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Influence of polysorbate 80 and cyclopropane fatty acid synthase activity on lactic acid production by *Lactobacillus casei* ATCC 334 at low pH

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Abstract Lactic acid is an important industrial chemical commonly produced through microbial fermentation. The efficiency of acid extraction is increased at or below the acid's p*K*a (pH 3.86), so there is interest in factors that allow for a reduced fermentation pH. We explored the role of cyclopropane synthase (Cfa) and polysorbate (Tween) 80 on acid production and membrane lipid composition in *Lactobacillus casei* ATCC 334 at low pH. Cells from wild-type and an ATCC 334 *cfa* knockout mutant were incubated in APT broth medium containing 3 % glucose plus 0.02 or 0.2 % Tween 80. The cultures were allowed to acidify the medium until it reached a target pH (4.5, 4.0, or 3.8), and then the pH was maintained by automatic addition of NH_4OH. Cells were collected at the midpoint of the fermentation for membrane lipid analysis, and media samples were analyzed for lactic and acetic acids when acid production had ceased. There were no significant differences in the quantity of lactic acid produced at different pH values by wild-type or mutant cells grown in APT, but the rate of acid production was reduced as pH declined. APT supplementation with 0.2 % Tween 80 significantly increased the amount of lactic acid produced by wild-type cells at pH 3.8, and the rate of acid production was modestly improved. This effect was not observed with the *cfa* mutant, which indicated Cfa activity and Tween 80 supplementation were each involved in the significant increase in lactic acid yield observed with wild-type *L. casei* at pH 3.8.

Keywords *Lactobacillus* · Membrane · Fermentation · Lactic acid

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Introduction

Lactic acid is an industrial chemical used worldwide in medicines, foods, and beverages, and has recently been used as a renewable chemical feedstock to replace petrochemicals in applications such as polymerization into bioplastics [20, 28, 32]. Acid production through microbial fermentation is the most common source of lactic acid, and the efficiency of acid extraction from the fermentation media is increased at lower pH [31, 32]. As a result, the pH at which the microbial cultures can live and efficiently produce lactic acid is an important property for strain selection. Aciduric *Lactobacillus* species such as *L. casei* have therefore been identified as having excellent potential as biocatalysts for L-lactic acid production [7, 30, 32].

Lactobacillus casei is an aciduric, rod-shaped, facultatively heterofermentative lactic acid bacterium that produces lactic acid (approximately 95 % L-lactate and 5 % D-lactate) as the major end-product of carbohydrate fermentation [14]. *L. casei* is reported to grow and produce lactic acid at pH values as low as pH 4.0, but much higher levels of acid are produced when the fermentation pH is maintained at pH 5.0 [1, 7]. As the pH of the medium (pH_o) decreases or the concentration of lactate increases, the concentration of protonated (undissociated) lactic acid in the medium will also increase. The undissociated form

of lactic acid is membrane soluble, and can therefore enter the cytoplasm by simple diffusion [17]. Metabolically active bacteria maintain a pH gradient (ΔpH) where the intracellular pH (pH_i) is more alkaline than pH_o [2, 15], so diffusion of acid into the cytoplasm results in rapid dissociation and release of protons and anions inside the cell. If the rate of proton accumulation exceeds the cytoplasmic buffering capacity and capabilities of efflux systems, pH_i begins to fall and eventually reaches a critical point where the ΔpH can no longer be maintained, and cellular functions are impaired [2, 12]. Furthermore, intracellular accumulation of acid anions may be of greater importance than proton release in the inhibition of cell growth [4, 22, 23]. Thus, acidurance in *L. casei* likely involves resistance to both low pH and intracellular accumulation of organic acid anions.

To increase the industrial efficiency of *L. casei* for production of lactic acid, it is important to understand physiological attributes of this species that promote resistance to low pH and intracellular anion accumulation. The first line of defense against these and many other stress conditions in bacteria is the cell envelope. Research has demonstrated that exposure to acid or other stress conditions will frequently induce changes in the cytoplasmic membrane fatty acid (CMFA) composition (e.g., chain length, saturation, and cyclopropanation), and deliberate modification of the membrane lipid content can impart a stress-resistant phenotype [6, 9, 19, 29, 34]. Acid adaptation in *L. casei*, for example, is accompanied by a dramatic increase in the cyclopropane C19:0 content [3, 9, 33] and other changes that in combination are predicted to result in a cell membrane that is more rigid and less permeable to organic acids [19, 33].

