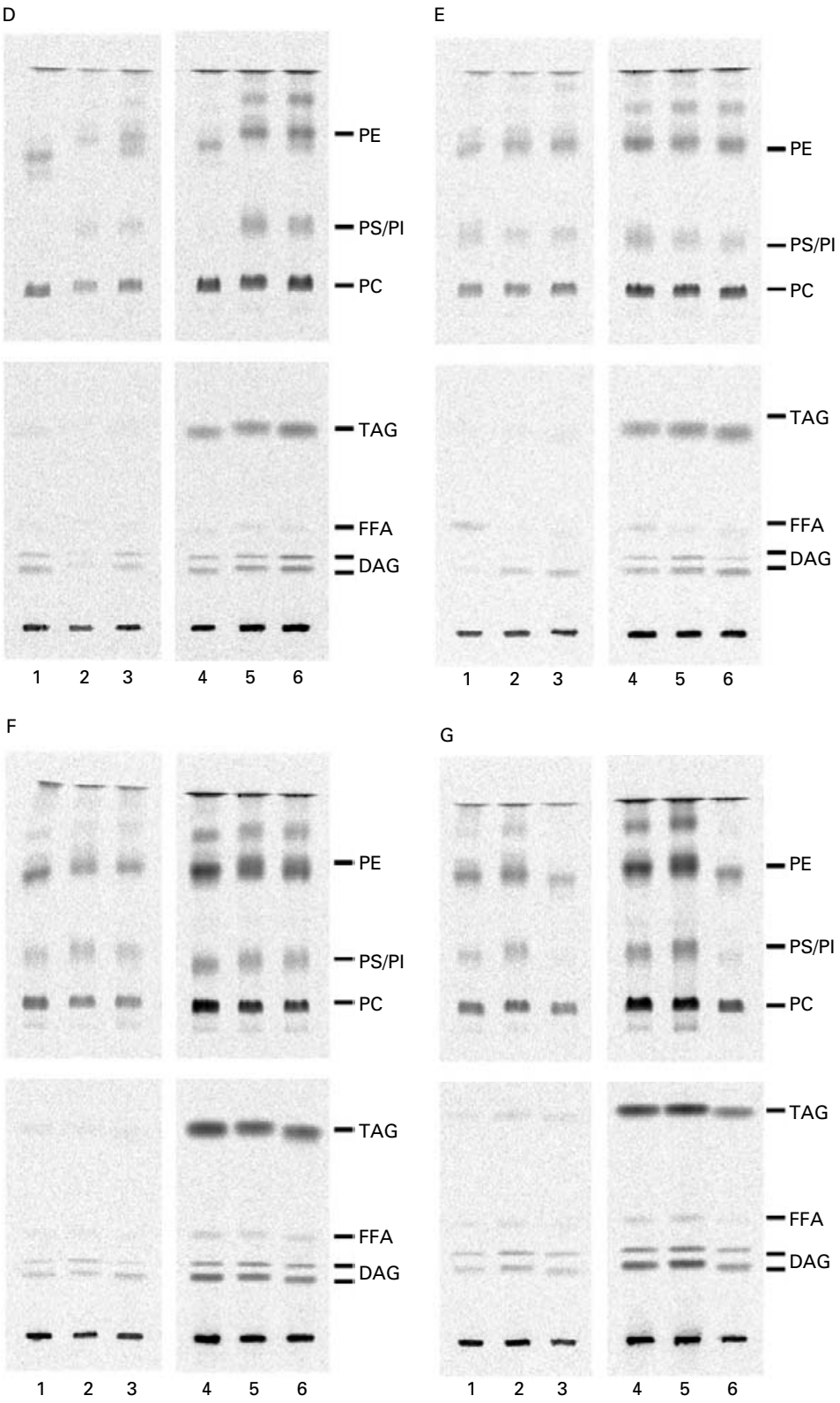

10 to 18 h (lanes 1–3) and from 26 to 34 h (lanes 4–6) during intraerythrocytic development. The following ^{14}C-fatty acids were used for metabolic labelling: (B) ^{14}C-$C_{16:0}$ (lanes 1 and 4), ^{14}C-$C_{18:0}$ (lanes 2 and 5), and ^{14}C-$C_{18:2,\ n\text{-}6}$ (lanes 3 and 6); (C) ^{14}C-$C_{14:0}$ (lanes 1 and 4), ^{14}C-$C_{18:0}$ (lanes 2 and 5), and ^{14}C-$C_{18:2,\ n\text{-}6}$ (lanes 3 and 6); (D) ^{14}C-$C_{14:0}$ (lanes 1 and 4), ^{14}C-$C_{18:0}$ (lanes 2 and 5), and ^{14}C-$C_{18:1,\ n\text{-}9}$ (lanes 3 and 6); (E) ^{14}C-$C_{18:0}$ (lanes 1 and 4), ^{14}C-$C_{18:1,\ n\text{-}9}$ (lanes 2 and 5), and ^{14}C-$C_{18:2,\ n\text{-}6}$ (lanes 3 and 6); (F) ^{14}C-$C_{16:0}$ (lanes 1 and 4), ^{14}C-$C_{18:1,\ n\text{-}9}$ (lanes 2 and 5), and ^{14}C-$C_{18:2,\ n\text{-}6}$ (lanes 3 and 6); and (G) ^{14}C-$C_{16:0}$ (lanes 1 and 4), ^{14}C-$C_{18:1,\ n\text{-}9}$ (lanes 2 and 5), and ^{14}C-$C_{14:0}$ (lanes 3 and 6). The polar and neutral lipids are shown in the upper and lower panels, respectively, and the positions of the standard lipids are indicated on the right side. PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; TAG, triacylglycerol; FFA, fatty acid; DAG, diacylglycerol.

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Table 2. Summary of the parasite-associated capacities for fatty acid desaturation and elongation

(Data shown are the averages of the conversion rates obtained from 3 independent cultures for each GC and metabolic labelling analyses and are expressed as percentages ±S.D. The conversion rate is calculated by dividing the amount of the corresponding product by the total amount of the substrate and product, and multiplying by 100. The conversion rate in metabolic labelling is quantified also by densitometry using radio-isotope labelled fatty acids as a standard, e.g. 80·4 and 1·8 pmole per h per 10^7 infected erythrocytes for desaturating the *n*-9 position of $C_{18:0}$ of the $C_{16:0}/C_{18:0}/C_{18:2,\ n\text{-}6}$ sample and for elongating $C_{14:0}$ into $C_{16:0}$ of the $C_{14:0}/C_{18:0}/C_{18:2,\ n\text{-}6}$ sample, respectively. n.d., not done; n. a., not applicable.)

