Changes in membrane lipid composition in ethanol- and acid-adapted *Oenococcus oeni* cells: characterization of the *cfa* gene by heterologous complementation

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Cyclopropane fatty acid (CFA) synthesis was investigated in *Oenococcus oeni*. The data obtained demonstrated that acid-grown cells or cells harvested in the stationary growth phase showed changes in fatty acid composition similar to those of ethanol-grown cells. An increase of the CFA content and a decrease of the oleic acid content were observed. The biosynthesis of CFAs from unsaturated fatty acid phospholipids is catalysed by CFA synthases. Quantitative real-time-PCR experiments were performed on the *cfa* gene of *O. oeni*, which encodes a putative CFA synthase. The level of *cfa* transcripts increased when cells were harvested in stationary phase and when cells were grown in the presence of ethanol or at low pH, suggesting transcriptional regulation of the *cfa* gene under different stress conditions. In contrast to *Escherichia coli*, only one functional promoter was identified upstream of the *cfa* gene of *O. oeni*. The function of the *cfa* gene was confirmed by complementation of a *cfa*-deficient *E. coli* strain. Nevertheless, the complementation remained partial because the conversion percentage of unsaturated fatty acids into CFA of the complemented strain was much lower than that of the wild-type strain. Moreover, a prevalence of cycC19:0 was observed in the membrane of the complemented strain. This could be due to a specific affinity of the CFA synthase from *O. oeni*. In spite of this partial complementation, the complemented strain of *E. coli* totally recovered its viability after ethanol shock (10 %, v/v) whereas its viability was only partly recovered after an acid shock at pH 3.0.

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INTRODUCTION

Most prokaryotes are able to adapt to and survive fluctuations of environmental conditions by using multiple strategies that can operate simultaneously. To survive and function in wine, *Oenococcus oeni* requires the activation of a proton-extruding ATPase (Drici-Cachon *et al.*, 1996; Salema *et al.*, 1996) and the proton-motive force generated by malolactic fermentation. Nevertheless, two main strategies are also required and these seem to be conserved in most bacteria. The first one involves the induction of the expression of specific genes called heat-shock genes (*hsp*). The second strategy leads to membrane fluidity adjustments by modifications in membrane composition,

generally affecting phospholipids and their fatty acid composition. Indeed, the cell membrane constitutes one of the first barriers against environmental stresses and consequently plays a major role in homeostasis and in adaptation to stress conditions (Jones, 1989; Sajbidor, 1997; Weber & de Bont, 1996). Bacteria can modify their fatty acid composition in response to atypical conditions (Cronan, 2002). The lipid structure and the portion of saturated, unsaturated, branched or cyclic fatty acids in individual phospholipids influences the fluidity of biological membranes.

Many studies have focused on the deleterious effect of ethanol on membrane modifications in micro-organisms (Dombek & Ingram, 1984; Guzzo *et al.*, 2000; Svobodova & Svoboda, 1988; Swan & Watson, 1997). In *Escherichia coli*, one of the changes that occur in membrane lipid

Abbreviations: CFA, cyclopropane fatty acid; QRT-PCR, quantitative reverse transcriptase PCR.

composition in response to ethanol is an increase in the amount of unsaturated fatty acid (Ingram, 1976). During acetone-butanol fermentation, modifications in the unsaturated/saturated fatty acid ratio were found in *Clostridium acetobutylicum* cell membranes (Lepage *et al.*, 1987). The unsaturated fatty acid acyl chains of bacterial membrane phospholipids have a major influence on membrane properties. In *cis-trans* isomerization, catalysed by a *cis-trans* isomerase, the double bond is reconfigured. The *cis* unsaturated chain contains a bend which increases membrane fluidity, whereas the *trans* isomer increases membrane rigidity. Loffeld & Keweloh (1996) suggested that the isomerization of *cis* into *trans* unsaturated fatty acids is an emergency action of cells of *Pseudomonas putida* to adapt membrane fluidity to drastic changes in environmental conditions.

