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ORIGINAL PAPER

Cyclopropane fatty acid synthase from *Oenococcus oeni*: expression in *Lactococcus lactis* subsp. *cremoris* and biochemical characterization

Thi Mai Huong To[1] · Cosette Grandvalet[1,2] · Hervé Alexandre[1] · Raphaëlle Tourdot-Maréchal[1]

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Abstract Bacterial cyclopropane fatty acid synthases (CFA synthases) catalyze the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to the double bond of a lipid chain, thereby forming a cyclopropane ring. CFAs contribute to resistance to acidity, dryness, and osmotic imbalance in many bacteria. This work describes the first biochemical characterization of a lactic acid bacterium CFA synthase. We have overexpressed *Oenococcus oeni* CFA synthase in *E. coli* in order to purify the enzyme. The optimum cyclopropanation activity was obtained at pH 5.6 and 35.8 °C. The high K_m (AdoMet) value obtained (2.26 mM) demonstrates the low affinity of *O. oeni* enzyme toward the *L. lactis* subsp. *cremoris* unsaturated phospholipids. These results explain the partial complementation of the *L. lactis* subsp. *cremoris cfa* mutant by the *O. oeni cfa* gene and suggest a probable substrate specificity of the *O. oeni* enzyme. The current study reveals an essential hypothesis about the specificity of *O. oeni* CFA synthase which could play a key function in the acid tolerance mechanisms of this enological bacterium.

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✉ Cosette Grandvalet
cosette.grandvalet@u-bourgogne.fr

[1] Unité Mixte de Recherche Procédés Alimentaires et Microbiologiques (UMR A 02.102 AgroSup Dijon-Université de Bourgogne), Equipe Vin Aliment Microbiologie et Stress, Institut Universitaire de la Vigne et du Vin "Jules Guyot", Rue Claude Ladrey, Campus Universitaire Montmuzard, BP 27877, 21078 Dijon, France

[2] Institut National Supérieur des Sciences Agronomiques de l'Alimentation et de l'Environnement, AgroSup Dijon, Site Epicure, 1 Esplanade Erasme, Campus Universitaire, 21000 Dijon, France

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Introduction

Lactic acid bacteria (LAB) are Gram-positive, low GC bacteria that produce lactic acid as their primary catabolic end product (Carr et al. 2002). They play an essential role in the preservation of agricultural resources and in the improvement of the nutritional and organoleptic properties of human foods. Moreover, at present these organisms are increasingly used to obtain health-promoting probiotics and enzymes, as well as being used as metabolite factories and vaccine delivery vehicles (Escamilla-Hurtado et al. 1996; Tarahomjoo 2012). Regardless of their natural habitat, bacteria are exposed to constant fluctuations in their growth conditions. Thus, during food processing or ingestion and storage, LAB experience adverse environmental conditions such as oxidative, heat, cold, acid, and osmotic stresses. To respond to these different stress conditions, bacteria must coordinate their gene and protein networks to counteract the perturbations caused by specific environmental stressors (van de Guchte et al. 2002; Spano and Massa 2006; Spano et al. 2007).

Several LAB species are important industrial microorganisms, which are employed as starters in most fermented foods. In particular, *Oenococcus oeni* is of fundamental importance in enology because of its ability to perform malolactic fermentation in wine (Liu 2002). *O. oeni* tolerates the presence of ethanol associated with a high acidity in wine; thus, it is an ideal candidate to improve our knowledge of the stress response in LAB. Indeed, many studies have highlighted the molecular and physiological bases of the stress response in *O. oeni*. Several mechanisms allow

O. oeni to withstand stress conditions, including the activation of malolactic fermentation to generate a proton-motive force and maintain intracellular pH (Salema et al. 1996a, b; Tourdot-Marechal et al. 1999), activation of membrane-bound H^+-ATPases (Carrete et al. 2002; Fortier et al. 2003), stress protein synthesis (Guzzo et al. 2000; Bourdineaud et al. 2003; Beltramo et al. 2004; Bourdineaud et al. 2004; Grandvalet et al. 2005; Beltramo et al. 2006; Bourdineaud 2006; Cecconi et al. 2009; Maitre et al. 2014), and membrane fluidity including changes in lipid composition (Tourdot-Marechal et al. 2000; Da Silveira et al. 2003; Grandvalet et al. 2008).

The modification in fatty acid composition of the cytoplasmic membrane represents one of the most important adaptive microbial responses to stress, where the bacterial response includes modifications in the degree of saturation (Hazel and Williams 1990), carbon chain length, branching position (Kaneda 1977), cis/trans isomerization (Heipieper et al. 1992; Loffeld and Keweloh 1996), and the conversion of unsaturated fatty acids (UFAs) into cyclopropane fatty acids (CFAs) (Broadbent and Lin 1999; Teixeira et al. 2002; Grandvalet et al. 2008; Pini et al. 2009). These CFAs are formed in situ via post-synthetic modification, which involves the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to the double bond of a UFA in a phospholipid molecule (Grogan and Cronan 1997). CFA synthase catalyzes this reaction, and it is encoded by the *cfa* gene (Taylor et al. 1981; Grogan and Cronan 1984, 1986). Early studies of CFA-producing bacteria have shown that these modified fatty acids first appear during the late exponential or early stationary phases of growth (Wang and Cronan 1994). This conversion plays a major role in the adaptation of bacteria in response to a drastic perturbation of the environment. In particular, it has been shown that the CFA content increases in response to a low pH in various bacteria (Brown et al. 1997; Alvarez-Ordonez et al. 2008; Grandvalet et al. 2008; Montanari et al. 2010), as well as in response to high temperature (McGarrity and Armstrong 1981; Dubois-Brissonnet et al. 2001), osmotic (Guillot et al. 2000), and ethanol stresses (Teixeira et al. 2002; Grandvalet et al. 2008; To et al. 2011). Physiological studies based on the construction of *cfa*-deficient cells in *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Brucella abortus* have demonstrated that the formation of CFAs in the membrane is a major factor that protects bacteria from low pH (Grogan and Cronan 1986; Kim et al. 2005; Palacios-Chaves et al. 2012). Moreover, an increased CFA content in the membrane of *Clostridium acetobutylicum* enhances resistance to acid and butanol (Zhao et al. 2003).