	16:0→16:1*n*-7		18:0→18:1*n*-9		14:0→16:0	16:0→18:0
	GC	Metabolic labelling	GC	Metabolic labelling	Metabolic labelling	Metabolic labelling
16:0/18:0/18:2*n*-6	0·6±0·0	0·5±0·1	36·1±2·8	31·2±1·1		0·0±0·0
14:0/18:0/18:2*n*-6			12·6±2·4	10·7±0·5	1·4±0·4	
14:0/18:0/18:1*n*-9			n.a.	24·6±0·6	1·6±0·4	
18:0/18:1*n*-9 /18:2*n*-6			n.a.	15·9±0·5		
14:0/16:0/18:1*n*-9	n.d.	1·2±0·1			0·8±0·3	0·0±0·0
16:0/18:1*n*-9/18:2*n*-6	n.d.	0·9±0·0				0·0±0·0
16:0/18:1 *n*-9	1·6±0·3	1·2±0·2				0·7±0·0
Human serum	n.a.	0·6±0·1	n.a.	29·4±0·1	0·0±0·0	0·0±0·0

Intraerythrocytic P. falciparum *can elongate and desaturate serum-derived fatty acids*

The fatty acids from the medium were mostly converted into the major lipid components of the parasite's membranes and lipid body (Figs 3A, B and 4A–G; Vial *et al*. 1982; Palacpac *et al*. 2004). To determine which fatty acid species were generated by metabolism, we analysed the metabolically labelled parasites by TLC. Analysis of the ^{14}C-$C_{18:0}$-labelled samples showed ^{14}C-labelled $C_{18:1}$ methyl ester was present in the infected erythrocytes but not in the uninfected erythrocytes (Fig. 5A). Even when the imaging plates for the uninfected erythrocyte samples were overexposed until the intensity of the $C_{18:0}$ signals reached the levels in the infected cells, a $C_{18:1}$ methyl ester signal could not be seen. A signal corresponding to ^{14}C-labelled $C_{16:1}$ methyl ester could also be specifically detected in the infected but not the uninfected ^{14}C-$C_{16:0}$-labelled erythrocytes. In contrast, we did not detect signals for fatty acids produced by Δ9-, Δ12-, or Δ15-desaturases from infected erythrocytes metabolically labelled with ^{14}C-$C_{14:0}$, ^{14}C-$C_{18:1,\ n\text{-}9}$, or ^{14}C-$C_{18:2,\ n\text{-}6}$ (data not shown). These results clearly indicate that the intraerythrocytic *P. falciparum* can desaturate mainly the *n*-9 position of $C_{16:0}$ and $C_{18:0}$ and that this activity is unaffected by the culture conditions (Table 2).