An opposite effect in response to ethanol is observed in other micro-organisms (Rigomier *et al.*, 1980; Teixeira *et al.*, 2002). An increase in the content of saturated fatty acids is observed in *Bacillus subtilis* (Rigomier *et al.*, 1980). A mechanism involved in the increase of saturated fatty acids is the conversion of monounsaturated fatty acids to cyclopropane fatty acids (CFAs). CFAs have been detected in membrane phospholipids of a variety of eubacteria (Grogan & Cronan, 1997). These CFAs are synthesized *in situ* by the transfer of a methylene group from S-adenosyl-L-methionine to a double bond of unsaturated fatty acid chains of membrane phospholipids by CFA synthase. Conversion of monounsaturated fatty acids to CFAs has been reported in many Gram-negative bacteria when the growth rate of cultures is markedly slowed, i.e. during the stationary phase of growth. In *E. coli*, the synthesis of CFAs is generally regarded as a means to reduce membrane fluidity and prevent the penetration of undesirable molecules in order to adapt the cells to adverse conditions (Chang & Cronan, 1999; Grogan & Cronan, 1997). The positive roles of CFAs have also been demonstrated in bacterial cells adapted to high acidity (Brown *et al.*, 1997; Teixeira *et al.*, 2002). *cfa*-deficient cells of *E. coli* and *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) are highly sensitive to acid stress (Chang & Cronan, 1999; Kim *et al.*, 2005). In the Gram-positive bacterium *C. acetobutylicum*, the CFA content detected in early-exponential-phase cells enhanced acid and solvent resistance (Zhao *et al.*, 2003). In *O. oeni*, cells respond to culture in the presence of ethanol by increasing their CFA content (Teixeira *et al.*, 2002). The presence of CFAs in the membrane could reduce proton permeability (da Silva *et al.*, 2002) and increase membrane rigidity (da Silva *et al.*, 2003). Nevertheless, the mechanisms involved in membrane fluidity adjustments in this bacterium remain unclear.

In *E. coli*, the onset of CFA synthesis as cultures enter stationary phase is due to increased transcription of *cfa* from the RpoS-dependent promoter, whereas a standard RpoD-dependent promoter is responsible for the low level of CFA synthesis in exponentially growing cultures (Chang

& Cronan, 1999). In *Lactococcus lactis*, transcriptional fusion experiments have demonstrated high induction of *cfa* gene expression by acidity as well as during entry into the stationary phase of growth (Budin-Verneuil *et al.*, 2005). The *cfa* gene of *C. acetobutylicum* appears to form an independent operon with a *marR*-homologous gene. The MarR-like gene product may act to reduce expression of the *cfa* gene directly or indirectly, based on the observation that the overexpression of *marR* resulted in decreased CFA accumulation (Zhao *et al.*, 2003).

In this work, we analysed the membrane fatty acid composition of *O. oeni* in three conditions of stress: entry into the stationary phase of growth, and growth in the presence of ethanol or in acidic conditions. In all three conditions, we observed a decrease of the ratio of unsaturated to saturated fatty acids and an increase of CFA content. These results led us to study the level of transcription of the *cfa* gene of *O. oeni* in stress conditions. By complementation of an *E. coli* *cfa* mutant, we investigated the functionality of the *cfa* gene of *O. oeni*.

METHODS

Bacterial strains and media. *O. oeni* ATCC BAA-1163 (formerly *O. oeni* IOB 8413) was cultured at 30 °C in modified FT80 (mFT80) medium (Cavin *et al.*, 1989) at pH 5.3. The *E. coli* strains used were derivatives of *E. coli* K-12. *E. coli* ER2738 [F' *proA*⁺*B*⁺ *lac*^q Δ *lacZ*M15 *zcf*::Tn10(Tet^R)/*fhuA2 glnV thi-1* Δ (*lac-proAB*) Δ (*hsdS-mcrB*)5] (New England Biolabs) was used for cloning procedures. *E. coli* MG1655 (F⁻ λ ⁻ *ilvG rfb-50 rph-1*) and *E. coli* YYC1273 (MG1655 *cfa*::*kan*) were used to confirm the function of the *O. oeni* *cfa* gene (Chang & Cronan, 1999; Jensen, 1993). *E. coli* strains were grown aerobically at 37 °C in Luria-Bertani (LB) medium or in Tryptone Soya Broth medium (TSB, Biokar) (Bertani, 1951). For recombinant strains, the medium was appropriately supplemented with ampicillin (100 µg ml⁻¹) or kanamycin (25 µg ml⁻¹).

Growth in sublethal stress conditions, and acid and ethanol shocks. Precultures of *O. oeni* were used to inoculate mFT80 medium at an initial OD₆₀₀ of 0.1. To obtain control cells, exponential-phase cells were harvested when an OD₆₀₀ of 0.7 was reached at pH 5.3. Stationary-phase cells were harvested after 36 h incubation in the same medium. For ethanol- and acid-grown cells, *O. oeni* was inoculated at the same OD₆₀₀ of 0.1 in mFT80 with 8 % (v/v) ethanol and at pH 3.5, respectively. Stress-grown cells were harvested from their exponential growth phase after 36 and 31 h of growth with 8 % ethanol and at pH 3.5, respectively.