Interestingly, addition of polysorbate (Tween) 80, a surfactant derived from polyethoxylated sorbitan and oleic acid (C18:1(n9)), to the growth medium has been shown to substantially increase survival of some lactobacilli, including *L. paracasei*, at pH 2.5 [5]. Membrane lipid analysis revealed cells grown in the presence of Tween 80 contained much higher levels of C18:1(n9) and cyclopropane than did cells grown in the absence of the surfactant [5]. This finding was in agreement with the work of others that found lactobacilli can incorporate at least some of the C18:1(n9) from Tween 80 into their cell membranes [13, 27], where it may be subsequently converted by cyclopropane synthase (Cfa) into the cyclopropane dihydrosterculic acid (C19:0 (n9)) [27]. As a whole, these studies support a prominent role for CMFA modification in acid resistance by *L. casei.* To the best of our knowledge, however, its effect on lactic acid production at low pH has not been described. Here, we evaluated the contribution of Tween 80 and Cfa to *L. casei* ATCC 334 lactic acid production and CMFA composition at low pH.

Materials and methods

Bacterial strains and growth conditions

Lactobacillus casei ATCC 334 and *Escherichia coli* strains DH5 alpha and EC1000 were maintained in a laboratory collection at −80 °C as glycerol stocks. Working cultures were prepared from stock cultures through two successive transfers (1 % inocula) in broth media at 37 °C. *L. casei* was propagated without shaking in All Purpose Tween (APT; Becton–Dickinson and Co., Franklin Lakes, NJ) or MRS (Becton–Dickinson) broth, and *E. coli* strains were grown with shaking in LB broth (Fisher Scientific, Pittsburgh, PA, USA).

Transformation of *Lactobacillus casei*

To make competent cells, a 10-ml fresh culture of *L. casei* ATCC 334 in MRS medium was prepared from a frozen cell stock by overnight (16–18 h) incubation at 37 °C. The cells were subcultured into 200 ml of pre-warmed MRS supplemented with 1 % glycine to an absorbance at 600 nm (A_{600}) of 0.1. The cells were grown to an A_{600} of 0.6, then harvested by centrifugation and rinsed twice in 200 ml cold (~4 °C), sterile distilled water. The pellet was gently stirred with a micropipette tip to form a cell slurry and 0.5-ml aliquots were transferred into sterile 1.5-ml microcentrifuge tubes. These cells were rinsed five more times with 1 ml of cold, sterile distilled water, then suspended in 0.5 ml of cold, sterile 30 % PEG-8000 and stored in 0.5-ml aliquots at −80 °C until needed.

Immediately prior to electroporation, competent cells were thawed and an equal volume of cold (4 °C), sterile water was added. The cells were held at room temperature for 30 min, then pelleted by centrifugation. The supernatant was discarded and the cells rinsed in 0.5 ml 30 % PEG-8000 then pelleted again. Fresh 30 % PEG-8000 was added to the final cell pellet to make a cell slurry with a total volume of 0.5 ml. DNA (0.5–10 μg in up to 20 μl TE buffer) was added to 0.1 ml of the cell slurry and the mix was transferred to a cold, sterile 0.2 cm electroporation cuvette (Bio-Rad, Hercules, CA, USA). Electrotransformation was performed in a Bio-Rad Gene Pulser set at 2,500 V, 25 μF, and 200 Ω. Following electroporation, 0.9 ml of cold (4 °C) recovery medium (0.5 M sucrose in MRS) was added and the cells were incubated in 1.5-ml microcentrifuge tubes at 37 °C for 4 h then plated on selective agar.

Construction of a *cfa* deletion mutant

To explore the contribution of Cfa on *L. casei* ATCC 334 membrane composition and acid production at low pH, the *cfa* gene was inactivated by gene replacement. A 456-bp

Table 1 PCR primers used in the study

Primer	Sequence (5′–3′)	Restriction site[a]
*cfa*5′-f	GTTTGAATTCATTGGCTAAAGCGGTCTGTG	*Eco*RI
*cfa*5′-r	GTTTCCCGGGGTCCTCAAAGTAGGCACAGGAATAAG	*Xma*I
*cfa*3′-f	GATTCACGGCATCACGCGACAACAAG	none
*cfa*3′-r	GTTTGAGCTCCATGGAAGAAGACCTTGTCGATGTCAG	*Sac*I, *Nco*I
*cfa*1	ATTTGTTATACACCTGTG	None
*cfa*2	AGTCATACCGAACATAAG	None
*cfa*3	AAGTGTGCGTTGATAATG	None
*cfa*4	ATGAAAGTATAAGGCAAG	None

[a] *Underlined sequence regions* depict introduced restriction enzyme recognition sites

