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Synthesis of cyclopropane fatty acids in *Lactobacillus helveticus* and *Lactobacillus sanfranciscensis* and their cellular fatty acids changes following short term acid and cold stresses

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ABSTRACT

An implemented GC method to separate and quantify the cell cyclopropane fatty acids lactobacillic (C19cyc11) and dehydrosterculic (C19cyc9) was used to study the adaptive response to sublethal acid and cold stresses in *Lactobacillus helveticus* and *Lactobacillus sanfranciscensis*. The comparison of the composition of cellular fatty acids of the two strains and their changes after 2 h of stress exposure under micro-aerobic and anaerobic conditions indicated that the aerobic biosynthetic pathway for unsaturated fatty acids is prevalent in *L. sanfranciscensis*, while the anaerobic pathway is prevalent in *L. helveticus*. Indeed in the latter strain, in the presence of a source of oleic acid and under micro-aerobic conditions, C18:1n11 and its post-synthetic derivative C19cyc11 accounted for overall proportion ranging from 52 to 28% of the total FAs. On the other hand *L. sanfranciscensis* synthesizes by aerobic pathway C18:1n9 and transforms it to C19cyc9. However in this species the cumulative level of these two FAs did not exceed 30%. The relevant proportion of dodecanoic acid in the latter species suggests that carbon chain shortening is the principal strategy of *L. sanfranciscensis* to modulate fluidity or chemico-physical properties of the membranes.

1. Introduction

Bacterial cytoplasmic membranes can be considered at the same time as the Achilles heel and the strong point for the microbial survival in the growth environment. In fact, one of the most important adaptive microbial response to stress exposure, in addition to the synthesis of specific proteins (Wouters et al., 1999, 2001), is related to changes in membrane fatty acid (FA) composition. The adaptive strategies include alterations in saturation degree, carbon chain length, branching position, *cis*/*trans* isomerisation and conversion of unsaturated fatty acids (UFAs) into cyclopropanes (Kaneda, 1977; Russell, 1984; Heipieper et al., 1992; Loffeld and Keweloh, 1996). In particular, the ratio of unsaturated vs saturated fatty acids (USFAs ratio) of the membrane lipids has been observed to vary both with the growth temperature and with the source of carbon and energy for growth. The changes relative to the unsaturation level are accomplished either by the oxygen independent synthesis of UFAs (the anaerobic mechanism) or by the oxygen dependent desaturation of saturated fatty acids (SFAs) (the aerobic mechanism) (Russell, 1984). In the first case vaccenic acid (*cis*-11-octadecenoic acid, C18:1n11) is formed by palmitoleic acid (*cis*-9-hexadecenoic acid, C16:1n9) (Keweloh and Heipieper, 1996). In the second case the double bond is introduced into the aliphatic acyl chains of SFAs, already integrated in the phospholipids, by specific desaturases (Keweloh and Heipieper, 1996). The so called anaerobic mechanism has been found not only in anaerobic bacteria (*Clostridium*, *Lactobacillus*) but also in aerobic ones while the aerobic mechanism has been observed in species of *Bacillus*, *Mycobacterium*, *Micrococcus* (Magnuson et al., 1993). There is some evidence that both mechanisms coexist in *Pseudomonas*, *Vilnius* and *Acinetobacter* (Härtig et al., 1999) and in *Lactobacillus helveticus* (Guerzoni et al., 2001).

Some specific FAs have a peculiar role in the bacterial growth or survival. In particular, *Lactobacillus* spp. are well known to need oleic acid (*cis*-9-octadecenoic acid, C18:1n9) for their growth (Johnsson et al., 1995). However it has been shown, at least in *Lactobacillus delbrueckii*, that only some strains require exogenous FAs for their growth (Partanen et al., 2001).

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Lactic acid bacteria (LAB), used as important starters in fermentation of plants and animal origin foods, are exposed during their proliferation and preservation, as well as during food fermentation, to unfavourable conditions such as oxidative, heat, cold, acid and osmotic stresses. Some specific FAs are reported to play an important role in stress responses: the proportions of palmitic acid (hexadecanoic acid, C16:0) and linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid, C18:2) increase in *Lactobacillus acidophilus* grown at low temperature (Fernandez Murga et al., 2000). The C18:1n9 concentration increases in response to low temperature in *Lactobacillus plantarum* (Russell et al., 1995), to acid pH in *Streptococcus termophilus* (Béal et al., 2001) and to osmotic stress in *Lactococcus lactis* (Guillot et al., 2000). On the contrary C18:1n9 concentration decreases in response to freezing in lactic streptococci (Gilliland and Speck, 1974) and to spray drying in *L. acidophilus* (Brennan et al., 1986).

Also cyclopropane fatty acids (CFAs), lactobacillic acid (11,12-methyleneoctadecanoic acid, C19cyc11) and dehydrosterculic acid (9,10-methyleneoctadecanoic acid, C19cyc9), are regarded as key FAs in stress tolerance of LAB. These unusual FAs are formed "*in situ*" with a post-synthetic modification, by means of a transfer of a methyl group from S-adenosyl-L-methionine (SAM) to a double bond of UFAs already integrated into a phospholipid molecule (Law, 1971; Huang et al., 2002; Zhao, 2002). It has been reported that CFAs favour the stress tolerance of *Lactobacillus bulgaricus*, *L. helveticus* and *L. acidophilus* (Gómez Zavaglia et al., 2000), as well as the response to acid, osmotic and ethanol stresses (Guillot et al., 2000; Béal et al., 2001; Teixeira et al., 2002). However the two CFAs are generally not well separated by the gas-chromatographic conditions commonly adopted and are reported as cumulative values in the current literature. Since C19cyc11 and C19cyc9 are derived from different precursors, C18:1n11 and C18:1n9, synthesized respectively by the anaerobic and aerobic pathways, the uncertain assessment of their absolute and relative extent does not allow a clear interpretation of the meaning and importance of their individual role in the stress response.