To test resistance to ethanol and acidity, *E. coli* strains were cultivated in TSB medium to late-stationary phase (15 h) and 1 ml samples of cultures were harvested by centrifugation at room temperature. The cells were then washed with TSB medium and resuspended in 1 ml TSB medium at pH 3 (adjusted with HCl) or TSB supplemented with ethanol 10 % (v/v) for challenge. The cell suspensions were shaken at 37 °C and aliquots were collected at timed intervals (1, 2 and 3 h), and viable counts were measured by serial dilution and plating on LB agar supplemented with ampicillin. Survival is defined as the ratio of colonies (c.f.u.) formed on LB agar medium after challenges to the initial number of viable colonies.

Lipid analysis. Bacterial cells were taken from exponential-phase cultures under optimal conditions, in the presence of 8 % ethanol,

and at pH 3.5. Total lipids were extracted with chloroform/methanol according to the method described by Bligh & Dyer (1959). Phospholipids were purified by TLC on silica gel plates (Merck) with hexane/diethyl ether/methanol/acetic acid (90/20/3/2, by vol.) for development. The phospholipid band was scraped off and extracted from the silica gel with chloroform/methanol/water (45/45/10, by vol.). The fatty acids of total lipids and phospholipids were directly transesterified with methanol/H₂SO₄ (95/5, v/v) at 80 °C for 2 h. Total fatty acids were quantified by adding C17:0 (heptadecanoic acid) as an internal standard. The fatty acid methyl esters (FAMES) were analysed by GLC using a Chrompack CP 9002 chromatograph equipped with a Varian Factor Four capillary column (30 m × 0.32 mm). The oven temperature increased after 1 min from 60 °C to 150 °C at 30 °C min⁻¹ for 3 min then to 220 °C at 2 °C min⁻¹. The FAMES were identified by comparing retention times with those of authentic standards (Nu Chek Prep., Elysian, MN, USA).

RNA extraction and analysis. RNA extraction was performed using Tri Reagent (Sigma) according to the manufacturer's instructions and 0.4 g of glass beads (70–100 µm) to disrupt cells with a FastPrep cell disintegrator (Bio 101). Samples were then treated as recommended by the manufacturer and used for Northern blotting, primer extension analysis, reverse transcriptase PCR (RT-PCR) or quantitative RT-PCR (QRT-PCR) experiments. Northern blotting was carried out as described by Sambrook *et al.* (1989). A DNA fragment corresponding to the *O. oeni* *cfa* gene was amplified by PCR using oligonucleotides L1 (TGGTATTACATTGAGCGAGGAG) and R1 (GGATTATCGTGATCTCAAAGACG) and used as a probe in Northern hybridization experiments. PCR was performed in a final volume of 50 µl containing *O. oeni* genomic DNA (1 µg ml⁻¹), dNTPs (0.2 mM each), oligonucleotides (1 mM each), 10 U ml⁻¹ of *Taq* DNA polymerase (Bioline), and the buffer supplied with the enzyme. Amplification was performed for 35 cycles consisting of 30 s denaturation at 92 °C, 30 s annealing at 60 °C, and 30 s elongation at 72 °C. The PCR products were purified by using the Qiaquick PCR purification kit (Qiagen) and probe was radiolabelled with [α -³²P]dATP using a random primers DNA labelling kit (Invitrogen). Primer extensions were performed as previously described (Grandvalet *et al.*, 2005) with oligonucleotides *cfa*2C (TTTGGCTTACCAGTCCCATAA) and *cfa*4C (CGGTCTTACC-ATCCCAATAAG). The corresponding DNA-sequencing reactions were carried out by using the same oligonucleotides and PCR-amplified DNA fragments with oligonucleotides *cfa*1C (CTTGTTTAAATTTTCACTTTTATTG) and *cfa*2C, carrying the *cfa* promoter region. Nucleotide sequences were determined by the dideoxy chain-termination method using the DNA sequencing cycle Reader kit (MBI Fermentas). RT-PCR and QRT-PCR were performed as previously described (Grandvalet *et al.*, 2005) using the primer pair L1 and R1. The specificity of QRT-PCR products was determined with a melting curve. The efficiency of real-time amplification is calculated by the formula $E = [10^{(1/s)} - 1] \times 100$, where s is the slope of standard curve. Three independent experiments were performed and the results were calculated by the comparative critical threshold ($\Delta\Delta C_T$) method, in which the amount of target RNA is adjusted to a reference relative to an internal calibrated target RNA. The *ldhD* gene of *O. oeni* was chosen as an internal control for these experiments (Desroche *et al.*, 2005).

