Temperature adaptation in *Lactobacillus fermentum*: interconversions of oleic, vaccenic and dihydrosterulic acids

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The interchange of octadecenoic acids and dihydrosterulic acid was a response of aerobically growing *Lactobacillus fermentum* to changes in growth temperature. Oleic and vaccenic acid contents decreased both at temperatures below 20 °C and above 26 °C, showing mirror image behaviour, with a concomitant increase in dihydrosterulic acid. A temperature-dependent shift from vaccenic to oleic acid synthesis, and the conversion of the latter to dihydrosterulic acid was responsible for the overall change. Consequently, the degree of fatty acid unsaturation decreased at temperatures above 26 °C, whereas the degree of cyclization increased. The converse occurred below 20 °C. The relative amount of lactobacillic acid, total cellular fatty acid content, and mean fatty acid chain length were practically temperature-independent. The occurrence of oleic acid is thought to be related to aerobic growth conditions.

Introduction

The membrane and mesosomal lipids of typical lactobacilli contain even-carbon, straight-chain saturated and monoenoic acids, together with cyclopropane acids (Thorne & Kodicek, 1962; Croom *et al.*, 1964; Veerkamp, 1971). Their relative amounts vary according to culture conditions such as growth phase and rate, medium composition, and temperature (Croom *et al.*, 1964; Henderson & McNeill, 1966; Veerkamp, 1971; Uchida & Mogi, 1973*b*; Smittle *et al.*, 1974; Uchida, 1975; Gill & Suisted, 1978). As a consequence, the physical properties of structural lipids vary, and influence many fundamental cellular functions, including membrane transport and the activity of membrane-bound enzymes involved with energy metabolism (Cronan & Gelmann, 1975; Sandermann, 1978; Brenner, 1984; Neidleman, 1987). An understanding of such lipid-associated phenomena is vital in maintaining cellular viability during the processing and preservation of many fermented products, e.g. low temperature storage (Mazur, 1970; Smittle *et al.*, 1972, 1974; Calcott, 1986).

The long-chain fatty acids of lactobacilli are formed in a similar way to those of *Escherichia coli*. The enzymes involved are acetyl-CoA carboxylase (ACC), which produces malonyl-CoA, and a complex of at least eight

different functional protein units called fatty acid synthetase (FAS II) (O'Leary, 1965; Birnbaum, 1969, 1970; Weeks & Wakil, 1970). The double bond of monounsaturated vaccenic, palmitoleic and shorter chain ω -7 acids is formed anaerobically during elongation by the enzyme β -hydroxyacyl- β,γ -dehydratase (Scheuerbrandt & Bloch, 1962; Norris *et al.*, 1964; O'Leary, 1965).

Oleic, and other monoenoic acids of the ω -9 series are rarely detected in lactobacilli (Nakano *et al.*, 1979; Uchida & Mogi, 1972, 1973*a*). However, in *Lactobacillus plantarum* oleic acid or its derivative regulates fatty acid synthesis by repressing the formation and activity of ACC and FAS II (Henderson & McNeill, 1966; Birnbaum, 1970; Weeks & Wakil, 1970). This does not occur in *E. coli* which contains only vaccenic acid (Silbert & Vagelos, 1967; Silbert *et al.*, 1968). In microbes, oleic acid can be formed by two routes, either anaerobically as presented above for ω -7 acids or by oxygen-dependent desaturases (Scheuerbrandt & Bloch, 1962). Monoenoic fatty acids can further be cyclized to cyclopropane acids by the enzyme cyclopropane synthetase, which adds a methylene carbon bridge from S-adenosyl-L-methionine (SAM) across the double bond. Thus, lactobacillic acid is formed from vaccenic acid (Chalk & Kodicek, 1961; Liu & Hofmann, 1962; Law, 1971; Goldfine, 1972; Cronan *et al.*, 1979) and, interestingly, when oleic acid is added to the growth medium of *L. plantarum*, it is cyclized to

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dihydrosterulic acid (Henderson & McNeill, 1966; Polachek *et al.*, 1966; Buist & McLean, 1981).

In this paper, we report the occurrence of oleic acid as a natural constituent of aerobically grown, heterofermentative *Lactobacillus fermentum*, and demonstrate its role in temperature adaptation via interconversions with vaccenic and dihydrosterulic acid.