(amino acids 144–295) in-frame deletion of the *S*-adenosylmethionine-binding site in the *cfa* ORF (LSEI_2108; NCBI accession number YP_807300) was generated from PCR products obtained with a Bio-Rad DNA Engine thermal cycler using a Phusion high-fidelity DNA polymerase kit (New England Biolabs, Ipswich, MA, USA) and Mastermix Taq DNA polymerase (5 Prime Inc., Gaithersburg, MD, USA). First, 5′ and 3′ fragments of the *cfa* gene were obtained by amplification of a 685-bp-long 5′ *cfa* DNA fragment with primers *cfa*5′-f × *cfa* 5′-r, which respectively contain *Eco*RI and *Xma*I restriction linkers, and a 674 bp *cfa* 3′ DNA fragment using primers *cfa* 3′-f × *cfa* 3′-r, with an *Nco*I linker in the 3′ reverse primer (Table 1). Ends of the *cfa*5′ and *cfa*3′ PCR products were filled in with Taq polymerase (5 Prime Inc.) then individually blunt-end ligated into the cloning vector pGEMT (Promega Inc., Madison, WI) and transformed by electroporation into *E. coli* DH5α. Transformants were selected by blue–white colony screening on LB agar with 50 μg/ml ampicillin, then plasmid DNA was collected from ampicillin-resistant (amp^R) colonies and sequenced to confirm the integrity of each *cfa* fragment and the presence of the desired restriction enzyme sites.

Next, the *cfa*5′ fragment was digested with *Eco*RI and *Xma*I and the *cfa*3′ fragment was cut with *Nco*I and *Xma*I (using the native *Xma*I site in *cfa* that lies 53 bp downstream of the *cfa*3′-f primer sequence). The two fragments were ligated together at the *Xma*I sites, and ligated into the multicloning region of vector pBS1, which had been double-digested with *Eco*RI and *Nco*I. Plasmid pBS1 is a smaller-sized derivative of the integration vector pCJK47 [16] that was created for this study by removing the *lacZ* gene. Briefly, a 3.5-kb fragment of pCJK47 carrying the origin of replication, multicloning site, and genes for erythromycin resistance (Ery^r) and the *Enterococcus faecalis* phenylalanyl-tRNA synthetase PheS (a counter-selectable marker that encodes sensitivity to 4-chloro-DL-phenylalanine) [16] was PCR-amplified using Phusion high-fidelity DNA polymerase (New England Biolabs), and the ends ligated at a newly generated *Cla*I restriction site. Like pCJK47, pBS1 will only replicate in a host cell that provides the RepA gene product in *trans* (e.g., *E. coli* EC1000) [18].

The ligated DNA was electroporated into *E. coli* EC1000 and transformants were selected on LB agar with 100 μg/ml erythromycin (Ery). Recombinant plasmid DNA was collected from Ery^r colonies and the purified plasmid's structure confirmed by PCR and by restriction enzyme digestion. Once confirmed, the recombinant plasmid, designated pBSΔ*cfa*, was transformed into *L. casei* ATCC 334 by electroporation and transformants were selected on MRS agar that contained 2.5 μg/ml Ery. Genomic DNA from Ery^r colonies was screened by PCR using primers *cfa*1 × *cfa*3 and primers *cfa*2 × *cfa*4 (Table 1) to identify merodiploids with pBSΔ*cfa* integrated into the wild-type *cfa* locus via a single-crossover event, generating a cell that produced both a wild-type and a deleted copy of the *cfa* gene. Several merodiploid isolates were individually propagated overnight in 1 ml MRS broth with 2.5 μg/ml Ery, then passaged three times in the absence of selection (10 μl of cell culture added to 1 ml of MRS without Ery), and incubated 24 h at 37 °C. Following the third non-selective passage, 100 μl from serial dilutions of the cells (10^{-5}, 10^{-4}, 10^{-3}) were plated on BHI (Becton–Dickinson) agar that contained 7 mM 4-chloro-DL-phenylalanine (BHI-ClPhe; Sigma-Aldrich, Inc. St. Louis, MO, USA), which selects against the PheS activity encoded by pBS1 (and pCJK47) [16]. Fifty microliters of the 10^{-5} dilution was plated on MRS agar as a control.

Lactobacillus casei colonies that had lost the integration vector pBS1 through a second crossover event were usually visible after 2 days incubation at 37 °C, so colonies that appeared after 2 days were transferred from BHI-ClPhe plates onto MRS and MRS with 2.5 μg/ml Ery agar plates to confirm loss of the Ery^r marker, and hence the vector. Approximately 30 Ery-sensitive colonies were collected and incubated separately in 1 ml MRS broth at 37 °C overnight. Frozen cell stocks were prepared from these cell cultures and stored at −80 °C. A 100-μl aliquot was collected from a freshly prepared sample of each isolate and the cells lysed by successive treatment with lysozyme (0.5 μg/ml, 30 min, 37 °C) and proteinase K (0.5 μg/ml, 1 h, 55 °C).