Oleic acid (C18:1n-9), which is the UFA precursor of the CFA lactobacillic acid (cycC19:0n-7), is often supplemented in the medium under the form of Tween 80 (polyethylene glycol sorbitan monooleate), and it is known to stimulate LAB growth as well as to protect cells toward stress (Williams and Fieger 1946; Goldberg and Eschar 1977; Guerrini et al. 2002; Corcoran et al. 2007). The CFAs lactobacillic acid and dihydrosterculic acid (cycC19:0n-9), which are derived from oleic acid and vaccenic acid (C18:1n-7), respectively, are regarded as key fatty acids for stress tolerance in LAB (Goldberg and Eschar 1977; Johnsson et al. 1995; Broadbent and Lin 1999; Montanari et al. 2010). Thus, Tween 80-supplemented cultures of *O. oeni* exhibit enhanced bacterial survival in wine which could be explained by a higher oleic acid and dihydrosterculic acid content and a lower level in palmitic (C16:0), vaccenic, and lactobacillic acids (Guerrini et al. 2002). Modifications of the CFA content have been demonstrated in *O. oeni* cells grown during sublethal stress conditions (Teixeira et al. 2002; Grandvalet et al. 2008). A previous characterization of the *O. oeni cfa* gene showed that the abundance of *cfa* transcripts increased when cells were harvested during the stationary phase and when cells were grown in the presence of ethanol or at low pH, thereby suggesting that transcriptional regulation of the *cfa* gene occurs under different stress conditions (Grandvalet et al. 2008). The function of the *O. oeni cfa* gene has been analyzed by *cfa*-deficient *E. coli* strain complementation where partial complementation was observed. In fact, the percentage conversion of UFAs into CFA by the complemented strain (18 %) was much lower than that by the parental strain (90 %). This may be attributed to some issues due to the expression of a heterologous gene in Gram-negative *E. coli* cells (protein misfolding) and/or to the intracellular pH of the host cell (7.2–7.8 for *E. coli* vs. 5.8–6.3 for *O. oeni*) unfavorable to the protein activity (Slonczewski et al. 1981; Salema et al. 1994; Augagneur et al. 2007). Alternatively, the substrate specificity of the *O. oeni* enzyme may also be involved.

Few bacterial CFA synthases have been well characterized, and the homologous enzyme from *E. coli* is the best known regarding synthesis and reaction mechanisms (Wang et al. 1992; Courtois et al. 2004; Courtois and Ploux 2005; Guianvarc'h et al. 2008; Guangqi et al. 2013). The *E. coli* CFA synthase is a peptide that comprises 382 amino acid residues, which is active in vitro as a homodimer (Wang et al. 1992). Similar to many methyltransferases, this enzyme has three motifs that comprise 8–10 amino acids, which are involved in the binding of AdoMet and *S*-adenosyl homocysteine (Kagan and Clarke 1994). Three cysteine residues are highly conserved, although mutations in these residues do not affect the enzyme activity significantly (Grogan and Cronan 1997). A previous biochemical analysis demonstrated the relative instability of the CFA synthase purified from *E. coli*, where its half-life was 16 h at 4 °C and only a few minutes at 37 °C (Grogan and Cronan 1984). This enzyme can be made relatively stable in vitro by adding lipid molecules to purified fractions.

The addition of bovine serum albumin (BSA) or dithiothreitol (DTT) also stabilizes the enzyme in vitro (Courtois et al. 2004). The CFA synthase from *E. coli* has a K_m with AdoMet of 80 μM and a k_{cat} of 4 min^{-1} (Guianvarc'h et al. 2006).

Therefore, to understand the partial complementation of a *cfa*-deficient *E. coli* mutant previously reported (Da Silveira et al. 2003), we studied the activity of the CFA synthase from *O. oeni* in a Gram-positive bacterium. We complemented the *cfa*-deficient *L. lactis* subsp. *cremoris* MG1363 strain, which was constructed previously by To et al. (2011). Moreover, the enzyme was overproduced in *E. coli* strain BL21(DE3) using the pET expression system for purification, and the biochemical characteristics of the *O. oeni* CFA synthase were determined in vitro. *In vitro* enzymatic activity measurements were taken by monitoring product appearance using gas chromatography (GC). The in vitro characterization of a CFA synthase from a lactic acid bacterium was described for the first time.

Materials and methods

Strains, plasmids, and media

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were cultured in liquid Luria–Bertani (LB) medium (Bertani 1951) at 37 °C on a rotary shaker at 150 rpm. *L. lactis* subsp. *cremoris* MG1363 strains were cultured at 30 °C in modified M17 medium, which was adjusted to pH 7 (M17/7) or pH 5 (M17/5), and 6 % ethanol (vol/vol) was added (To et al. 2011). Kanamycin, spectinomycin, and ampicillin were used at concentrations of 50, 50, and 100 μg/ml, respectively, with *E. coli*. For the *L. lactis* subsp. *cremoris* MG1363 strains, 1 mg/ml of spectinomycin was added to the medium as necessary.

Complementation of the *L. lactis* subsp. *cremoris* *cfa*-deficient strain

Standard procedures such as purification, ligation, restriction analysis, and gel electrophoresis were performed as described by Sambrook and Pollack (1974). *L. lactis* subsp. *cremoris* chromosomal DNA was prepared as described previously (Leenhouts et al. 1989). The PCR products and restriction products were purified using a Gene Elute PCR kit (Sigma, St Quentin Fallavier, France). Conventional electroporation was used for *E. coli*. *L. lactis* subsp. *cremoris* was transformed by electroporation, as described by Dower (1990).