Methods

Strain and growth conditions. The bacterium used was *Lactobacillus fermentum* ATCC 14931. The strain was stored in 10% (v/v) glycerol (Merck) at -60°C , and grown in 250 ml Erlenmeyer flasks containing 50 ml MRS-broth (Difco). The flasks were shaken at 240 r.p.m. in a Gallenkamp orbital shaker/incubator (Model INR 200 010V), and growth was monitored with a Klett-Summerson colorimeter (filter no. 66). For preparation of inocula, cells were grown to late exponential growth phase (150–200 Klett units); 1 ml of this culture was transferred into a shake flask containing the same medium and allowed to grow to the exponential phase (70–80 Klett units). This culture (1 ml) was inoculated into four parallel flasks to be used for analyses. All the stages were carried out at 15, 20, 26, 30, 35 and 40°C . The fatty acid composition was determined in the early-exponential (50 Klett units), exponential (100 Klett units), late-exponential (150 Klett units) and stationary (>200 Klett units) phases. The total fatty acid content of cells was determined in the exponential phase.

To determine the growth temperature maximum, the microbe was grown on agar in a cuvette of a plate-type temperature gradient incubator (Gradiplate). The medium used was MRS-broth solidified with 2% (w/v) agar.

Analyses. To determine the fatty acid composition, samples (5 ml) of culture suspensions were centrifuged for 10 min (5000 g). The supernatant was removed and the cells were resuspended in 5 ml of tap water. Centrifugation was repeated, the water was removed, and cells were frozen under an N_2 stream and stored (-20°C) for 1–10 days. To determine the cellular fatty acid content, the remaining bacterial culture suspension was harvested and washed as described above. The bacterial cells were lyophilized and weighed, an internal standard (heptadecanoic acid methyl ester, Sigma) was added, and the fatty acid composition was determined as follows.

Bacterial fatty acid esters were saponified, and extracted as methylesters, as described by Suutari *et al.* (1990). The major fatty acids were identified from their GLC peak retention times relative to fatty acid methyl ester standards (Supelco) and by GLC-MS. GLC was done using a Hewlett-Packard model 5890A gas chromatograph equipped with a flame ionization detector, a capillary inlet system and a model 7673A high-speed automatic liquid sampler with a 10 μl syringe. The conditions were as follows: HP-FFAP (25 m $\times$ 0.2 mm $\times$ 0.3 μm) column; carrier gas helium, column flow-rate approx. 1.0 ml min $^{-1}$; total hydrogen flow-rate to the detector 40 ml min $^{-1}$; make-up gas, helium, flow-rate 30 ml min $^{-1}$; septum purge flow-rate 1–2 ml min $^{-1}$; split ratio 1:20; column inlet pressure, 150 kPa; injector temperature, 250°C ; detector temperature, 250°C ; oven temperature, programmed from 70°C to 200°C at the rate of $25^{\circ}\text{C min}^{-1}$. Peak areas were measured using a Hewlett-Packard model 3396A integrator. Combined GLC-MS of the fatty acid methyl esters was carried out using the GLC chromatograph described above, and equipped with a Hewlett-Packard mass selective detector 5971A. GLC conditions were as presented above, except that column inlet pressure was 70 kPa. The mass spectra were recorded at an electron energy of 70 eV and a trap current of 300 μA . The ion source temperature was 180°C , and the molecular separator temperature 135°C .

Calculations. The growth rate was determined from the slope of the growth curve in the exponential growth phase. The relative fatty acid composition was estimated as a percentage of the total peak area, and expressed as a mean value from exponential and late exponential growth phases. The total fatty acid content of the cells was defined as the proportion of cell dry weight (w/w) consisting of fatty acid methyl esters. The degree of fatty acid unsaturation (Δmol^{-1}) in the lipid fraction was calculated as $[\Sigma(\% \text{ monoene})]/100$, and the degree of cyclization (Δmol^{-1}) as $[\Sigma(\% \text{ cyclic fatty acid})]/100$. The mean chain length of the fatty acids was expressed as the mean number of carbon atoms in the fatty acid chain. The standard deviation ($n=8$) was $<0.9\%$.