The lysed cell suspension was twice extracted with 1 volume of chloroform, and ethanol precipitated to recover genomic DNA. The DNA was screened once more using PCR primers *cfa*1 × *cfa*3 and *cfa*2 × *cfa*4 to distinguish clones that had retained the wild-type *cfa* gene (1.5- and 1.4-kb amplicons, respectively) from those in which the wild-type gene had been replaced with the deleted version of *cfa* (1.0- and 0.9-kb fragments, respectively). DNA sequencing from the 5′-flanking DNA upstream of the deletion to a point downstream using primers *cfa*1 and *cfa*3, and from the 3′-flanking DNA downstream of the deletion to a point upstream using primers *cfa*2 and *cfa*4 (Table 1) confirmed the desired in-frame deletion was the only change to the *L. casei cfa* coding sequence. After growth curves for three independently isolated mutants and the WT strain in APT broth were found to be similar, one of the mutants was selected for further use in the study and designated *L. casei* 334:Δ*cfa*.

Lactic acid production under pH control

Batch cultures of wild-type (WT) *L. casei* ATCC 334 and the 334:Δ*cfa* mutant were prepared by dilution of a fresh working culture to an A_{600} of 1.0 in APT medium modified to contain 3 % glucose (mAPT), then inoculated at 1 % (vol/vol; initial cell concentration ~5 × 10^6 to 1 × 10^7 cfu/ml) into 1 l of mAPT and 1 l mAPT plus 1.8 % Tween 80 (Sigma-Aldrich), adjusted to pH 6.5, and both vessels placed in a Sartorius Stedim Biostat Bplus dual controlled biofermenter (Sartorius AG, Goettingen, Germany). The two culture samples were incubated at 37 °C with an agitation rate of 100 rpm to prevent sedimentation and a 5 % CO_2 and 95 % N_2 gas mixture was continuously passed over the headspace of the fermenter to maintain anaerobic conditions. The cells were allowed to acidify the medium until the desired pH of 4.5, 4.0, or 3.8 was achieved, after which it was maintained at that set point by automatic addition of 15 % (vol/vol) NH_4OH. The cultures were incubated to substrate exhaustion as indicated by the cessation of base addition. The entire experiment was repeated on three separate occasions, and average time to substrate exhaustion calculated. Student's *t* test was used for pairwise comparisons between means to determine significant differences ($\alpha = 0.05$) between samples [11].

Organic acid measurement

For organic acid measurement, 10-ml samples were collected at time 0 (inoculation) and after substrate exhaustion. Samples were centrifuged at 5,000×*g* and the supernatant passed through a 2.5-μm syringe-mounted filter (VWR International, Radnor, PA, USA) to remove cellular debris and stored at −20 °C until needed. D-lactic acid/L-lactic acid or acetic acid concentrations were measured using commercial enzymatic test kits (R-Biopharm AG, Darmstadt, Germany). Samples were thawed and diluted 1:100 in sterile HPLC-grade water, then assayed according to the manufacturer's instructions. A Student's *t* test was used for pairwise comparison of means from triplicate experiments to determine significant differences ($\alpha = 0.05$) in acid concentrations between treatments or cell types. The final acid concentration and time for substrate exhaustion were fitted into a least-squares linear regression model to determine the acid production rate, then the 95 % confidence interval of the slopes was used to determine significant differences between the strains [11].

CMFA Analysis Cells were grown in batch culture as described, on two separate occasions, and 20-ml samples were taken at the midpoint of base addition (between time zero and the time for substrate exhaustion). The cells were collected by centrifugation at 5,000×*g* for 5 min and then washed twice with phosphate buffered saline. The CMFAs were isolated from the cell mass according to the protocol of Sasser [25] and identified using gas chromatography as described previously [21]. Mean amounts of individual fatty acids (FA) were calculated as a percent of total from replicate extractions, and a two-tailed Student's *t* test was used to determine if there were significant differences in mean FA content of different samples.

Results

Influence of Tween 80 supplementation on lactic acid production

There were no significant differences ($p > 0.05$) in the yields of L- and D-lactate or acetate by *L. casei* WT or 334:Δ*cfa* cells grown in mAPT broth after fermentation at any of the three pH values tested (Fig. 1). However, the time period before cessation of acid production by WT cells (i.e., the time when automatic base addition in the fermenter ceased) increased significantly ($p < 0.05$) as pH was reduced (Table 2). The rate of acid production by WT cells also declined significantly with each successive drop in the fermentation pH (Table 3). Similar trends were noted with 334:Δ*cfa* cells, which also showed the lowest rates for acid production, but the effects were not statistically significant (Tables 2, 3).