The entire *cfa* gene of *O. oeni* ATCC BAA 1163 (including the promoter region) was amplified by PCR with Platinum® Taq DNA Polymerase High Fidelity (Life Technologies, Saint-Aubin, France) using the primers cfa4MT (5′CCCGGATCCTTCATTT-

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or marker: characteristics and uses[a]	Sources or references
Escherichia coli strains		
BL21 (DE3)	Host strain for expression vector pET28a(+)	Novagen-Merck KGaA
BL21/pET28a	BL21(DE3) harboring pET28a(+), Km^R	This study
BL21/pETcfa1-2	BL21(DE3) harboring pETcfa1-2, Km^R	This study
K12 ER2738	General cloning strain	New England Biolabs
Lactococcus lactis subsp. *cremoris*		
MG1363	Plasmid-free derivative of *S. lactis* NCDO 712 cured of ΦT712 prophage	Wang et al. (2014)
MG1363/pDL278	MG1363 harboring pDL278, sp^R	This study
MG1363 Δ*cfa*	MG1363 *cfa*-deficient strain obtained by deleting an internal part of the *cfa* gene	Heipieper et al. (1992)
MG1363 Δ*cfa*/pDL278	MG1363 Δ*cfa* harboring pDL278, sp^R	This study
MG1363 Δ*cfa*/pGC20	MG1363 Δ*cfa* harboring pGC20, sp^R	This study
Plasmids		
pUC18	Cloning vector, ap^R	Our laboratory
pET28a(+)	Expression vector containing the T7 promoter, km^R	Novagen-Merck KGaA
pETcfa1-2	pET28a(+) containing *O. oeni cfa* gene under control of the T7 promoter and fused to 6-His codons, km^R	This study
pDL278	*E. coli–L. lactis* shuttle vector (6.6 kbp), sp^R	Oku et al. (2004)
pGC20	pDL278 derivative vector used to express the *O. oeni cfa* gene under an endogenous promoter, sp^R	This study

[a] km^R, sp^R, and ap^R indicate resistance to kanamycin, spectinomycin, and ampicillin, respectively

TAATTAAAAAAAATAAAATTTT3′) and cfa5MT (5′GGGGAATTCTCTTGTTTCCTTTTTTTTA-GAAAATT3′), which include *Bam*HI and *Eco*RI restriction sites, respectively (the restriction sites are underlined). The PCR product was first subcloned into pUC18, and then the 1140-bp fragment isolated by *Bam*HI and *Eco*RI (Life Technologies, Saint-Aubin, France) digestion was ligated into the corresponding sites of pDL278 to yield pGC20. After verifying the sequence of the *cfa* gene (Beckman Coulter Genomics, Essex, UK), the pGC20 recombinant vector was introduced into the *L. lactis* subsp. *cremoris cfa*-deficient strain as the heterologous expression system.

RNA extraction and analysis

L. lactis subsp. *cremoris* cells in the exponential growth phase were centrifuged and washed with 0.1 % dimethyl-pyrocarbonate-treated water before autoclaving to inactivate RNases. The cell pellets were resuspended in 1 ml TRI Reagent (Sigma-Aldrich, St Quentin Fallavier, France), and the cells were broken using a Precellys Disruptor (Bertin Technologies, Montigny-le-Bretonneux, France), with two cycles for 30 s at 6000 rotations per minute in the presence of 200 mg of glass beads (diameter 70–100 µm, Scientific Industries Inc., NY, USA). Next, the total RNA was extracted with phenol–chloroform, precipitated with isopropanol, and resuspended in RNase-free water. Reverse transcription-PCR was performed using the reverse transcriptase iScript™ (Bio-Rad, Marnes-la-Coquette, France), as recommended by the supplier. Quantitative real-time PCR (qRT-PCR) was performed using IQ™ SYBR® Green Supermix (Bio-Rad, Marnes-la-Coquette, France) on a Bio-Rad iCycler with the SYBR Green system. Thermal cycling conditions included the following steps: initial denaturation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 30 s. Fluorescence measurements were recorded during each annealing step. The primers used for amplification were 1163cfaF (5′GCTGGCAGCTTTTTGACTGATA3′) and 1163cfa (5′TATCGTAGTGACTTTGAACG3′) for the *O. oeni cfa* gene; MG1363cfaF (5′GGACAGTTTTTTAACTGACCAATC3′) and MG1363cfaR (5′CGAGATTGTTTTTGAGAATGATT3′) for the *L. lactis* subsp. *cremoris cfa* gene. The specificity of each primer pair was determined with a melting curve. The threshold cycle value (C_T) was determined for each measurement. The mRNA levels were quantified using the threshold method ($\Delta\Delta C_T$), where the quantity of target cDNA was adjusted to a reference based on the amount of cDNA, as previously described (To et al. 2011). The results were normalized using the *L. lactis* subsp. *cremoris butB* gene, which encodes 2,3-butanediol dehydrogenase, as reference gene. Primers RTF-butB (5′GGATATCATGCTGTTGAACGTGC3′) and RTR-butB (5′TGAGCTTTGGCAACTGCAGCT3′) target an internal region of the *butB* coding sequence. Previously, we verified that *butB* gene transcription level was independent of the growth conditions tested in this study (To et al. 2011).

Cloning of the *O. oeni cfa* gene into the pET-28a(+) vector

The *O. oeni cfa* gene was cloned and expressed in *E. coli* in order to raise antibodies production and biochemical characterization. To facilitate the purification of CFA synthase, a His-tag was added to the N-terminal using the pET expression system. The entire coding sequence of the *O. oeni cfa* gene was amplified by PCR using the primers cfa1M (GGGGCCCCCATATGTTAGAGAAAACCATTTATCG) and cfa2 (CCCGAATTCTTAACTTAATTCATGTTCATAAATAT), which contain *Nde*I and *Eco*RI restriction sites, respectively (the restriction sites are underlined). The PCR product was subcloned into pUC18, before the 1180-pb fragment isolated by *Nde*I and *Eco*RI (Life Technologies, Saint-Aubin, France) digestion was ligated into the corresponding sites of pET-28a(+) to yield the pETcfa1-2 plasmid. The sequence of *O. oeni cfa* gene was verified by DNA sequencing (Beckman Coulter Genomics, Essex, UK).