DNA isolation, manipulation and transformation

Cloning of the *cfa* gene from *O. oeni*. The *cfa* gene was cloned by PCR amplification from *O. oeni* ATCC BAA-1163 chromosomal DNA using oligonucleotides *cfa*4M (CCCGGATCCTTCATTTTAAATTA-AAAAAATAAAATTTT) and *cfa*5M (GGGGAATTCTCTGTGTTCC-TTTTTTTAGAAAATT). *Bam*HI and *Eco*RI sites (italic) were included to aid subsequent manipulations. The PCR was performed

by using *Taq* ADN polymerase high-fidelity Platinum (Invitrogen) as recommended by the manufacturer in order to minimize errors during polymerization. The resulting fragment carrying the whole *cfa* gene, including the promoter region and the terminator, was ligated to the corresponding cloning sites of pUC18 (Invitrogen) after digestion with *Bam*HI and *Eco*RI, to form pMT1. This construct put the *O. oeni* *cfa* gene under control of its own promoter region. The sequence of the cloned PCR amplification product was confirmed by DNA sequencing (GENOME express). Plasmid pMT1 was used to transform the *cfa*-deficient *E. coli* strain YYC1273 and the wild-type *E. coli* strain MG1655. Transformation of *E. coli* was conducted by electroporation.

Sequence and statistical analysis. Sequence alignments were performed with CLUSTAL W (Kohli & Bachhawat, 2003). The significance of the difference between cell viability percentages and total fatty acid amounts was determined by a two-tailed Student's *t* test. The confidence interval for a difference in the means was set at 95 % ($P \leq 0.05$) for all comparisons.

RESULTS

Changes in membrane fatty acid composition in stationary-phase and in stress-grown *O. oeni*

Two sublethal stress conditions were chosen to obtain stress-grown *O. oeni* cells: cultures in the presence of 8 % (v/v) ethanol and at pH 3.5. The concentration of 8 % ethanol was chosen as a hinge value: the main changes observed normally occurred in cells grown in the presence of at least 8 % ethanol in the medium (Teixeira *et al.*, 2002). The control cells were harvested in the exponential phase of growth, when an OD₆₀₀ of 0.7 was reached after 12 h of incubation. Stationary-phase cells were harvested after 36 h of incubation (maximal OD₆₀₀ of 1.6). Stress-grown cells were harvested from their exponential growth phase (OD₆₀₀ of 0.7) after 36 and 31 h of growth with 8 % ethanol and at pH 3.5, respectively. The maximal OD₆₀₀ values reached by cultures in the presence of ethanol or at pH 3.5 were 1.0 and 1.2, respectively. The Neperian specific growth rate (μ) of cells cultured under optimal conditions was 0.16 h⁻¹ whereas that of cells cultured in the presence of 8 % ethanol and at pH 3.5 significantly decreased to the same value of 0.05 h⁻¹. Nevertheless, the final biomass obtained by growth in sublethal stress conditions represented approximately 75 % of the final biomass obtained in optimal conditions (data not shown).

We determined the fatty acid composition of the total lipids and the phospholipid fraction extracted from *O. oeni* cells grown under optimal conditions. The fatty acid profile of phospholipids was similar to that of total fatty acids and accounted for nearly 94 % (w/w) of the total fatty acids. From then on, fatty acid analyses were carried out only on the total lipid extract. In *O. oeni* control cells (mid-exponential growth phase), eight main fatty acids were identified (Fig. 1); these represented 90 mol% of total fatty acids. The higher amount of oleic (C18:1 n-9) and dihydrosterculic (cycC19:0 n-9) acids compared to that of *cis*-vaccenic (C18:1 n-7) and lactobacillic (cycC19:0 n-7)

Fig. 1. Fatty acid composition of *O. oeni* cells during growth in mFT80 medium under optimal conditions (exponential-phase cells, stationary-phase cells), in the presence of 8% (v/v) ethanol (ethanol-grown cells) or in medium at pH 3.5 (acid-grown cells). Stress-grown cells were harvested for analyses from their exponential growth phase. Means $\pm$ SD are presented ($n=3$).

acids was in accordance with the presence of Tween 80 in the *O. oeni* culture medium (Guerrini *et al.*, 2002; Lonvaud-Funel & Desens, 1990).

In the stationary phase of growth, the levels of major saturated fatty acids [myristic (C14:0) and palmitic (C16:0)] remained constant. In contrast, we observed a decrease in unsaturated fatty acids, particularly oleic acid, which is the main unsaturated fatty acid present in the medium. Moreover, the main effect on the fatty acid profile was an increase in the molar percentage of dihydrostercularic acid.

In cells grown in the presence of 8% ethanol, the membrane fatty acid profile dramatically altered compared to that of control cells. There was an increase in the molar percentage of the saturated palmitic (C16:0) and

cyclopropane dihydrostercularic (cycC19:0 n-9) acids, and a decrease in that of the monounsaturated palmitoleic (C16:1) and oleic acids (Fig. 1). The amount of total fatty acids dropped by twofold in comparison with that of control cells (Table 1).