Increasing the Tween 80 content of mAPT broth from 0.02 % (the amount normally present in APT) to 0.2 % did not affect acid yields or improve the rate of acid production from WT cells at pH 4.5 or 4.0 (Fig. 1; Table 3). At pH 3.8, however, the amount of lactic acid produced by WT cells was significantly higher than any other treatment (Fig. 1), and the rate of acid production also improved

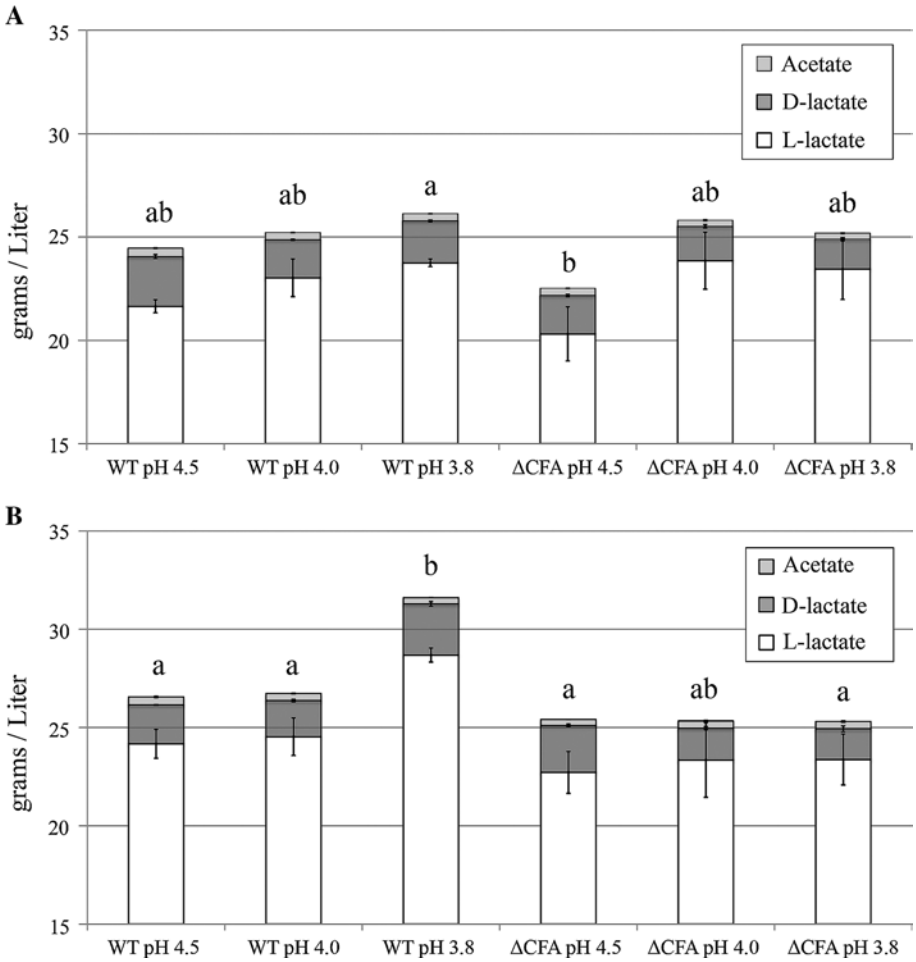

Fig. 1 Concentrations of organic acids produced by *Lactobacillus casei* strains ATCC 334 (WT) and 334:Δ*cfa* (ΔCFA) grown at different pH in mAPT broth with 0.02 % (**a**) or 0.2 % Tween 80 (**b**). *Error bars* correspond to the standard error of the mean, and *different letters* for the same treatment across panels denote treatments that produced significantly different levels of lactic acid ($p < 0.05$)

Table 2 Time before acid production stopped (h)

Treatment	pH		
	4.5	4.0	3.8
L. casei ATCC 334 in mAPT broth*	24.6 ± 6.2^{a}	47.6 ± 9.1^{b}	125.5 ± 6.2^{df}
L. casei ATCC 334 in mAPT with 0.2 % Tween 80	33.6 ± 8.2^{ab}	71.0 ± 8.1^{c}	97.0 ± 5.0^{ef}
L. casei 334:Δ*cfa* in mAPT broth	73.9 ± 6.2^{d}	100.6 ± 16.4^{ef}	154.8 ± 33.8^{df}
L. casei 334:Δ*cfa* in mAPT broth with 0.2 % Tween 80	54.73 ± 1.1^{c}	90.0 ± 14.0^{cef}	130.8 ± 36.7^{def}

As indicated by the cessation of automatic base addition in the fermenter. Values across columns and rows with different superscript letters are significantly different ($p < 0.05$)

* APT broth normally contains 0.02 % Tween 80

over that seen in mAPT at this pH (Table 3). In contrast, lactate yields from the *cfa* mutant grown at 4.0 or 3.8 in mAPT with 0.02 or 0.2 % Tween 80 were not significantly different (Fig. 1). However, increasing the Tween 80 content of mAPT to 0.2 % did result in a significant increase in acid yield from the mutant at pH 4.5, but the final level was not different from that obtained from these or WT cells at pH 4.0 or 3.8 in medium with either Tween concentration (Fig. 1). Moreover, the rate of acid production by the Δ*cfa* mutant was slower than that for WT cells at all three pH values (Table 3). Collectively, these data demonstrate that the stimulatory effect of Tween 80 supplementation on lactic acid production by *L. casei* ATCC 334 at pH 3.8 requires Cfa activity.