CFA synthase overproduction and purification

The pETcfa1-2 plasmid was transformed into *E. coli* BL21(DE3) (Novagen-Merck KGaA, Darmstadt, Germany). The recombinant *E. coli* was grown aerobically at 37 °C into 1 liter of LB broth supplemented with 50 µg/ml kanamycin for 2 h (OD_{600} = 0.7). Expression of the *cfa* gene was induced by adding 50 µM isopropyl-β-D-1-thiogalactoside (IPTG), and the cells were incubated for 19 h at 16 °C on a rotary shaker at 150 rpm. The cells were harvested by centrifugation (6000×*g*, 15 min at 4 °C), washed with lysis buffer (KH_2PO_4/K_2HPO_4 50 mM, pH 7.4; NaCl, 300 mM), and resuspended in the same buffer (20 UDO_{600nm}/ml). The cells were then disrupted using a One Shot Cell Disrupter (Z plus Series Cell, Constant Systems Ltd, Northants, UK) with one cycle at 1.4 kbar. Cell debris were removed by centrifugation (7000×*g*, 30 min at 4 °C), and the supernatant was applied directly to a 2-ml nickel-nitrilotriacetate column (Ni-NTA agarose, Qiagen, Courtaboeuf, France), which had been pre-equilibrated with 10 column volumes of wash buffer (NaH_2PO_4/Na_2HPO_4, 100 mM; Tris–HCl, 10 mM; pH 8, NaCl 300 mM) at a flow rate of 1 ml/min using a peristaltic pump. The column was washed with 10 column volumes of wash buffer, and the recombinant protein was eluted with four column volumes gradient of 10–400 mM imidazole in wash buffer (NaH_2PO_4/Na_2HPO_4 100 mM, pH 8; Tris–HCl, 10 mM; NaCl, 300 mM). The flow rate was 0.5 ml/min for

loading, washing, and eluting. The eluate was collected in 2-ml fractions, and 10-µL aliquots of each fraction were subjected to SDS-PAGE (12.5 % polyacrylamide) to verify the purity and apparent molecular mass of the eluted protein. The protein content was quantified using the Bradford method (Bradford 1976). Fractions that produced a single intense band on the gel (fractions eluted by the imidazole gradient from 100 to 200 mM) were pooled and dialyzed to remove imidazole and salts using an Amicon Ultra filter 15 kDa (Merck Millipore, Darmstadt, Germany). The purification yield obtained was around 4 µg/mg wet cell pellet. After adding glycerol (50 %, vol/vol) and BSA (80 µM), the enzyme was stored at −20 °C until use.

Western blotting experiments

L. lactis subsp. *cremoris* stationary phase cells were harvested by centrifugation at 6300×*g* for 10 min, washed with lysis buffer (10 mM Tris–HCl, pH 8; 300 mM NaCl), and resuspended in 1/100th of the volume of original culture volume using lysis buffer. The cells were disrupted using a Precellys Disruptor, with four cycles for 30 s at 6500 rotations per minute in the presence of 200 mg of glass beads (diameter 70–100 µm; Scientific Industries Inc., NY, USA). The lysate was centrifuged at 17,900×*g* for 20 min at 4 °C, and the protein concentration of the supernatant (crude extract) was determined using the Bradford method (Bio-Rad reagent, Richmond, CA, USA) with BSA as the standard (Bradford 1976). SDS-PAGE (12.5 % polyacrylamide) was performed according to Laemmli's method (1970). The proteins were transferred to an Amersham Hybond-ECL membrane (GE Healthcare Life Sciences, Velizy-Villacoublay, France) using a Trans-Blot SD semi-dry Transfer Cell (Bio-Rad, Marnes-la-Coquette, France) and the following buffer: 25 mM Tris; 192 mM glycine; and 20 % methanol. *O. oeni* CFA synthase protein was detected using serum from a rabbit (AGRO-BIO, La Ferté Saint-Aubin, France) immunized with the *O. oeni* CFA synthase–His-tag purified protein and the Amersham ECL detection system (GE Healthcare Life Sciences, Velizy-Villacoublay, France).

Preparation of phospholipids

L. lactis subsp. *cremoris* cells (80 UDO_{600nm}) collected in the stationary growth phase after growth in M17 media were washed and resuspended in NaCl 0.9 %. Cell suspension was used to extract cellular lipids with chloroform/methanol (1:2) according to Bligh and Dyer (1959).

For the in vitro enzymatic assay, lipids were extracted from stationary phase *L. lactis* subsp. *cremoris* Δ*cfa* cells grown in M17/7 medium. A film of extracted lipids was obtained by evaporation of chloroform with a rotavapor at 65 °C (Rotary Evaporator RE100 Bibby, Odil) under nitrogen-reduced pressure for 45 min followed by rehydration with pre-warmed buffer 20 mM potassium phosphate buffer (pH 7.4) at 65 °C. The liposome suspension was gently mixed and sonicated 2 × 30 s (ELMA D-78224, VWR) prior to its use as the substrate in the CFA synthase assay.

Esterification of fatty acids and gas chromatography (GC) analysis

The fatty acid of total lipids and phospholipids was directly transesterified with methanol/H_2SO_4 (95/5, v/v) at 80 °C for 2 h. The fatty acid methyl esters (FAMEs) were extracted with hexane and analyzed by GC using a Chrompack CP 9002 chromatograph equipped with a Varian FactorFour™ capillary column (30 m × 0.32 mm) (Middelburg, the Netherlands). The detector and injector temperatures were 230 and 220 °C, respectively. Initially, the oven temperature was 160 °C for 10 min, before increasing to 180 °C at 2 °C/min. The FAMEs were identified by comparing retention times with those of authentic standards (Nu Check Prep., Elysian, MN, USA). The percentage of each component was calculated based on the peak area obtained (peak area of component/total area of all peaks). The absolute quantification of each component was achieved by using a defined amount of pentadecanoic acid (C15:0) as an internal standard.

CFA synthase assay

The CFA synthase GC-based assay was adapted from the radiometric method described previously by Taylor et al. (Taylor et al. 1981) for *E. coli* enzyme characterization, with the following modifications. The assay consisted in 1 mg/ml phospholipids extracted from *L. lactis* subsp. *cremoris* Δ*cfa* cells, 0.5 mg/ml bovine serum albumin (BSA), 10 % (vol/vol) glycerol, 2 mM dithiothreitol (DTT), 0.75 mM AdoMet, 20 mM potassium phosphate buffer, pH 5.6, in a final volume of l00 µl. After pre-incubation at 35 °C for 5 min, the reaction was initiated by adding the CFA synthase (20 µg/ml) and incubated at 35 °C for a maximum of 45 min. The reaction was stopped by adding 1 ml 10 % (vol/vol) trichloroacetic acid. The total lipids were extracted and transesterified directly after adding 200 µg of pentadecanoic acid (C15:0, Sigma-Aldrich, St Quentin Fallavier, France) as an internal standard in each assay. The methyl esters obtained were then quantified by GC. The activity measured under these conditions was linear with time over a period of 1 h and linear with enzyme concentration up to 100 µg/ml of protein (data not shown).

