Transcriptional analysis of the cyclopropane fatty acid synthase gene of *Lactococcus lactis* MG1363 at low pH

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Abstract

Cyclopropane fatty acid synthase (cfa) catalyses the transfer of a methyl group from S-adenosylmethionine (SAM) to unsaturated fatty acids. Northern blot experiments demonstrated that the *Lactococcus lactis* MG1363 *cfa* gene is mainly expressed as a bicistronic transcript together with *metK*, the gene encoding SAM synthetase, and is highly induced by acidity. The *cfa* promoter was characterized by 5′-RACE PCR, and fused to β-galactosidase by cloning into the pAK80 plasmid. This transcriptional fusion was highly induced by acidity (23-fold at pH 5) as well as during entry into the stationary phase (8-fold) in *L. lactis*. Interestingly, the *cfa* promoter expression is repressed in a *L. lactis* relA* mutant which accumulates (p)ppGpp, whereas its induction by acidity appeared independent of (p)ppGpp in *L. lactis* and in *Escherichia coli*.

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1. Introduction

Bacterial membranes constitute the interface between the neighbouring environment and the intracellular medium, and consequently play a major role in homeostasis and in adaptation to atypical conditions. Accordingly, bacteria modify their fatty acid composition in response to changing temperature, pH, osmolality, exposure to solvents or other stress conditions in order to modify the membranes physico-chemical properties [1]. One of these modifications involves adding a methylene group, derived from the methyl group of S-adenosyl-L-methionine (SAM), to unsaturated fatty acids to form a cyclopropane ring, a reaction catalysed by a cyclopropane fatty acid (CFA) synthase [2].

The changes in the physico-chemical properties of membranes conferred by CFAs are not well characterized [2]. However, evidence exists to suggest that CFAs might play a role in bacterial adaptation to environmental stresses. Indeed, the proportion of CFAs is modified in stressed cells, and strains with altered CFA synthase activity exhibit altered phenotype towards diverse stressing situations. For example, the CFA content increases during heat shock [3] or osmotic stress [4] in *Lactococcus lactis*, and during acid stress in *Pectinatus* sp. and *Escherichia coli* [5,6].
Furthermore, CFA defective mutants of *E. coli* and *Salmonella enterica* sv. *typhimurium* are highly sensitive to acid stress [7,8].

We recently carried out a proteomic analysis of the acid tolerance response of *L. lactis* MG1363, and showed that the CFA synthase was induced during exposure to pH 5 [9]. Therefore, we have characterized the corresponding *cfa* gene, studied its expression in acid conditions and in a *relA* mutant (VI6963) which was previously shown to accumulate ppGpp and to be acid tolerant [10].

### 2. Materials and methods

#### 2.1. Bacterial strain, plasmids and growth conditions

*L. lactis* ssp. *cremoris* strain MG1363 [11] and derivatives were grown at 30 °C in M17 [12] or M17/7 (see below) supplemented with 0.5% glucose. M17/7 corresponded to M17 in which β-glycerophosphate was replaced by MOPS (200 mmol l⁻¹) and with the pH adjusted to 7.2 with NaOH. M17/5 corresponded to M17/7 acidified with HCl to pH 5. *E. coli* NM522 and EC101 were grown at 37 °C on LB medium adjusted to pH 7 (LB/7) or eventually acidified with HCl at pH 5.5 (LB/5.5). For solidified media, agar was added at 15 g l⁻¹. The plasmids used were pAK80 [13], pALS10 and pALS14 [14]. Antibiotics were added when necessary at the following concentrations: ampicillin, 100 µg ml⁻¹; erythromycin (Em), 150 µg ml⁻¹ for *E. coli* and 2.5 µg ml⁻¹ for *L. lactis*. Growth was monitored spectrophotometrically at 600 nm.

#### 2.2. DNA manipulations and cloning

The *cfa* promoter was amplified by PCR using oligonucleotides Ptcfa_F: 5'-CTTGTCGGTCTAAATT-TAGC-3' and Ptcfa_R: 5'-CTGTTTTTTCAAAATAC-CAAG-3', and cloned upstream of β-galactosidase in the pAK80 plasmid using the *SmaI* restriction site [13]. The resulting plasmid (pAK80_Ptcfa) was used to transform *E. coli* EC101 and transformants were selected on LB medium agar plates containing Em and X-Gal (100 µg ml⁻¹). The orientation of the *cfa* promoter insert was verified by PCR amplification using oligonucleotide Ptcfa_R and the oligonucleotide pAK801: 5'-AAGCTTTCGGCAGGACTCG-3'. The pAK80_Ptcfa plasmid was then transformed into *L. lactis* MG1363 or VI6963 strains and transformants were selected on M17 medium agar plates containing Em and X-Gal. Putative positive clones were verified by PCR amplification using oligonucleotides Ptcfa_R and pAK801. Control strains were obtained by transformation of the pAK80 plasmid into *L. lactis* MG1363 or VI6963.