Table 3 Acid production rate

Treatment	pH		
	4.5	4.0	3.8
L. casei ATCC 334 in mAPT broth*	1.04 ± 0.30^{a}	0.52 ± 0.07^{g}	0.21 ± 0.01^{bef}
L. casei ATCC 334 in mAPT with 0.2 % Tween 80	0.73 ± 0.10^{a}	0.37 ± 0.03^{h}	0.32 ± 0.01^{cde}
L. casei 334:Δ*cfa* in mAPT broth	0.30 ± 0.03^{de}	0.25 ± 0.03^{de}	0.16 ± 0.04^{bef}
L. casei 334:Δ*cfa* in mAPT broth with 0.2 % Tween 80	0.46 ± 0.13^{bcd}	0.29 ± 0.07^{bcde}	0.19 ± 0.13^{be}

Calculated as grams of lactic acid per hour from time zero until the cessation of acid production. Values across columns and rows with different superscript letters are significantly different ($p < 0.05$)

* APT broth normally contains 0.02 % Tween 80

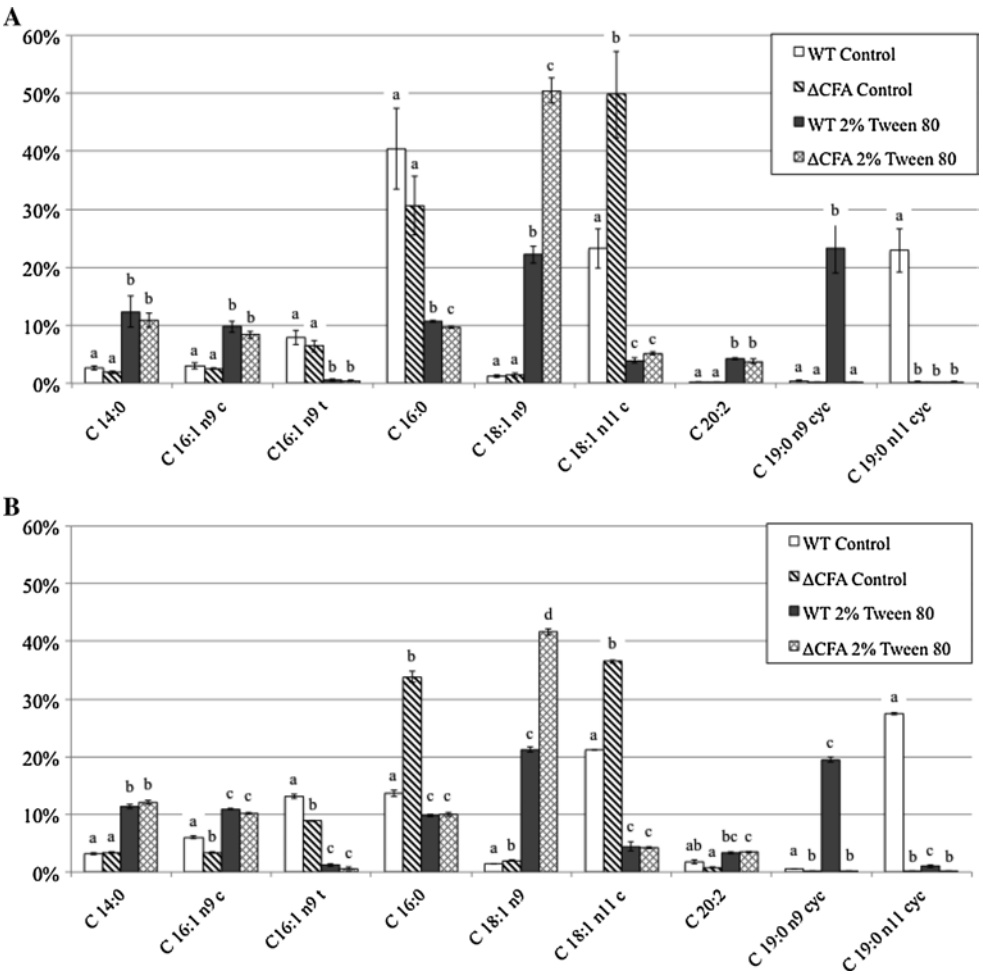

Fig. 2 Membrane fatty acid composition for *Lactobacillus casei* strains ATCC 334 (WT) and 334:Δ*cfa* (ΔCFA) grown in mAPT broth at pH 4.0 (**a**) or 3.8 (**b**). *Error bars* correspond to the standard error of the mean, and *different letters* within each lipid type indicate that the means differ significantly ($p < 0.05$)

Influence of *cfa* inactivation and Tween 80 addition on membrane lipid composition