Experimental design and statistical analyses

The experiments were designed using NEMRODW (LPRAI, University of Marseille, France) to determine the maximal rate of CFA conversion in the enzymatic reaction at different pH values (5–6) and temperatures (30–37 °C). We employed a square domain and a second-degree polynomial model in this study. Data are represented as mean ± SD for replicate experiments. To determine whether two sets of data were significantly different, paired Student's *t* tests were performed using the T.TEST function in Excel. A value of $p < 0.05$ was considered statistically significant.

Results

The *O. oeni cfa* gene partially complemented the *L. lactis* subsp. *cremoris* MG1363 *cfa*-deficient strain

The *L. lactis* subsp. *cremoris* MG1363 *cfa*-deficient strain constructed previously by To et al. (2011) was complemented with the pGC20 plasmid derived from the low-copy number vector pDL278 (Chen and LeBlanc 1992), which carried the entire *O. oeni cfa* gene under the control of its own promoter. Analysis of total phospholipids revealed that in the presence of pGC20, we detected cycC19:0n-7 and we observed a slight decrease in the level of its precursor C18:1n-7 in the *L. lactis* subsp. *cremoris* complemented strain, MG1363 Δ*cfa* pGC20 compared to *cfa*-deficient strain (Table 2).

In optimal growth conditions (M17/7), we observed that only 3.7 % of the CFA was converted by the complemented strain, whereas 36 % was converted by the parental strain MG1363. Although there were multiple copies of the vector, complementation of the *L. lactis* subsp. *cremoris* MG1363 strain did not restore the amount of CFA converted compared with that obtained with the parental strain. Based on transcriptional fusion constructions, Budin-Verneuil et al. (2005) reported the induction of the *L. lactis* subsp. *cremoris* MG1363 *cfa* gene under moderate acidity. Similarly, we observed almost 90 % CFA conversion when the cells were cultured in acidic conditions. In agreement with Budin-Verneuil et al. (2005), our results confirm that there was a significant increase in C18:1n-7 cyclization by the parental strain in acidic conditions (M17/5). We also observed an increase in the CFA conversion rate by the complemented strain. However, the level of CFA conversion in the complemented strain (15 %) was still lower than that in the parental strain. In acidic conditions, the CFA conversion level increased by four times in the complemented strain compared with that in the optimal conditions, whereas the CFA conversion level increased by 2.5 times in the parental strain. Previously, we reported that the addition of ethanol (6 %) to the medium increased the CFA level in *L. lactis* subsp. *cremoris* MG1363 by two times (To et al. 2011). The CFA level increased three times in the complemented strain. These results confirm that the *O. oeni cfa* gene complemented the *L. lactis* subsp. *cremoris cfa*-deficient strain in terms of its fatty acid composition, although the CFA level was partial. Moreover, in stressful conditions (growth at pH 5 or with 6 % ethanol) we observed an increase in the CFA conversion ratio in the complemented strain, which suggests an induction of the *O. oeni* cfa gene expression in *L. lactis* subsp. *cremoris* complemented strain.

Expression of the *O. oeni cfa* gene in the complemented *L. lactis* subsp. *cremoris* strain compared with the endogenous *cfa* gene in *L. lactis* subsp. *cremoris*

To understand the partial complementation of the *cfa*-deficient mutant, qRT-PCR experiments were performed in order to compare the expression level of the *cfa* gene in *O. oeni* with that of the *cfa* gene in *L. lactis* subsp. *cremoris* (Table 3). In optimal growth conditions (pH 7), the *O. oeni cfa* expression was increased sixfold in the complemented strain compared with the *cfa* transcript level in the parental strain. In acidic growth conditions (pH 5), the *cfa* transcript level was increased by over 36 times in the complemented strain, compared with an increase of only 3.5 times in the parental strain. However, these results did not explain the partial complementation of the *cfa*-deficient mutant because the *O. oeni cfa* transcript levels were six times and 71 times higher than that of *L. lactis* subsp. *cremoris cfa* in the optimal growth condition and acidic conditions, respectively. However, the incomplete cyclization of UFAs in the complemented *cfa*-deficient strain may be attributable to a low translation level or to the suboptimal enzymatic activity of *O. oeni* CFA synthase in the heterologous host. The production of heterologous CFA synthase in *L. lactis* subsp. *cremoris* strains was estimated by immunodetection using specific antibodies directed against *O. oeni* CFA synthase. As shown in Fig. 1 (lane 2), an overproduced protein was detected in the complemented strain. No protein corresponding to the same molecular size was displayed in the wild-type and the *cfa*-deficient strains (Fig. 1, lanes 1 and 3), indicating that this band represents the *O. oeni* CFA synthase. The anti-CFA synthase antibodies detected another minor band for the *L. lactis* subsp. *cremoris* wild-type strain, which was not detected in the *cfa*-deficient strain (Fig. 1, lane 1). This band, corresponding to a slightly smaller size protein, was probably attributable to a cross-reaction with the CFA synthase from *L. lactis* subsp. *cremoris*. Thus, an enzymatic approach

Table 2 Fatty acid compositions (molar percentage) of *Lactococcus lactis* subsp. *cremoris* MG1363 strains grown at 30 °C on M17/7 or in sublethal stress conditions