#### 2.3. β-Galactosidase assays

β-Galactosidase assays were performed with cultures maintained in logarithmic growth for more than 14 h, which were obtained as follows. Dilutions (10⁻²–10⁻⁸) of a resuspended fresh colony were used to inoculate cultures in M17/7 for *L. lactis* and LB/7 for *E. coli*. After overnight growth at 30 °C, the culture with an OD₆₀₀ below 0.3 was diluted to a final OD₆₀₀ of 0.01 in fresh medium and incubated until 0.1. Cells were harvested by centrifugation and resuspended in the same volume of M17/7, M17/6 or M17/5 for *L. lactis* and LB/7 or LB/5.5 with or without 100 µmol l⁻¹ IPTG for *E. coli*. The β-galactosidase activity was determined by the method of Miller [15]. At different time points, aliquots of cells were adjusted to an OD₆₀₀ of about 10, harvested by centrifugation for 10 min at 5000 rpm at 4 °C and resuspended in 1 ml of Z-Buffer. Cells were disrupted using a One Shot Cell Disrupter (Constant Systems) at 2.7 kbar and debris were removed by centrifugation (13,000 rpm, 1 min). The β-galactosidase activity was measured by adding 200 µl of ONPG (4 mg ml⁻¹) to 50 µl of supernatant and 750 µl of Z-Buffer. After 2 min, the OD was recorded at 420 nm during 10 min (UVikon 940 Spectrophotometer, Kontron Tek Instruments). The β-galactosidase activity was expressed in nmol min⁻¹ mg⁻¹ of proteins. Bradford proteins assays were performed in the same time [16].

#### 2.4. Transcriptional analyses and 5'RACE PCR

Total RNA was extracted from *L. lactis* MG1363 cells cultured in M17/7 or M17/5 using the RNeasy Midi Kit (Qiagen), and treated with DNase. For northern blot experiments, 5 µg of total RNA was electrophoresed and blotted onto Hybond-N+ membranes according to standard procedures [17]. Membrane-bound nucleic acids were hybridized with a *cfa* single-strand labelled probe prepared by PCR as described by Rince et al. [18], using 10 ng of the PCR product obtained with *cfa_F*: 5'-CACCTGCAATGTGCTTCGTCAATGTC-3' and *cfa_R*: 5'-TCCAGGATCGTCAAAATAC-3' as template DNA, only the reverse primer (*cfa_R*), and 2 µCi of [α-³²P]dATP. Dried membranes were exposed to a storage phosphor screen (Packard Instrument Company) for 2–5 h and quantification was carried out using the OptiQuant Image Analysis Software (Packard Instrument Company). 5'-RACE PCR was carried out as described previously [19].
3. Results and discussion

3.1. The cfa and metK genes form an operon

In the genomic sequence of *L. lactis* IL1403 [20] available at www.tigr.org, the cfa gene appears to be divided into two contiguous orfs (TIGR loci names: NT01LL2285 and NT01LL2286), both annotated as cfa-2. We amplified and sequenced this locus in the IL1403 and MG1363 strains, and determined that this apparent frameshift was due to the absence of a Guanine residue at position 881 in the published sequence (data not shown).

The cfa gene is flanked by birA2, encoding a biotin-acetylCoA-carboxylase ligase, transcribed divergently, and metK, encoding S-adenosylmethionine synthetase, transcribed in the same orientation. We identified a putative ρ-independent transcription terminator (T1) in the 427-bp cfa-metK intergenic region \(ΔG^0 = -13 \text{ kcal mol}^{-1}\), and another one upstream of metK (T2, \(ΔG^0 = -18.8 \text{ kcal mol}^{-1}\)).

The genetic structure and the expression of the cfa locus was first analyzed by northern blot experiments. Total RNA was extracted from *L. lactis* MG1363 cultivated in M17 buffered medium adjusted with HCl to pH 7 or 5. Hybridization of a cfa probe revealed a main transcript of 3 kb, which strongly increased under acidic conditions (Fig. 1). At pH 5, a minor 1.2 kb transcript appeared, which would correspond to the size expected for a monocistronic expression of cfa (Fig. 1).

The size of the main transcript suggested that (i) cfa would be mainly cotranscribed with metK or (ii) the cfa promoter would be located 1.8 kb downstream of the translational start. Therefore, we both mapped the cfa promoter and hybridized a metK probe to the membranes. 5′RACE PCR led us to demonstrate that the cfa mRNA begins at a G residue, located 28 nucleotides downstream of the TTG translational start point (Fig. 2). This nucleotide is preceded by both a putative −35 (TTGACT) and a −10 (TATAGT) box (Fig. 2).

Hybridization of a metK probe on the northern blots revealed both a 3 kb- and a 1.5 kb-transcript (data not shown). The 1.5 kb transcript was more abundant than the 3 kb one at pH 7, but the amounts of both mRNAs increased sharply after exposure to pH 5. Together, these results demonstrated that (i) cfa and metK form an operon, (ii) metK is mainly transcribed monocistronically, and (iii) all transcripts appeared induced by acid growth.