The most abundant CMFAs in WT cells incubated in mAPT broth at pH 4.0 were palmitic acid (C:16:0), vaccenic acid (C18:1 (n11)), and lactobacillic acid (C19:0 (n11) (Fig. 2a). Membranes from these cells also contained low levels of cis- and trans-palmitoleic acid (C16:1 (n9)), myristic acid (C14:0), and a very small percentage of oleic acid (C18:1 (n9)), the lipid present in Tween 80. Propagation at pH 4.0 with 0.2 % Tween produced essentially stoichiometric shifts in the CMFA percentages of C18:1 (n11) and C19:0 (n11) into C18:1 (n9) and dihydrosterculic acid (C19:0 (n9)), respectively, as well as dramatic and significant reductions in the C:16:0 and C16:1 (n9 trans) contents (Fig. 2a). Additionally, CMFAs from WT cells grown in

Table 4 Net membrane lipid composition of *L. casei* cells grown in mAPT broth at pH 4.0 or 3.8

	L. casei ATCC 334		*L. casei* 334:Δ*cfa*	
	Control	0.2 % Tween 80	Control	0.2 % Tween 80
pH 4.0				
% Cyclopropane	21.8 ± 5.5	23.8 ± 4.3	ND	ND
% Saturated	41.8 ± 3.2	25.9 ± 4.0	35.0 ± 5.9	23.2 ± 1.0
% Unsaturated	58.2 ± 3.2	74.1 ± 4.0	65.0 ± 5.9	76.8 ± 1.0
Saturated/unsaturated	0.72 ± 0.09	0.35 ± 0.07	0.54 ± 0.14	0.30 ± 0.02
pH 3.8				
% Cyclopropane	28.2 ± 0.3	20.9 ± 0.2	ND	ND
% Saturated	18.3 ± 0.5	25.3 ± 0.5	40.5 ± 0.9	26.3 ± 1.0
% Unsaturated	81.7 ± 0.5	74.7 ± 0.5	59.5 ± 0.9	73.7 ± 1.0
Saturated/unsaturated	0.22 ± 0.01	0.34 ± 0.01	0.68 ± 0.03	0.36 ± 0.02

Percentage of cyclopropane fatty acids (FAs) (C19:0[n9cyc] and C19:0[n11cyc]), saturated FAs, and unsaturated FAs (including cyclopropanes) in the membrane, and ratio of saturated to unsaturated FAs

ND not detected

mAPT with 0.2 % Tween showed significantly higher levels of C16:1 (n9 cis) and C14:0 (Fig. 2a). Overall, the CMFA content of cells incubated in mAPT with 0.2 % Tween had a substantially lower saturated FA content than cells grown in mAPT with only 0.02 % Tween, and correspondingly higher unsaturated FA content (cyclopropanes were included in this category), so that the saturated:unsaturated FA ratio was reduced by 50 % (from 0.72 to 0.35, respectively) (Table 4).

As expected, membrane lipid profiles from 334:Δ*cfa* cells grown at pH 4.0 or 3.8 in the presence of 0.02 or 0.2 % Tween 80 confirmed *cfa* inactivation in *L. casei* ATCC 334 abolished cyclopropane (C19:0 (n9) and C19:0 (n11)) formation (Fig. 2; Table 4). Otherwise, lipid profiles from 334:Δ*cfa* cells grown in mAPT with 0.02 or 0.2 % Tween 80 at either pH showed their CMFA content largely mimicked that of their WT counterparts, except that the percentage of C18:1 (n11) or C18:1 (n9) in mutant cells was roughly equal to the sum of that lipid plus its cognate cyclopropane in the respective WT cell treatment (Fig. 2). As is shown in Table 4, the observed changes in CMFA content of 334:Δ*cfa* cells grown at pH 4.0 in mAPT with 0.02 or 0.2 % Tween 80 each resulted in a lower saturated:unsaturated FA ratio than was seen in WT cells grown under parallel conditions.

When *L. casei* cells were grown at pH 3.8 instead of pH 4.0, most of the CMFA changes noted in response to the different Tween 80 concentrations at the higher pH were retained. The only prominent change was a dramatic and significant decrease in the percentage (37 versus 14 %) of C16:0 in WT cells incubated at pH 3.8 in mAPT with 0.02 % Tween (Fig. 2). A decrease in *L. casei* CMFA C16:0 content at lower pH was also reported by Fozo et al. [9]. Interestingly, this change was not observed in the mutant strain, 334:Δ*cfa*, when it was incubated under the same conditions. The net effect of all CMFA changes in WT cells grown in mAPT with 0.02 % Tween was such that the saturated:unsaturated FA ratio fell from 0.72 to 0.22, while the saturated:unsaturated FA ratio in cells grown in mAPT with 0.2 % Tween was virtually unchanged (0.35 versus 0.34) (Table 4). In contrast, the saturated:unsaturated FA ratio in 334:Δ*cfa* cells was higher at pH 3.8 versus pH 4.0 at both Tween 80 levels (Table 4).