In cells grown under acid conditions (pH 3.5), we observed similar changes in membrane fatty acid profile as those observed in ethanol-grown cells but of smaller amplitude (Fig. 1). The ratio of unsaturated to saturated fatty acids dropped by fivefold compared to that of control cells. The total fatty acid content was only one-third of that of control cells (Table 1).

Expression of the *O. oeni* *cfa* gene increases at the onset of stationary phase and in stress growth conditions

The biosynthesis of CFAs from unsaturated fatty acid phospholipids is catalysed by CFA synthases (Grogan & Cronan, 1997). The genome sequence of *O. oeni* ATCC BAA-1163, (accession no. NZ_AAUUV00000000) revealed an ORF (locus_tag: OENOO_64048) encoding a 45 kDa protein sharing 45% amino acid sequence identity with CFA synthase from *Clostridium acetobutylicum* (Zhao *et al.*, 2003) and 29% identity with CFA synthase from *E. coli* (Grogan & Cronan, 1984) and *S. typhimurium* (Kim *et al.*, 2005). This putative *O. oeni* *cfa* gene is flanked by two genes (locus_tag OENOO_64049 and OENOO_64046) transcribed in the same orientation as *cfa* (Fig. 2b). The gene upstream of *cfa* (OENOO_64049) encodes a putative permease protein of an ABC transporter system, whereas the downstream gene (OENOO_64046 annotated *ubiD*) encodes a 3-octaprenyl-4-hydroxybenzoate carboxy-lyase. We noted that the genome sequence of *O. oeni* PSU-1 (accession no. NC_008528.1) contains an ORF (OEOE_1175) between *cfa* and *ubiD*, transcribed divergently. This putative gene encodes a 304 aa protein similar to a transcriptional regulator of the LysR family. This ORF is also present in the *O. oeni* ATCC BAA-1163 genome (OENOO_64047), but it has not been annotated because of the presence of a frameshift which is due to a sequencing

Table 1. Analysis of fatty acid profiles of *O. oeni* cells grown under different conditions

Cells were cultured in mFT80 medium under optimal conditions (exponential-phase cells, stationary-phase cells), or under stress conditions (8%, v/v, ethanol or pH 3.5).

Parameter	Exponential-phase cells (control)	Stationary-phase cells (36 h)	Ethanol-grown cells	Acid-grown cells
Saturation degree*	0.50	0.62	0.87	0.82
Unsaturated/saturated fatty acids†	0.99	0.61	0.15	0.23
Total fatty acids‡	5.6 $\pm$ 1.3	ND	2.8 $\pm$ 0.2	1.8 $\pm$ 0.1

*Saturation degree = $\Sigma(\text{C14:0} + \text{C16:0} + \text{C18:0} + \text{cycC19:0})/100$ (cycC19:0 is considered as a saturated fatty acid).

†Ratio of unsaturated to saturated fatty acids = $\Sigma(\text{C16:1} + \text{C18:1})/\Sigma(\text{C14:0} + \text{C16:0} + \text{C18:0} + \text{cycC19:0})$.

‡The values represent quantities of total fatty acids (μg per OD₆₀₀ unit of cells) and are means $\pm$ SD for three independent cultures. ND, Not determined.

Fig. 2. Determination of the transcription initiation site of the *cfa* gene by primer extension analysis. Total RNA was extracted from *O. oeni* cells harvested in the exponential phase. (a) Primer extension product corresponding to the *cfa* gene (lane ext) is shown alongside DNA-sequencing reaction products (lanes T, G, C and A). The corresponding nucleotide sequence is shown on the left. (b) Map of the *cfa* genomic region. The transcriptional start site is indicated by an asterisk, the -10 sequence is boxed, the TG sequence element is underlined, the presumed ATG translational start codon is shaded and the putative rho-independent transcription terminators of *cfa* and OENOO_64049 genes are labelled T_{cfa} and T_{64049} , respectively.