Results and Discussion

Effect of temperature on fatty acid composition

The temperatures used (15 – 40°C) covered most of the growth range of *L. fermentum*. Growth was extremely slow at 15°C (Fig. 1), and there was no growth at 10°C . According to Buchanan & Gibbons (1974), *L. fermentum* does not grow even at 15°C . Thus, the growth temperature minimum of our organism may be estimated to be near 15°C . The highest temperature (40°C) in the shake flask cultivations was 6 – 7°C below the growth temperature maximum (46 – 47°C), as determined by the temperature gradient technique. This agrees well with the earlier observation that *L. fermentum* grows at 45°C (Buchanan & Gibbons 1974).

Within the temperature range used, seven fatty acids, accounting for over 95% of total fatty acid in all growth phases were found (Fig. 2). They were identified as myristic, palmitic, palmitoleic, oleic, *cis*-vaccenic, dihydrosterulic (*cis*-9,10-methyloctadecanoate) and lactobacillic (*cis*-11,12-methyloctadecanoate) acids. Temperature markedly affected their relative amounts. However, the cellular fatty acid content in the exponential growth phase was low (1.5–2.5%), and showed no significant temperature dependence. Therefore, the temperature-induced changes in the proportions of fatty acids did not greatly reflect changes in the overall efficiency of fatty acid synthesis.

The major temperature response in fatty acid composition was limited to the interchange of the octadecenoic acids and dihydrosterulic acid (Fig. 2). Oleic and vaccenic acid decreased when temperatures were either reduced below 20°C or increased above 26°C . Their fatty acid contents also showed mirror image behaviour, with a concomitant increase in dihydrosterulic acid. In addition, the proportion of myristic acid increased slightly at the temperature extremes.

Oleic acid and its cyclization in temperature adaptation

The shifts from vaccenic acid to oleic acid, and further to dihydrosterulic acid, occurred at both the lowest and

Fig. 1. Dependence of growth rate of *L. fermentum* on temperature.

Fig. 2. Effect of temperature on fatty acid profiles of exponential cultures of *L. fermentum*. Symbols: ●, dihydrosterulic acid; ○, palmitic acid; ▲, oleic acid; △, lactobacillic acid; ■, vaccenic acid; □, palmitoleic acid; ▼, myristic acid.

highest temperatures (Fig. 2). Oleic acid might be formed either aerobically by the desaturase from stearic acid or its derivative, or anaerobically during fatty acid elongation by the enzyme β -hydroxyacyl- β,γ -dehydratase (Scheuerbrandt & Bloch, 1962; Norris *et al.*, 1964). The importance of oxygen in oleic acid formation supports the presence of a desaturase such as those found in yeasts and certain bacteria. Neither we nor Rizzo *et al.* (1987) detected significant amounts of oleic acid in anaerobically grown *L. fermentum*. The β -hydroxyacyl- β,γ -dehydratase of our organism might shift between vaccenic and the stearic acid formation depending on tempera-

ture, and stearic acid would then be further desaturated to oleic acid. However, an anaerobic route for the synthesis of ω -7 and ω -9 olefinic fatty acids cannot be ruled out. This has been proposed earlier e.g. for *Clostridium butyricum* (Goldfine & Bloch, 1961), and for the genus *Pediococcus* which has a fatty acid pattern similar to that of lactobacilli (Uchida & Mogi, 1972). In the latter case, the specificity of the β -hydroxy-acyl- β,γ -dehydratase to the chain length of the substrate might be temperature-dependent.

The proportion of the other cyclic fatty acid, lactobacillic acid, was low (3.5–4.5%) and practically temperature independent even in the presence of high amounts of vaccenic acid. The membrane bound cyclopropane synthetase has been reported to have a strong preference for ω -7 monoenes (Goldfine & Panos, 1971; Goldfine, 1972; Cox *et al.*, 1973), which are acylated in phospholipids in a correct micellar state (Zalkin *et al.*, 1963; Chung & Law, 1964; Thomas & Law, 1966; Law, 1971). However, the present data on *L. fermentum* favours a preference for ω -9 monoenic substrates especially at extreme growth temperatures. Alternatively, multiple cyclopropane synthetases may exist in lactobacilli. Inducible and constitutive enzymes have been detected in *Pseudomonas denitrificans* and *E. coli*, respectively (Jaques, 1981; Cronan, 1968). The enzymes synthesizing oleic, *cis*-vaccenic and dihydrosterulic acids are clearly under some form of coordinate control.