In contrast to the desaturase activity, the ability to elongate fatty acids taken up from the surroundings can be detected only under limited culture conditions. We were able to detect the production of $C_{18:0}$ from ^{14}C-$C_{16:0}$ when the parasites were cultured in serum-free medium supplemented with lipid-rich BSA containing $C_{16:0}/C_{18:1,\ n\text{-}9}$ (Fig. 5B) but not when they were cultured in standard or serum-free medium supplemented with lipid-rich BSA containing $C_{16:0}/C_{18:0}/C_{18:2,\ n\text{-}6}$, $C_{14:0}/C_{16:0}/C_{18:1,\ n\text{-}9}$, or $C_{16:0}/C_{18:1,\ n\text{-}9}/C_{18:2,\ n\text{-}6}$ (data not shown). No $C_{18:0}$ signals could be observed in uninfected erythrocytes even when the images were overexposed. Similarly, in the presence of ^{14}C-$C_{14:0}$, a small but significant production of $C_{16:0}$ was observed in parasites grown in serum-free medium supplemented with lipid-rich BSA containing $C_{14:0}/C_{16:0}/C_{18:1,\ n\text{-}9}$, $C_{14:0}/C_{18:0}/C_{18:1,\ n\text{-}9}$, or $C_{14:0}/C_{18:0}/C_{18:2,\ n\text{-}6}$ but not in standard medium (data not shown). These results suggest that the

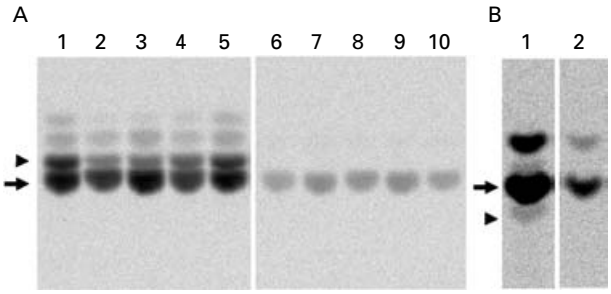

Fig. 5. Analysis of the parasite-associated capacities for desaturating and elongating serum-derived fatty acids. (A) Desaturation. Silanized TLC of FAMEs prepared from metabolically ^{14}C-$C_{18:0}$-labelled infected (lanes 1–5) and uninfected (lanes 6–10) erythrocytes. The fatty acid combinations used to supplement serum-free medium metabolic labelling were as follows: $C_{16:0}/C_{18:0}/C_{18:2,\ n\text{-}6}$ (lanes 1 and 6), $C_{14:0}/C_{18:0}/C_{18:2,\ n\text{-}6}$ (lanes 2 and 7), $C_{14:0}/C_{18:0}/C_{18:1,\ n\text{-}9}$ (lanes 3 and 8), and $C_{18:0}/C_{18:1,\ n\text{-}9}/C_{18:2,\ n\text{-}6}$ (lanes 4 and 9). Human serum (lanes 5 and 10) was also used for metabolic labelling. The arrowhead and arrow indicate the position of methyl ethers of $C_{18:1,\ n\text{-}9}$ and $C_{18:0}$, respectively. (B) Elongation. FAMEs prepared from metabolically ^{14}C-$C_{16:0}$-labelled infected (lane 1) and uninfected (lanes 2) erythrocytes. The pair of $C_{16:0}/C_{18:1,\ n\text{-}9}$ was used to supplement serum-free medium metabolic labelling. The arrowhead and arrow indicate the position of methyl ethers of $C_{18:0}$ and $C_{16:0}$, respectively.