error (data not shown). Using the software application 'Mfold web server' (<http://www.bioinfo.rpi.edu/applications/mfold>) (Mathews *et al.*, 1999; Zuker, 2003), we identified a putative rho-independent transcription terminator downstream of *cfa* (T_{cfa} , $\Delta G^0 = 11.2$ kcal mol $^{-1}$; -46.9 kJ mol $^{-1}$) and another one downstream of OENOO_64049 (T_{64049} , $\Delta G^0 = -15.6$ kcal mol $^{-1}$; -65.3 kJ mol $^{-1}$), suggesting monocistronic expression of *cfa* (Fig. 2b). The expression of the *cfa* gene was investigated by Northern blotting. A 1.2 kb transcript was detected, corresponding to the size expected for monocistronic expression of *cfa* (data not shown). The 5'-end mRNA of *cfa* was mapped by primer extension analysis (Fig. 2a). The transcriptional start site was identified at nucleotide position -22 , with reference to the presumed ATG translational start codon. This transcriptional start site was preceded by a putative -10 (TACGAT) hexamer,

which showed similarity to the consensus of -10 sequences usually described for *O. oeni* promoters. No putative -35 hexamer was identified at an appropriate distance from this putative -10 box. Nevertheless, a supplementary sequence element, 5'-TG-3', is located one base upstream of the -10 hexamer (Fig. 2b). To examine whether expression of *cfa* is induced during growth in stress conditions, a QRT-PCR was set up. Total RNA was extracted from exponential- (control) and stationary-phase *O. oeni* cells grown under optimal growth conditions and from exponential-phase cells grown in the presence of 8% (v/v) ethanol (ethanol-grown cells) and at pH 3.5 (acid-grown cells). By using the comparative critical threshold ($\Delta\Delta C_T$) method with the *ldhD* gene of *O. oeni* as an internal control (Desroche *et al.*, 2005), we found that the *cfa* mRNA levels increased by three-, six- and twofold in stationary-phase, ethanol- and acid-grown cells, respectively, in comparison with that of control cells.

Expression of the *O. oeni cfa* gene restores the synthesis of CFA in an *E. coli cfa* mutant

Because no genetic tool adapted to carry out gene inactivation in *O. oeni* is yet available, the function of the *O. oeni cfa* gene was explored in the *cfa*-deficient mutant strain *E. coli* YYC1273 (Chang & Cronan, 1999). The *O. oeni cfa* gene, its promoter region included, was cloned into the pUC18 vector to generate pMT1, which was introduced into strain YYC1273 (YYC1273/pMT1). Before analysing the phenotype of the complemented strain, the heterologous expression of the *O. oeni cfa* gene in *E. coli* was verified. *cfa* transcripts were detected in *E. coli* YYC1273/pMT1 (data not shown) by RT-PCR analysis. Moreover, primer extension analysis confirmed that the *cfa* promoter of *O. oeni* was recognized by the *E. coli* transcriptional machinery (data not shown).

The viabilities of late-stationary-phase cultures of *E. coli* strains exposed to ethanol shock (10%, v/v) and acid shock (pH 3.0) were measured. As previously described (Chang & Cronan, 1999; Grogan & Cronan, 1986), the *E. coli cfa*-deficient strain poorly survived ethanol or acid shock (Table 2). The control strains MG1655/pMT1 and YYC1273/pUC18 were also examined and no significant differences in percentage survival were noticed after

Table 2. Ethanol and acid survival of *E. coli* strains

<i>E. coli</i> strain	Survival (%) 3 h after challenge*	
	10% (v/v) ethanol	pH 3.0
MG1655 (wild-type)	93.2 $\pm$ 7.7	70.6 $\pm$ 2.5
YYC1273 (<i>cfa::kan</i>)	46.3 $\pm$ 5.2	11.0 $\pm$ 1.7
YYC1273/pMT1	94.1 $\pm$ 11.5	43.3 $\pm$ 3.8

*Percentage survival was defined as [(c.f.u. ml $^{-1}$ 3 h after challenge)/(c.f.u. ml $^{-1}$ at t_0)] $\times$ 100. The data represent the means $\pm$ SD of triplicate independent experiments.

ethanol and acid shocks in comparison with strains MG1655 and YYC1273, respectively (data not shown). As shown in Table 2, pMT1 could complement the *cfa*-deficient strain of *E. coli* when cells were exposed to ethanol. However, the presence of pMT1 only partially restored the acid resistance observed in the wild-type strain.

Previous studies showed that *E. coli* strains carrying the *cfa::kan* mutation lacked detectable CFA synthase activity and made no CFA detectable by gas chromatography (Cronan *et al.*, 1974; Grogan & Cronan, 1986). So, to explore the capacity of the *O. oeni* CFA synthase to promote the cyclopropanation of double bonds of *E. coli* unsaturated fatty acids, the composition of membrane fatty acids of *E. coli* strains was analysed (Table 3). The analysis of fatty acid composition of the wild-type strain *E. coli* (MG1655) revealed the presence of myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1) and *cis*-vaccenic acid (C18:1, n-7). We also noted the presence of CFAs such as cycC17:0 and lactobacillic acid (cycC19:0 n-7). Palmitic acid (C16:0) accounted for 58 % of the total fatty acids identified whereas the monounsaturated fatty acid proportion was low (about 1 %). The CFAs accounted for 31 % of the total fatty acids identified; cycC17:0 alone accounted for more than 24 % of the total. Consistent with previous reports, no CFA was detected in the *E. coli cfa*-deficient strain YYC1273 and the proportion of mono-unsaturated precursors was higher (41 %) than in the wild-type strain (1 %). Fatty acid analysis of the complemented strain YYC1273/pMT1 revealed a shift in the fatty acid composition of the cell membrane compared to the mutant strain. This result confirmed the capacity of the *O. oeni* CFA synthase to convert unsaturated fatty acids to CFAs in *E. coli*. However, the CFA level in the complemented strain (7 %) was lower than that of the wild-type strain (31 %). Thus, the incomplete restoration of acid resistance may be due to the deficiency of CFA in membranes of cells or to the modification of the unsaturated/saturated fatty acids