Fatty acids/strains	Fatty acids compositions (molar percentages)[a]								
	M17/7			M17/5			M17/7 + 6 % ethanol		
	MG1363 pDL278	MG1363Δcfa pDL278	MG1363Δcfa pGC20	MG1363 pDL278	MG1363Δcfa pDL278	MG1363Δcfa pGC20	MG1363 pDL278	MG1363Δcfa pDL278	MG1363Δcfa pGC20
C15:0 iso	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.84 ± 8.44	6.46 ± 3.19	6.82 ± 4.01
C14:0	9.21 ± 0.35	9.91 ± 0.75	9.64 ± 0.34	7.46 ± 1.09	6.86 ± 0.97	6.55 ± 0.27	10.45 ± 2.13	8.92 ± 0.73	9.64 ± 0.56
C15:0 anteiso	5.15 ± 1.43	3.65 ± 0.28	3.81 ± 0.28	7.22 ± 0.50	5.71 ± 0.16	5.87 ± 0.67	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C16:0	22.63 ± 2.08	24.04 ± 1.32	23.50 ± 1.31	29.53 ± 1.47	28.34 ± 0.95	28.06 ± 0.39	23.12 ± 3.27	26.20 ± 2.93	23.60 ± 1.79
C16:1n-7	2.42 ± 0.53	2.16 ± 0.18	2.31 ± 0.25	2.13 ± 1.58	2.60 ± 1.84	2.51 ± 1.70	17.36 ± 5.76	16.45 ± 5.15	13.29 ± 0.83
A4[b]	7.59 ± 0.60	8.33 ± 0.46	8.53 ± 0.87	1.76 ± 1.44	16.23 ± 1.57	13.22 ± 0.15	0.40 ± 0.41	2.06 ± 0.81	2.45 ± 0.35
C18:0	1.33 ± 0.51	1.63 ± 0.79	1.95 ± 0.88	3.44 ± 0.71	3.93 ± 0.64	4.28 ± 0.08	6.31 ± 1.62	8.14 ± 3.29	5.74 ± 0.63
C18:1n-7	32.29 ± 5.67	50.28 ± 1.69	47.94 ± 2.42	3.60 ± 0.95	36.32 ± 1.91	31.53 ± 1.43	9.93 ± 1.72	31.77 ± 7.47	34.40 ± 1.94
A5[b]	1.23 ± 0.71	0.00 ± 0.00	0.49 ± 0.44	13.74 ± 0.67	0.00 ± 0.00	2.40 ± 0.44	0.27 ± 0.47	0.00 ± 0.00	0.00 ± 0.00
cycC19:0n-7	18.15 ± 3.23	0.00 ± 0.00	1.83 ± 0.84	31.11 ± 0.43	0.00 ± 0.00	5.57 ± 1.53	25.32 ± 2.41	0.00 ± 0.00	4.06 ± 0.56
UFA/SFA ratio[c]	0.68	1.47	1.37	0.08	1.03	0.77	0.44	1.18	1.19
CFA conversion (%)[d]	35.99	0.00	3.67	89.62	0.00	15.00	71.83	0.00	10.56

UFA unsaturated fatty acids, *SFA* saturated fatty acids

[a] Samples were used during the stationary growth phase. Each value represents the mean of three separate analyses. The summed data represent the mean ± SD of triplicate independent experiments. C14:0 myristic acid; C16:0: palmitic acid; C16:1n-7: palmitoleic acid; C18:0: stearic acid; C18:1n-7: *cis*-vaccenic acid; cycC19:0n-7: lactobacillic acid

[b] No fatty acids identified

[c] UFA/SFA ratio = Σ(C16:1 + C18:1n-7)/Σ(C14:0 + C16:0 + C18:0 + cycC19:0n-7) (cycC19:0n-7 is considered to be a saturated fatty acid)

[d] Cyclopropane fatty acids (CFA) conversion = 100 × Σ(cycC19:0n-7)/Σ(cycC19:0n-7 + C18:1n-7)

Table 3 Determination of transcription levels of *cfa* genes by qRT-PCR

Gene	Strain	Growth in optimal conditions (pH 7)	Growth in acidic conditions (pH 5)
L. lactis cfa	MG1363/pDL278	1.61 ± 1.03	5.66 ± 0.73
O. oeni cfa	MG1363Δ*cfa*/pGC20	10.94 ± 2.06	403.15 ± 15.13

Total RNA was extracted from the stationary phase *L. lactis* subsp. *cremoris* MG1363 cells growing in M17/7 or M17/5 medium at 30 °C. Quantitative real-time PCR experiments were performed on *cfa* genes expressed in parental (MG1363/pDL278) and complemented (MG1363Δ*cfa*/pGC20) strains. The relative transcription levels were calculated using the comparative critical threshold ($\Delta\Delta C_T$) method, and they were normalized against that of the *butB* gene as an internal control. The data represent the mean ± SD of triplicate independent experiments

was employed to further study the in vitro CFA synthase activity of *O. oeni*.

Overproduction and purification of the CFA synthase

As described by Courtois et al. (2004) in *E. coli*, the *O. oeni* CFA synthase was overproduced as an N-terminal His_6-tagged recombinant protein using the plasmid pET28a(+). Overexpression in *E. coli* BL21(DE3), which harbored the recombinant plasmid pETcfa1-2, was optimized in the following conditions: induction at $OD_{600nm} = 0.7$, with 50 mM IPTG; and overnight incubation at 16 °C. The enzyme was purified by immobilized metal affinity chromatography using a Ni-NTA agarose column. As shown in Fig. 2, the *O. oeni* protein product had the expected molecular mass of ca 45 kDa.

Biochemical characterization of the recombinant CFA synthase

The conditions described previously (Guianvarc'h et al. 2008) were adapted to the CFA synthase GC assay (see "Materials and methods"). To determine the effects of pH and temperature on the activity of CFA synthase, the experimental design of the complete model was generated using NEMRODW. The constraints on the two parameters were predetermined experimentally, i.e., the temperature ranged from 30 to 37 °C and the pH ranged between 5.0 and 6.0. The second-degree polynomial model was calculated. We generated a response surface that displayed the variation in the two factors. The response surface and the corresponding projection with iso-response curves are shown in Fig. 3. The numbers along the curves indicate the predicted response, and the circle delimits the experimental domain where the prediction is reliable. Analyzing these iso-response surfaces showed that a pH value of 5.6 and a temperature of 35.8 °C would obtain the highest CFA conversion rate.