The principal aims of this paper were: i) the improvement of a specific gas-chromatographic method to separate and quantify the two CFAs; ii) the use of this implemented method to study the FAs composition and its modification in response to acid and cold stresses in *Lactobacillus sanfranciscensis* and *L. helveticus*. *L. sanfranciscensis*, whose exclusive habitat is wheat and other cereals sour dough (Ehrmann and Vogel, 2005), is predominant during the continuous dough propagation under acid stress (Gänzle and Ehrmann, 1998; De Angelis et al., 2001). The cellular FAs composition of the latter specie has not been previously studied according to our knowledge. On the contrary, the FAs composition of *L. helveticus*, which is usually propagated under selective conditions as natural whey starter during manufacturing of Swiss and Italian hard and extra-hard cheeses (Di Cagno et al., 2006), has been widely investigated (Dionisi et al., 1999; Guerzoni et al., 2001).

In this paper the two species were exposed to the cold and acid sublethal stresses which are frequently associated with cell FAs adaptation and particularly cyclopropanation (Gómez Zavaglia et al., 2000; Béal et al., 2001; Zhao et al., 2009).

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. helveticus CNBL 1156 from the collection of the Institute of Microbiology of the Catholic Sacred Heart University, Piacenza (Italy), stored at −80 °C, was pre-cultured for 24 h and subsequently inoculated and incubated for 24 h in MRS broth without Tween 80 at 45 °C under anaerobic conditions. The MRS broth medium was obtained by weighting single components as described by Oxoid (Unipath Ltd. Basingstoke, Hampsire, UK).

L. sanfranciscensis Bb12 from the collection of the Department of Food Science of the University of Teramo (Italy), stored at −80 °C, was pre-cultured for 24 h and subsequently inoculated and incubated for 24 h in SDB medium (Kline and Sugihara, 1971) without Tween 80 at 32 °C under anaerobic conditions. The pH of the medium was adjusted to 5.6 by addition of HCl 1 N.

2.2. Acid and cold stress exposure

The cells of both strains, obtained as above described, were used for the stress exposure experiment. *L. helveticus* culture was centrifuged at 9000 × g for 15 min at 20 °C, while *L. sanfranciscensis* culture, due to its difficult sedimentation, was centrifuged at 12 000 × g for 30 min at 20 °C. Cells were collected, inoculated (inoculum level 8 log CFU/ml) in the same fresh medium and exposed for 2 h to different sublethal stresses, under static micro-aerobic (250 ml of broth in conical flasks of 500 ml of volume) or anaerobic conditions (250 ml of broth in conical flasks of 250 ml of volume, subsequently introduced in anaerobic jar), in the presence or absence of Tween 80 (0.3 g/l). Different pH values (5.0, 4.0, 3.0 for *L. helveticus* and 4.0, 3.6, 3.0 for *L. sanfranciscensis*) were obtained by addition of lactic acid. Cold stress was applied incubating the cells at 10 °C, in comparison with the strains optimal growth temperatures (45 °C and 32 °C respectively). Two independent experiments, with three repetitions for each experiment, were performed according to Table 1. After 2 h cell suspensions were centrifuged, as above described, and the cells were harvested for FAs extraction.

Cell viability was determined by spread plating following serial dilutions, on MRS and SDB agar media (18 g/L agar concentration), for *L. helveticus* and *L. sanfranciscensis* respectively, immediately after resuspension and at different time intervals, after 1, 2 or 3 h. Numbers of CFU were determined after 48 h incubation at the respective temperatures.

2.3. Development of separation and identification method for cyclopropane fatty acids (CFAs)

L. helveticus was pre-cultured for 24 h and subsequently inoculated and incubated for 24 h in MRS broth (250 ml) under anaerobic conditions at 45 °C. Tween 80 (0.3 g/l) was added to the culture in order to obtain cells rich in CFAs (Johnsson et al., 1995). The total broth (10 ml) containing the cells was resuspended in two flasks containing 250 ml of fresh medium and adjusted to obtain micro-aerobic and anaerobic conditions. The culture was centrifuged as above described and cells were harvested for FAs extraction.

Since lipids of LAB are widely associated with the plasma membrane (Kates, 1964) cell pellets were directly analysed. Cellular fatty acids extraction and methylation was performed using the Microbial Identification System (MIS) protocol produced by Microbial ID (MIDI, Newark, DE, USA) also described in Welch (1991).

To evaluate the presence of CFAs hydrogenation and bromination were performed using the method of Brian and Gardner (1968) modified as follows. After extraction 0.2 ml of fatty acid methyl esters were evaporated with N_2 and hydrogenated for 30 min in 10 ml of chloroform:methanol (2:1 v/v, both from Sigma–Aldrich, Milan, Italy) with 100 mg of 5% Pt on charcoal (Fluka, Milan, Italy). In this selective procedure UFAs are converted to saturated ones, and CFAs are not affected. After paper filtration, samples were evaporated with N_2, diluted in 1 ml of diethyl ether (reagent grade, Sigma–Aldrich, Milan, Italy) and cooled to 0 °C. A 0.5-ml amount of bromine in diethyl ether (1:5 v/v, both from Sigma–Aldrich, Milan, Italy) was added to each sample to break the cyclopropane ring. After 30 min at room temperature by stirring, ether and excess bromine were evaporated at 50 °C with N_2. The final sample was collected with a 0.1-ml amount of hexane and transferred to a GLC vial for injection.