ratio. Moreover, in contrast to the wild-type strain, the membrane of the complemented strain contained more cycC19:0 (n-7) than cycC17:0.

DISCUSSION

In this study, we found that, like other bacteria, *O. oeni* produces CFAs at the beginning of the stationary phase. Moreover, we showed that environmental factors such as ethanol- and acid-growth conditions induce CFA synthesis. Biomembranes are the site of the primary contact of cells with their environment: the flexibility and the adaptation capability of the cells thus determine their survival ability. Membrane lipid and protein compositions are recognized as some of the main factors involved in stress tolerance (da Silveira *et al.*, 2003, 2004; Jones, 1989). Membrane lipid composition changes are induced in response to temperature, pH, solvents and other forms of stress (Dombek & Ingram, 1984; Gennis, 1989; Jones, 1989). In the present work, we observed significant increases in palmitic and dihydrosterculic acids in both ethanol- and acid-grown cells. According to the mechanisms for cyclopropane ring formation in membrane lipids proposed by Grogan & Cronan (1997), we can suggest that oleic and vaccenic acids would be converted into cyclopropane dihydrosterculic and lactobacillic acids, respectively. In *E. coli*, strains with high levels of CFAs survived acid shock much better than those with low CFA levels (Brown *et al.*, 1997). The cyclization of fatty acyl chains is generally considered a means of reducing membrane fluidity, thereby reducing proton permeability and preventing the penetration of undesirable molecules in order to adapt the cells to adverse conditions (Chang & Cronan, 1999; Cronan, 2002; Grogan & Cronan, 1997). Teixeira *et al.* (2002) observed an increase in the proportion of lactobacillic acid at the expense of *cis*-vaccenic acid in *O. oeni*. This increase protected *O. oeni* against the toxic effect of ethanol, balancing the increase in membrane fluidity normally

Table 3. Effect of *cfa*-bearing plasmid pMT1 on *E. coli* fatty acid composition

Fatty acid	Fatty acid composition (% of total identified)		
	MG1655 (wild-type)	YYC1273 (<i>cfa::kan</i>)	YYC1273/pMT1
C14:0	9.1 ± 0.8	6.6 ± 0.3	7.8 ± 0.1
C16:0	58.2 ± 0.8	52.0 ± 1.9	53.9 ± 0.4
C16:1	0.4 ± 0.1	31.1 ± 1.9	27.5 ± 0.7
cycC17:0	24.1 ± 0.9	0	2.4 ± 0.7
C18:1 (n-7)	0.7 ± 0.4	10.3 ± 0.3	3.7 ± 0.5
cycC19:0 (n-7)	7.5 ± 0.6	0	4.7 ± 0.9
U/S ratio*	0.01	0.71	0.45
CFA conversion (%)†	96.7	0	18.5

*U/S ratio: unsaturated to saturated fatty acids ratio.

†Percentage conversion was calculated by the ratio (% CFA)/(% CFA + % monounsaturated fatty acid precursors).

The data represent the means ± SD of triplicate independent experiments.

attributed to ethanol. In our study, the increases in palmitic and dihydrosterculic acids led to an increased degree of fatty acid saturation in both ethanol- and acid-grown cells. It should be noted that da Silveira *et al.* (2003) observed an increase in the degree of unsaturation in cells grown with ethanol. However, in that work, CFAs were not detected. This discrepancy could reflect the variability among *O. oeni* strains, with other resistance mechanisms to the presence of ethanol as previously observed in *E. coli* (Ingram, 1976) and *Saccharomyces cerevisiae* (Swan & Watson, 1997).