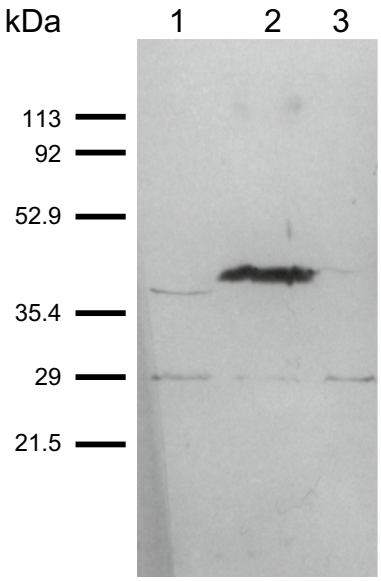

Fig. 1 Western blot analysis of *Lactococcus lactis* subsp. *cremoris* Δ*cfa* complemented by *Oenococcus oeni cfa* gene. Crude extracts from the *L. lactis* subsp. *cremoris* MG1363 strain harboring pDL278 (*lane 1*), *L. lactis* subsp. *cremoris* Δ*cfa* strain complemented by pGC20 (*lane 2*), and *L. lactis* subsp. *cremoris* Δ*cfa* strain harboring pDL278 (*lane 3*) were separated on a 12.5 % SDS-polyacrylamide gel. The separated proteins were transferred onto a Hybond membrane, and the immunoblot was probed with antibodies raised against *O. oeni* CFA synthase. Equal amounts of protein (20 μg) were applied to each lane. The molecular mass markers are indicated on the *left*

O. oeni CFA synthase exhibited saturation kinetics with AdoMet in the presence of a saturating concentration of phospholipids (1 mg/ml), where $K_m = 2.26$ mM for AdoMet, $V_{max} = 64.5$ μM/min, and $k_{cat} = 6000$/min. Compared with the values reported previously for *E. coli* CFA synthase (Taylor et al. 1981; Courtois et al. 2004; Iwig et al. 2004; Guianvarc'h et al. 2006), the *O. oeni* enzyme exhibited a lower cyclization activity with the unsaturated phospholipids from *L. lactis* subsp. *cremoris* in the test conditions. These results explain the partial cyclization

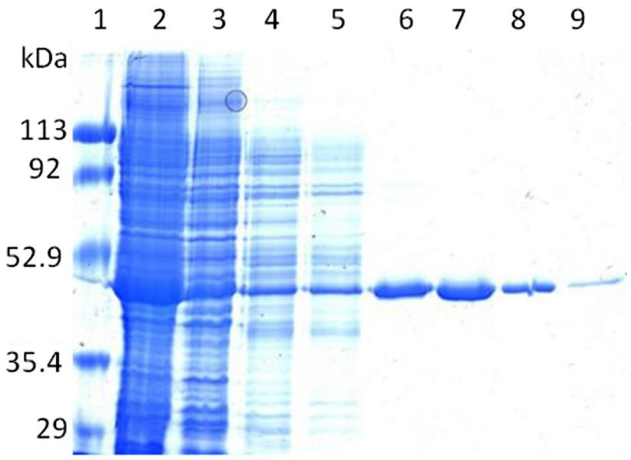

Fig. 2 Overproduction and purification of *Oenococcus oeni* cyclopropane fatty acid synthase. SDS-PAGE analysis of crude extract from *E. coli* BL21 λ DE3 carrying pETcfa1-2 (*lane 2*), the protein fraction not retained on Ni-NTA agarose (*lane 3*), and the fractions eluted with purification buffer containing 10 (*lane 4*), 20 (*lane 5*), 50 (*lane 6*), 100 (*lane 7*), 200 (*lane 8*), and 400 mM imidazole (*lane 9*). The protein samples were separated on a 12.5 % SDS-polyacrylamide gel, which was stained with Coomassie Brilliant Blue after electrophoresis. Ten micrograms of protein crude extract (*lane 2*) or 10 µl aliquots of each fraction (*lanes 3–9*) were loaded. The molecular mass standards were loaded in *lane 1*

of the UFAs in the *L. lactis* subsp. *cremoris cfa*-deficient mutant complemented with the *O. oeni cfa* gene.

Discussion

Our characterization of *O. oeni* CFA synthase was driven by the need to explain the previously described partial complementation of an *E. coli cfa*-deficient mutant (Grandvalet et al. 2008). This partial complementation was possibly caused by the use of a Gram-negative bacterium as the host, the identity of the homologous CFA synthase, or the reduced substrate specificity of the *O. oeni* enzyme. Thus, we complemented a *L. lactis* subsp. *cremoris cfa*-deficient strain with the *O. oeni cfa* gene, which encodes a protein that shares a 62 % amino acid sequence identity with the homologous protein in *L. lactis* subsp. *cremoris* MG1363 (versus a 29 % shared identity with *E. coli* CFA synthase). Unfortunately, CFA cyclization remained partial in the complemented strain under optimal as well as stressful conditions (Table 2). This partial complementation was not a consequence of the faulty expression of the expression gene in *L. lactis* subsp. *cremoris*. Moreover, the immunodetection assay confirmed the presence of the heterologous CFA synthase in the complemented strain. Furthermore, in a previous study, the *L. lactis* subsp. *cremoris cfa*-deficient strain was totally complemented by its homologous *cfa* gene using the same expression vector (pDL278) and 100 % CFA conversion was obtained (To et al. 2011). Thus, differences in the fatty acid precursor levels may have been responsible for the partial conversion observed in the complemented strain. Surprisingly, although the complemented strain grown on Tween 80-supplemented medium incorporated a large amount of C18:1n-9 (30 %), only a small amount of cycC19:0n-9 (dihydrosterculic acid) was produced compared with cycC19:0n-7 (data not shown). The predominance of cycC19:0n-7 (lactobacillic acid) instead of cycC19:0n-9 (dihydrosterculic acid) suggests that the partial cyclization was not linked to the tails of the fatty acids or to the position of the double bond. Finally, the biochemical characterization of *O. oeni* CFA synthase was performed in vitro. For the first time, we purified and characterized a recombinant CFA synthase from a lactic acid bacterium with an N-terminal His_6-tag based on a previous observation