Discussion

Fermentation is the most common method for industrial production of lactic acid, and the efficiency of end product extraction increases at lower pH [31, 32]. For this reason, aciduric bacteria such as *L. casei* show exciting potential as biocatalysts for industrialized L-lactic acid production [7, 30, 32]. This study sought to explore physiological attributes of *L. casei* that might promote acid-producing ability at low pH.

Results show that *L. casei* ATCC 334 can produce lactic acid in mAPT broth at pH 3.8, and that this ability does not require Cfa activity (Fig. 1a). Total acid yields also were not significantly affected by *cfa* deletion (Fig. 1), but the rate of acid production in mAPT broth by 334:Δ*cfa* cells was lower than that seen with WT cells under comparable conditions (Table 3). Interestingly, supplementation of the growth medium with 0.2 % Tween 80 slowed the rate of acid production by WT cells at pH 4.5 or 4.0, but this effect was not observed with 334:Δ*cfa* cells incubated at pH 4.5, 4.0 or 3.8 (Table 3). More importantly, addition of 0.2 % Tween significantly enhanced lactic acid yield from WT cells at pH 3.8, but not from the mutant strain 334:Δ*cfa* (Fig. 1), and also provided a modest (but not significant) increase to the rate of acid production in both strains at pH 3.8 (Table 3). Collectively, these observations indicate both Cfa activity and an extracellular surplus of oleic acid contributed to the significant increase in lactic acid yield and improved rate of lactic acid production that were observed with *L. casei* ATCC 334 at pH 3.8.

Because the cell envelope is the first line of defense against acid stress and Cfa and Tween 80 are each known to influence CMFA composition in lactobacilli [27], we analyzed the membrane lipid content of *L. casei* WT and 334:Δ*cfa* cells incubated under the different treatment conditions. As expected, *L. casei* WT cells incubated in mAPT with 0.2 % Tween 80 at pH 4.0 or 3.8 showed a CMFA content that was significantly enriched in oleic acid (C18:1(n9) and its cyclopropane derivative dihydrosterculic acid (C19:0 (n9)) versus WT *L. casei* grown in mAPT with 0.02 % Tween (Fig. 2). However, these changes did not produce a notable increase in the CMFA cyclopropane content of *L. casei* because WT cells grown in mAPT with 0.02 % Tween instead contained similarly high percentages of vaccenic acid (C18:1 (n11)) and the cyclopropane derivative of that FA, lactobacillic acid (C19:0 (n11). In *E. coli*, the ability to synthesize cyclopropane has been shown to decrease membrane permeability to protons as well as increase the bacterium's ability to extrude H^+ [26]. Data in Tables 2 and 3 suggest Cfa inactivation in *L. casei* had a negative effect on cell fitness at low pH and, as noted above, the ability to synthesize cyclopropane was essential for the significant boost in lactate production by WT cells at pH 3.8 in mAPT with 0.2 % Tween (Fig. 1).

In addition to the shift from vaccenic to oleic acids (and the accompanying shift in their respective cyclopropanes), incubation at pH 4.0 or 3.8 in the presence of 0.2 % Tween produced other significant changes in the CMFA profile that dramatically altered saturated:unsaturated FA ratio relative to WT cells grown in mAPT with 0.02 % Tween (Fig. 2; Table 4). Alteration of the saturated:unsaturated FA ratio via modification of the CMFA profile is the primary mechanism by which bacteria adjust cytoplasmic membrane fluidity [8, 10, 24], and our data suggest that this ratio in *L. casei* was influenced more by the presence of 0.2 % Tween 80 than by fermentation pH or Cfa activity (Table 4).

Changes in the CMFA composition in bacteria occur through a combination of de novo FA biosynthesis and modification of existing lipid membrane phospholipid acyl chains [34, 35]. The alterations noted in this study (Fig. 2) can be attributed to: (1) incorporation of exogenous oleic acid when cells were incubated with 0.2 % Tween; (2) modification of oleic or vaccenic acids into their cognate cyclopropane derivatives by Cfa; and (3) de novo FA biosynthesis. Because de novo bacterial FA biosynthesis is an energy intensive process [34], wholesale replacement of endogenously made vaccenic acid with freely available exogenous oleic acid would afford a substantial energy savings to cells grown in mAPT with 0.2 versus 0.02 % Tween. In previous work, we presented evidence that acid tolerance in *L. casei* was energetically costly due to the need for de novo FA biosynthesis in order to alter CMFA composition [3]. Results from this work suggest that the energy savings afforded by an abundance of exogenous oleic acid drives the saturated:unsaturated FA ratio of *L. casei* at pH 4.0 or 3.8 and, together with Cfa, allows these cells to modify their cell envelope in a manner that enabled a significant increase in lactic acid yield and modest improvement in the rate of lactic acid production at pH 3.8 (Fig. 1; Table 3).

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