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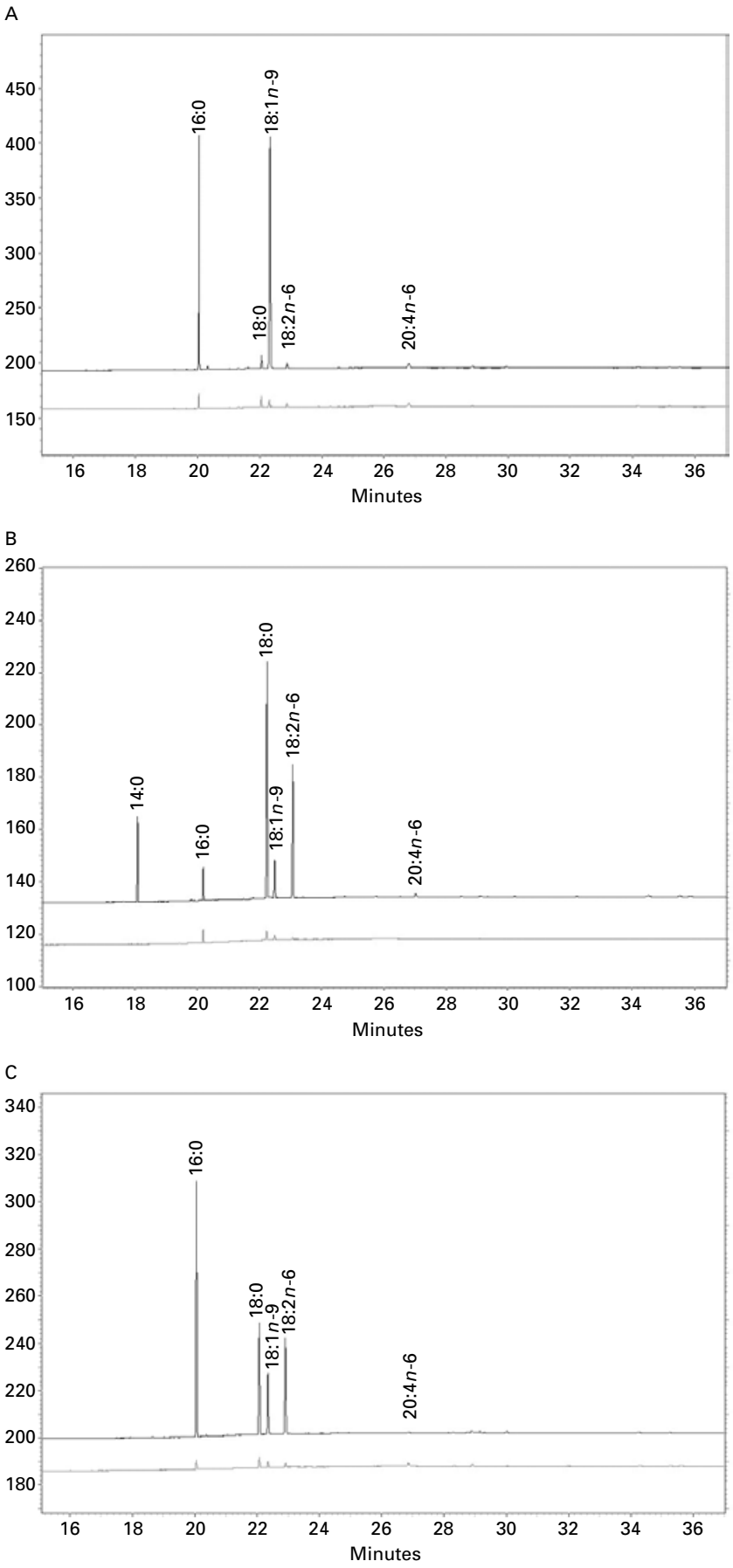

Fig. 6. GC analysis of the fatty acid composition and profile in *Plasmodium falciparum*-infected erythrocytes. Representative chromatograms from at least 4 injections are shown with black lines (infected erythrocytes) and grey lines (uninfected erythrocytes). The combinations of fatty acids used for the cultures were (A) $C_{16:0}/C_{18:1,\ n\text{-}9}$, (B) $C_{14:0}/C_{18:0}/C_{18:2,\ n\text{-}6}$, and (C) $C_{16:0}/C_{18:0}/C_{18:2,\ n\text{-}6}$.