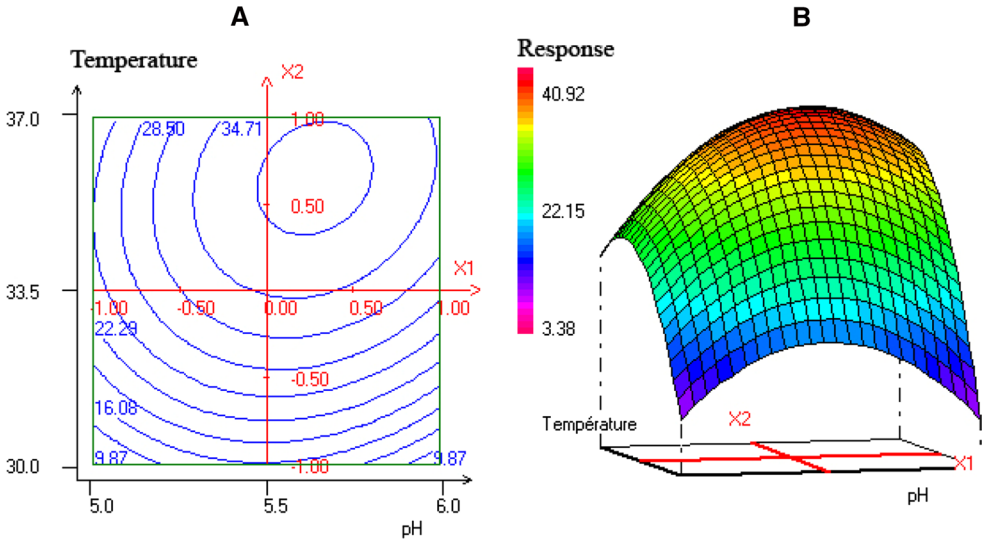

Fig. 3 Biochemical characterization of the activity of *Oenococcus oeni* CFA synthase using the iso-response method. Two-dimensional curves (**a**) and a three-dimensional response surface (**b**) showing CFA synthesis (µg/ml of cycC19:0n-7) as functions of temperature and pH. Enzymatic assays were performed as described in "Materials and methods"

that the presence of a His_6-tag did not perturb the catalytic activity of the *E. coli* CFA synthase (Courtois et al. 2004). The recombinant enzyme was produced in *E. coli* BL21(DE3) using the pET expression system, and nickel affinity chromatography facilitated the rapid purification of *O. oeni* CFA synthase. The enzymatic activity measurements were taken in vitro using GC. The highest CFA conversion rate was obtained at pH 5.6 and 35.8 °C (Fig. 3). Thus, the behavior of this enzyme differs from that of the recombinant CFA synthase of *E. coli*, which has an optimal pH of 7.5 (Courtois et al. 2004). However, previous biochemical characterizations of many intracellular enzymes in *O. oeni*, such as glucosidase (Michlmayr et al. 2010), arabinofuranosidases (Michlmayr et al. 2011), esterase (Matthews et al. 2007), ornithine decarboxylase (Bonnin-Jusserand et al. 2011), and malolactic enzyme (Schumann et al. 2013; Wang et al. 2014), have determined optimum pH values between 5 and 6, which match the intracellular pH (pH_i) found in *O. oeni* cells (5.8–6.3 in optimal conditions) (Salema et al. 1994; Augagneur et al. 2007). Given the differences between the pH_i values determined for the hosts used for complementation and *O. oeni* (7.73 in *E. coli* and 7.24 in *L. lactis* subsp. *cremoris* with an external pH of 7.0), the partial complementation may be explained by a weak activity due to an excessively basic environment (Slonczewski et al. 1981; Hickey and Hirshfield 1990; O'Sullivan and Condon 1997; Olsen et al. 2002).

The pH_i decreased in a linear manner as the extracellular pH decreased. In *L. lactis* subsp. *cremoris*, the pH_i decreased from 7.24 to 5.19 as the extracellular pH declined from 7.0 to 4.0 (O'Sullivan and Condon 1997). Thus, with the M17/5 medium, the pH_i value of the complemented strain was closer (pH_i 5.9) to the optimum pH required for the activity of *O. oeni* CFA synthase. Therefore, independent of the transcriptional regulation of the *cfa* gene, the modulation of the pH_i value explains why the CFA conversion rate of the complemented strain increased by four times in acidic conditions compared with that in optimal conditions, whereas that of the parental strain only increased by 2.5 times (Table 2).

In optimal conditions (pH 5.6 and 37 °C), the activity of *O. oeni* CFA synthase was low compared with that of *E. coli* CFA synthase, although the same expression system was employed and the same purification method was used for enzyme characterization (Courtois et al. 2004; Iwig et al. 2004; Guianvarc'h et al. 2006). We suggest that the affinity for the substrate could be linked to the phosphoric acid head groups of the membrane phospholipids. The lipid content varies among bacterial species. For example, in *B. subtilis*, phosphatidylglycerol (PG) accounts for 75 % of the total membrane lipid content and the remainder mainly comprises lysyl-PG and cardiolipin, whereas up to 38 % of the membrane lipid is lysyl-PG in *S. aureus* (Peschel et al. 2001). There is a great difference between the phosphoric acid head groups in the *O. oeni* membrane and those of *L. lactis* (Exterkate et al. 1971; Teixeira et al. 2002). The *O. oeni* membrane contains approximately 40 % phosphatidylethanolamine (PE), 20 % sphingomyelin, and 20 % PG (Teixeira et al. 2002). The *L. lactis* membrane contains 65 % PG, 4 % diphosphatidylglycerol (DPG), and 23 % lysyl-PG, but no PE (Exterkate et al. 1971). In fact, the phospholipids differ in terms of the substituents attached to phosphoric acid, which determine the charges of the phospholipids. Moreover, Gram-positive bacteria can reduce the overall negative charge on cell membranes by producing positively charged lysine esters of PG, i.e., lysyl-PG (Lennarz et al. 1967; Peschel et al. 2001; Oku et al. 2004). Steric hindrance and the hydration capacity of the substituent can also determine the characteristics of the phospholipid. For example, ethanolamine has a low steric hindrance and low hydration, thereby implying that a PE head group is smaller than a glycerol group, which has the same steric hindrance but greater hydration. Thus, PE has a cone shape and PG has a cylindrical shape. The wide variety of polar and nonpolar components is responsible for the high diversity of phospholipids and their physical properties. We suggest that differences in the phosphoric acid head groups in the *O. oeni* lipid bilayer and that of the host bacterium could hinder accessibility to *O. oeni* CFA synthase. Further investigations are needed to identify the specificity of different phospholipids with various substrates to optimize the CFA synthase activity in this LAB. *O. oeni* CFA synthase enzyme seems to be strongly tailored to its native membrane environment and should not be suitable for expression in other microorganisms. The use of phospholipids from *O. oeni* as positive control could support this hypothesis. Preliminary tests for obtaining phospholipids devoid of CFAs from *O. oeni* cells harvested in early log phase were not conclusive. The construction of *O. oeni cfa*-deficient strain would increase the extraction rate of phospholipids devoid of CFA. However, tools to delete genes in *O. oeni* are currently under development.

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