Effect of temperature on fatty acid unsaturation and cyclization, and the mean chain length

The degree of fatty acid unsaturation decreased with increasing temperature above 26 °C, while the degree of cyclization increased (Fig. 3a). The proportion of unsaturated fatty acids is often reduced with increasing growth temperature to maintain ideal membrane fluidity (Farrell & Rose, 1967; Fulco, 1974; Cronan & Gelmann, 1975; Neidleman, 1987; Suutari *et al.*, 1990). However, the formation of cyclopropane acids from unsaturated fatty acids does not necessarily alter the physicochemical properties of lipids (Cullen *et al.*, 1971; Van Deenen, 1971; Cronan *et al.*, 1979). Nevertheless, cyclopropane fatty acids are thought to make membranes more stable during exposure to adverse environmental conditions (Cronan, 1968; Dufourc *et al.*, 1984). In any case, the strong shift between the degree of fatty acid unsaturation and cyclization in response to temperature changes indicates its physiological significance to *L. fermentum*. Interestingly, opposite behaviour occurred in these proportions at temperatures below 20 °C, near the growth minimum, and concomitantly the growth rate fell markedly.

Fig. 3. Effect of temperature on (a) the degree of fatty acid unsaturation (■) and cyclization (▲), (b) the mean chain length, both in exponential cultures.

The mean fatty acid chain length (Fig. 3b) was practically temperature-independent. The increments near both extreme temperatures can be explained by the increasing proportion of dihydrosterulic acid at the expense of octadecenoic acids. Thus, the steps in fatty acid biosynthesis determining the mean chain length of fatty acids are not effected by temperature in *L. fermentum*.

The roles of cyclic fatty acids and oxygen in temperature adaptation

This study demonstrates that the interplay of oleic, vaccenic and dihydrosterulic acids is a physiologically significant response of lactobacilli to changing growth

Fig. 4. Effect of culture age on the fatty acid composition of *L. fermentum*. Symbols as Fig. 2.

temperature. Although the existence of lactobacillic and other cyclic fatty acids is long established (Hofmann *et al.*, 1952; Hofmann & Sax, 1953), their role is still poorly understood (Cronan *et al.*, 1979; Dufourc *et al.*, 1984; Schweizer, 1989). Their appearance is often connected with disadvantageous growth conditions such as extreme temperatures, entering stationary growth phase and decreasing pH (Knivett & Cullen, 1965; Cronan, 1968; Law, 1971; Veerkamp, 1971; Uchida & Mogi, 1973b; Cronan *et al.*, 1974; Uchida, 1975; Gill & Suisted, 1978). Similarly, the data presented showed that dihydrosterulic acid was formed in *L. fermentum* at extreme temperatures (Fig. 2) concomitantly with decreasing growth rates (Fig. 1). Furthermore, the proportions of dihydrosterulic and lactobacillic acid increased at the expense of oleic acid (Fig. 4) upon ageing of the cells. This suggests that cyclic fatty acids might protect lactobacilli from adverse environmental effects. Indeed lactobacillic acid has been shown to stabilize lactobacilli in liquid nitrogen (Smittle *et al.*, 1974). Nor can it be ruled out that cyclopropane acids protect lactobacilli against other microbes and viruses. When *E. coli* is infected with phage T3 the formation of cyclic fatty acids is inhibited by a phage-encoded SAM cleavage enzyme (O'Leary, 1965; Cronan *et al.*, 1979). Being energetically expensive to synthesize (Taylor & Cronan, 1976), cyclic acids might also serve as energy stores under starvation conditions created by factors other than those related to energy or lipid metabolism.

The occurrence of oleic acid and, accordingly, dihydrosterolic acid was clearly dependent on oxygen. Therefore, despite the fact that *L. fermentum* maintains a fermentative metabolism under aerobic conditions (Buchanan & Gibbons, 1974; Condon, 1987), oxygen has an important effect on metabolism related to membrane structure and function. No cyclic fatty acids were detected in obligately anaerobic rumen lactobacilli (Miyagawa, 1982). The induction of oleic acid synthesis might be related to the protection of the cells against the deleterious effects of oxygen.

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