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Table 3. Fatty acid content in *Plasmodium falciparum*-infected erythrocyte

(The samples were prepared from the cultures maintained in serum-free medium supplemented with reconstituted lipid-associated BSA containing a combination of fatty acids. Data shown are the averages of the results obtained from three independent cultures and are expressed as nmole per 10^7 infected erythrocytes ± S.D.)

	14 : 0	16 : 0	16 : 1*n*-7	18 : 0	18 : 1*n*-9	18 : 1*n*-7	18 : 2*n*-6	18 : 3*n*-3	20 : 4*n*-6	22 : 6*n*-6
16 : 0/18 : 1*n*-9	0·05 ± 0·03	22·51 ± 1·79	0·36 ± 0·03	0·16 ± 0·23	28·03 ± 1·42	0	0·31 ± 0·01	0	0·37 ± 0·21	0
14 : 0/18 : 0/18 : 2*n*-6	10·08 ± 7·61	1·75 ± 0·15	0	19·37 ± 1·50	2·80 ± 0·62	0	11·66 ± 2·28	0	0·18 ± 0·23	0
16 : 0/18 : 0/18 : 2*n*-6	0·06 ± 0·06	24·31 ± 5·25	0·16 ± 0·04	9·89 ± 1·45	5·58 ± 0·87	0	9·05 ± 5·98	0	0·14 ± 0·24	0

intraerythrocytic *P. falciparum* can elongate $C_{14:0}$ and $C_{16:0}$ into $C_{16:0}$ and $C_{18:0}$, respectively, and that this ability is regulated by the fatty acids available in their surroundings (Table 2).

Effect of the fatty acid composition in the medium on the fatty acid content of intraerythrocytic P. falciparum

We next used GC to examine the effects of the fatty acid composition of the surroundings on that of the parasites. In standard medium, the fatty acid species in the infected erythrocytes and the pooled human serum were similar (Table 1). The fatty acid composition in the infected erythrocytes is similar to that of parasites overall lipids described in the previous study (Hsiao *et al*. 1991). Likewise, in serum-free medium supplemented with reconstituted lipid-associated BSA containing $C_{16:0}$/ $C_{18:1,\ n\text{-}9}$, $C_{14:0}$/ $C_{18:0}$/$C_{18:2,\ n\text{-}6}$, or $C_{16:0}$/$C_{18:0}$/$C_{18:2,\ n\text{-}6}$, the fatty acid composition of the infected erythrocytes largely reflected the supplements (Fig. 6A–C and Table 3). These results confirm metabolic labelling results, namely, that intraerythrocytic *P. falciparum* can utilize a broad range of fatty acids from their surroundings for growth, suggesting that the majority of plasmodial factors involved in uptake/trafficking and metabolism of serum-derived fatty acids have broad specificity.

The results of GC analyses also support the idea that intraerythrocytic *P. falciparum* can carry out Δ9-desaturation. In serum-free media supplemented with reconstituted lipid-associated BSA containing $C_{14:0}$/$C_{18:0}$/$C_{18:2,\ n\text{-}6}$ or $C_{16:0}$/$C_{18:0}$/$C_{18:2,\ n\text{-}6}$, we detected a significant amount of $C_{18:1,\ n\text{-}9}$, which was not supplied in the medium (Fig. 6B, C and Table 3). GC analyses also indicated the presence of elongase activity in intraerythrocytic *P. falciparum*, although it was difficult to quantify due to a high background level of $C_{16:0}$ and $C_{18:0}$ in uninfected erythrocytes (Fig. 6A, B; data not shown).

DISCUSSION

Scavenging of fatty acids from serum is thought to be necessary for the growth of *P. falciparum* (Holz, 1977; Vial and Ancelin, 1998; Mitamura and Palacpac, 2003), and accumulating evidence suggests that these parasites possess unique metabolic pathways (Surolia and Surolia, 2001; Krishnegowda and Gowda, 2003). Therefore, determination of the human serum-derived fatty acid species essential for intraerythrocytic proliferation of *P. falciparum* and characterization of their metabolism is important in developing new strategy for controlling malaria. Here, we comprehensively analysed the effects of fatty acids present in human serum on the growth of *P. falciparum*. We found that particular combinations of three fatty acids support the continuous