We cloned a *cfa* gene, predicted to encode a CFA synthase, from *O. oeni*. To understand the mechanisms involved in the conversion of monounsaturated fatty acids to CFAs in stationary-phase or in stress-grown cells, we quantified the mRNA of the *cfa* gene in *O. oeni* at two phases of growth in optimal conditions (exponential and stationary phases) and in cells grown in the presence of ethanol (8 %, v/v) and at low pH (pH 3.5). The increased amounts of *cfa* mRNA transcripts correlated with the increased amount of CFA in the membrane and suggest that the expression of this gene could be regulated at the transcriptional level as previously described in *E. coli* (Chang & Cronan, 1999). The *E. coli cfa* gene is controlled by the σ^S factor, which governs the general stress response to a number of environmental stimuli, including starvation, acid shock or osmotic stress. In *L. lactis*, the expression of *cfa* is induced by acidity as well as during entry into the stationary phase of growth (Budin-Verneuil *et al.*, 2005). The mechanism involved in the control of *cfa* expression has not yet been elucidated. In *C. acetobutylicum*, Zhao *et al.* (2003) identified a *marR*-homologous gene preceding *cfa* whose overexpression resulted in reduced CFA accumulation in cell membranes. The majority of MarR protein family members are transcriptional repressors. So, the *cfa* gene of *C. acetobutylicum* could be controlled by the product of the *marR*-like gene. Analysis of the *O. oeni cfa* promoter did not identify a putative –35 hexamer at an appropriate distance from the putative –10 hexamer that was identified. Nevertheless, a supplementary sequence element, 5'-TG-3', is located one base upstream of the –10 hexamer. A number of activator-independent promoters have been reported where specific –35 hexamer contacts are not required for transcription initiation (Barne *et al.*, 1997). Transcription initiation at these promoters is dependent on an 'extended' –10 element, 5'-TGnTATAAT-3', which appears to create alternative contact points for the σ subunit of RNA polymerase. In *O. oeni* ATCC BAA-1163, the ORF downstream of *cfa* (locus tag OENOO_64047 vs OEOE_1175 in *O. oeni* PSU-1), transcribed divergently, encodes a putative transcriptional regulator similar to LysR family members. This ORF could constitute a potential transcriptional regulator of *cfa*. Future studies will focus on the characterization of this gene.

No genetic tool adapted to carry out gene inactivation in *O. oeni* is yet available. In order to confirm the functionality of the *O. oeni cfa* gene, complementation

experiments with an *E. coli cfa*-deficient mutant were conducted. The presence of CFAs significantly increased stress tolerance of the complemented strain in comparison to the *cfa*-deficient strain. The complemented strain totally recovered its viability after ethanol shock, whereas its viability was only partly recovered for acid shock. These results suggest that the stabilizing effect of CFAs on cell membranes could differ depending on the nature of the shock. Kim *et al.* (2005) suggested that the incomplete restoration of acid resistance they observed in *S. typhimurium* might have been due to a deficiency of unsaturated fatty acids (UFAs) in the cell membrane due to the overexpression of CFA synthase. We considered this hypothesis for *O. oeni*. Nevertheless, our results suggest that the complementation in our experiments remained partial because the percentage conversion of UFAs into CFAs of the complemented strain was much lower than that of the wild-type strain. This could be explained by several factors. (i) The CFA synthase level was too low for a total conversion of UFAs into CFAs. The reduced level of CFA synthase could be caused by the expression of a heterologous gene in *E. coli* cells. (ii) Another explanation could be linked to substrate specificity. In the *E. coli* mutant strain complemented with the *O. oeni cfa* gene, the major CFA of the membrane was lactobacillic acid (cycC19:0 n-7) whereas the major membrane CFA of the wild-type *E. coli* strain was cycC17:0. We did not detect the presence of cycC17:0 in the *O. oeni* membrane. Its unsaturated fatty acid precursor (C16:1) was converted preferentially into the corresponding saturated fatty acid (C16:0), most probably by a saturase enzyme. It is noteworthy that cycC19:0 was the major CFA in the *O. oeni* membrane; it could account for 25 % of membrane fatty acids of *O. oeni* cells cultured under stress conditions. In contrast, cycC17:0 was the major CFA in *E. coli*: most palmitoleic acid (C16:1) was cyclized into cycC17:0 (Brown *et al.*, 1997). We detected two CFAs in *O. oeni* (cycC19:0 and cycC17:0), of which cycC19:0 accounted for the major part. In spite of the high proportion of C16:1, only a low amount of cycC17:0 was produced by the *O. oeni* CFA synthase. The prevalence of cycC19:0 in the membrane of the complemented strain could be due to a higher affinity of the *O. oeni* enzyme for its natural substrate.

This study demonstrates clearly the functionality of the *cfa* gene and suggests that it is regulated at the transcriptional level. Further investigation is needed in order to identify the regulators involved. The substrate specificity of the CFA synthase in *O. oeni* also needs to be elucidated.

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