3.2. The cfa promoter is transcriptionally up-regulated by acidity and growth phase in *L. lactis* MG1363

Northern blot experiments demonstrated that the amounts of the cfa- and cfa-metK-mRNAs increased
during exposure to acidic pH. To determine whether the induction occurred at the transcriptional level, and to facilitate the expression study, we cloned the promoter region of cfa upstream of the promoterless β-galactosidase gene contained in the pAK80 plasmid, thus creating a transcriptional fusion (pAK80_Ptcfa). The resulting plasmid was transformed into L. lactis MG1363, and the β-galactosidase activity was monitored during the growth in buffered M17 media adjusted to pH 7, 6 and 5.

None of the control strains (containing the pAK80 without the cloned promoter) displayed significant β-galactosidase activity (Fig. 3). When cultured at pH 7, the activity remained low during the exponential phase of growth, and increased 8-fold at the onset of the stationary phase (Fig. 3). It is noteworthy that the pH of the buffered M17/7 medium did not vary along the bacterial growth in our experimental conditions. In cells cultured at pH 6 and pH 5, the activity at 180 min of growth was 20- and 23-fold higher than at neutral pH, respectively (Fig. 3), and decreased in stationary phase (but remained higher than that measured in stationary phase cells at pH 7) (Fig. 3). These data demonstrate that the cfa promoter of L. lactis MG1363 is regulated at the transcriptional level by both acidity and the growth phase.

3.3. ppGpp regulates the cfa promoter expression but not its induction by acidity in L. lactis

In E. coli and several other bacteria, the abundance of CFAs increases in membranes when cells enter the stationary phase, or when subjected to various stress conditions including acid shock [1]. The stress and growth phase regulation mechanisms have been best studied in E. coli, in which different regulation levels were identified, including increased expression of the cfa gene [7], and the stability of the CFA synthase protein [21,22]. The transcriptional up-regulation of the cfa gene in E. coli was shown to be indirectly dependent on ppGpp, via the ppGpp-dependent growth-phase- and stress-activation of the σS sigma factor [23].

Interestingly, while cloning the cfa promoter in pAK80, we first transformed the resulting plasmid into E. coli EC101, and observed that the resulting strain degraded X-Gal. We examined the strain more closely, and determined an approximate 5.5-fold increase in β-galactosidase activity in acid-adapted cells (data not shown). This observation was particularly surprising because L. lactis lacks global stress sigma factors homologous to σS, so the acid regulation of cfa in L. lactis necessarily differs from that of the E. coli cfa gene. The lone regulatory mechanisms established to date for acid and general stress response in L. lactis are related to the Phosphate and GP pools, including ppGpp [10], which are known to indirectly regulate cfa transcription in E. coli [23]. As a consequence, we attempted to determine whether the acid induction of the L. lactis cfa promoter, observed in both E. coli and L. lactis, was dependent on (p)ppGpp. We transformed the pAK80_Ptcfa plasmid into E. coli NM522 containing pALS10 which allows IPTG-dependent relA overexpression and subsequent (p)ppGpp accumulation in E. coli, or pALS14 (in which a non functional truncated relA gene was cloned) as a control [14]. After 60 min, the acid induction of the fusion was observed in E. coli NM522 harbouring pALS10 and pALS14 plasmids, but we did not observe a significant difference between the two strains, even after IPTG addition (induction factors of 3.4- and 3.6-fold, respectively) (Fig. 4). These results led us to conclude that (p)ppGpp does not influence the activity of the L. lactis cfa promoter (directly or indirectly) in E. coli.
To verify if (p)ppGpp influences the expression of the cfa promoter in *L. lactis*, the pAK80_Ptcfa plasmid was transformed into *L. lactis* VI6963, an acid-resistant mutant previously obtained by Rallu *et al.* [10]. This mutant expresses a truncated active RelA protein, leading to the accumulation of (p)ppGpp [10]. As shown in Fig. 5, the activity of the fusion was 2-fold lower in the VI6963 strain than in the wild-type strain, both in neutral and in acid conditions. However, the induction by acidity was revealed to be quite similar (about 30-fold) in the VI6963 and MG1363 strains (Fig. 5). Altogether, our results suggest that the cfa locus is an essential component of the acid stress response of *L. lactis* MG1363.

Several changes in cell membrane composition were previously shown to be involved in adaptation of Lactic Acid Bacteria (LAB) to acidity [24]. However, although cyclopropanation of fatty acids was shown to be essential for acid resistance in *E. coli* and *S. enterica* [7,8], and CFA amounts in membranes of numerous LABs increase under various other stress situations [3,4], the link between acid resistance and CFA synthesis in LAB was not so obvious. Indeed, an increase in CFA amount during acid adaptation was only demonstrated in *Pectinatus* Sp. and *Lactobacillus casei* [5,25], while this was not detected in *S. mutans* and other streptococci [25,26].

As an understanding of the acid-stress regulation of genes is of particular importance in LAB [27,28], future efforts will aim to identify regulators implied in the acid regulation of the studied promoter.

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### References