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culture of *P. falciparum* in serum-free medium. Metabolic labelling and GC analyses revealed that the fatty acids essential for parasite growth were metabolized similarly in media supplemented with human serum or with lipid-rich BSA containing the fatty acids. These studies also showed that the fatty acid composition of the infected erythrocytes largely reflect that of the growth medium. These two independent biochemical analyses suggest that the parasite can desaturate and elongate serum-derived fatty acids to a limited extent. These results imply that intraerythrocytic *P. falciparum* utilizes serum-derived fatty acids with little modification to form membranes and the lipid body. Furthermore, the results suggest that, in the parasite, *de novo* fatty acid synthesis makes a very limited contribution to acyl groups for lipid metabolism. These features of fatty acid metabolism in *P. falciparum* are unique because cells usually control their fatty acid composition by coordinating *de novo* biosynthesis, scavenging, and modification (e.g., desaturation and elongation).

Among 84 combinations of saturated and unsaturated fatty acids present in human serum, only 11 ($C_{14:0}/C_{16:0}/C_{18:1,\ n\text{-}9}$, $C_{14:0}/C_{18:0}/C_{18:1,\ n\text{-}9}$, $C_{14:0}/C_{18:0}/C_{18:2,\ n\text{-}6}$, $C_{16:0}/C_{16:1,\ n\text{-}7}/C_{18:1,\ n\text{-}9}$, $C_{16:0}/C_{18:1,\ n\text{-}9}/C_{18:1,\ n\text{-}7}$, $C_{16:0}/C_{18:1,\ n\text{-}9}/C_{18:2,\ n\text{-}6}$, $C_{16:0}/C_{18:1,\ n\text{-}9}/C_{18:3,\ n\text{-}3}$, $C_{16:0}/C_{18:1,\ n\text{-}7}/C_{18:2,\ n\text{-}6}$, $C_{16:0}/C_{18:1,\ n\text{-}7}/C_{18:3,\ n\text{-}3}$, $C_{16:0}/C_{18:0}/C_{18:2,\ n\text{-}6}$, and $C_{18:0}/C_{18:1,\ n\text{-}9}/C_{18:2,\ n\text{-}6}$) could sustain long-term *in vitro* culture of *P. falciparum* in serum-free medium. There was not a single specific fatty acid species included in all of the combinations, but there was a trend for a combination of $C_{16:0}$ and either $C_{18:1,\ n\text{-}9}$ or $C_{18:1,\ n\text{-}7}$, and $C_{18:0}$. Because $C_{16:0}$, $C_{18:1,\ n\text{-}9}$, and $C_{18:0}$ can be generated by *de novo* biosynthesis in humans, it is interesting to speculate that *P. falciparum* evolved to adapt to human hosts in which the other fatty acids vary according to diet and health.

The *P. falciparum* genome contains candidate orthologues of a Δ-9 desaturases (gene ID in Plasmo DB (http://v5-0.plasmodb.org/plasmo-release5-0/home.jsp): PFE0555w) and elongases (gene IDs in PlasmoDB (http://v5-0.plasmodb.org/plasmo-release5-0/home.jsp): PFA0455c, PFF0290w, and PFI0980w). In agreement with this, our results suggest that intraerythrocytic *P. falciparum* can desaturate and elongate fatty acids taken up from their surroundings. Our results, however, do not agree with a previous report that the parasite has little or no ability to elongate or otherwise modify fatty acids scavenged from the external medium (Krishnegowda and Gowda, 2003). This discrepancy could be due to differences in the culture conditions.

Metabolic labelling and GC analyses also revealed that the parasite can produce $C_{16:1,\ n\text{-}7}$ and $C_{18:1,\ n\text{-}9}$ from $C_{16:0}$ and $C_{18:0}$, respectively. Because only one candidate gene for a putative *P. falciparum* desaturase could be found in its genome, it is likely that both $C_{16:1,\ n\text{-}7}$ and $C_{18:1,\ n\text{-}9}$ are produced by a single desaturase, although different levels may be generated due to substrate specificity of the plasmodial enzyme. Further, our results suggest that the capacity to desaturate $C_{18:0}$ into $C_{18:1,\ n\text{-}9}$ in the intraerythrocytic parasite is not affected by the culture conditions. From this, we infer that $C_{18:1,\ n\text{-}9}$ is always present in the parasites regardless of the environmental conditions and that it is therefore necessary for intraerythrocytic proliferation.

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