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Fatty Acid Composition of L-Forms of *Streptococcus faecalis* Cultured at Different Osmolalities

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The fatty acid composition of the membranes of three different penicillin-produced L-forms of *Streptococcus faecalis* was determined: (i) a stable (nonreverting) L-form (T_{53}) cultured in brain heart infusion (BHI) with 0.5 M sucrose; (ii) a stable L-form (T_{531}) cultured in BHI without sucrose; and (iii) an unstable L-form (T_9) cultured in BHI with 0.5 M sucrose and 1,000 U of penicillin per ml. L-forms were obtained by centrifugation and lysed by washing in 1 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer. The parent *S. faecalis* was also cultured in BHI and BHI containing 0.5 M sucrose, and washed with buffer. The fatty acid composition of L-forms of *S. faecalis* cultured in BHI without sucrose (370 mosmol) had higher $C_{18:1}$ and lower C_{18} than L-forms cultured in the same media with added 0.5 M sucrose (950 mosmol) in both exponential and stationary cultures. In the stationary phase of growth, C_{19} was reduced in the L-forms cultured without sucrose. Similar changes were seen in the parent *S. faecalis* cultured in the two types of media. These changes in membrane fatty acids may relate to osmo-regulation of the L-forms.

L-forms of *Streptococcus faecalis* are osmotically fragile and usually require sucrose or some other osmotic stabilizer in the growth medium, but may be adapted to grow in nutrient broth without an osmotic stabilizer (1, 5). This adaptation has occurred without obvious change in morphology of the L colony or cell membrane, and no cell wall was detectable on electron microscopy (5). Because of the possibility that changes in membrane lipid composition may have been a factor that permitted the L-forms to survive at lower osmolalities, we examined the fatty acid composition of the L-forms of *S. faecalis* cultured at different osmolalities. The fatty acid composition of the parent *S. faecalis* bacteria cultured in the same media has also been compared.

MATERIALS AND METHODS

The stable L-forms of *S. faecalis* (GK) T_{53} cultured in brain heart infusion (BHI) broth (Difco) with 0.5 M sucrose (950 mosmol) and T_{531} cultured in the same medium without sucrose (370 mosmol) have been described previously (5). An unstable L-form (designated T_9) after nine transfers in BHI 0.5 M sucrose agar containing 1,000 U of penicillin per ml and 2% (wt/vol) bovine serum albumin (Armour Co.) was transferred to biphasic liquid media as described previously (4). After two transfers in biphasic medium, it readily transferred to liquid medium without an agar slope. Experiments have been carried out with T_9 L-forms transferred an additional seven times. All cultures were stored at −15 C in liquid medium.

Prior to their use in these studies, all frozen cultures were thawed and 0.1 ml was inoculated into 9 ml of fresh medium. The organisms were used after two additional transfers in fresh medium. T_{531} was cultured in BHI, and T_{53} and T_9 were cultured in BHI with 0.5 M sucrose. Penicillin (1,000 U/ml) was added to T_9 cultures.

Cells in the exponential and late stationary phases of growth were obtained by centrifugation as described previously (5). The L-forms were lysed by washing seven times in 1 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.6) at 4 C. The *S. faecalis* parent bacteria was washed seven times in the same buffer at 4 C. The membrane preparation of L-forms and the washed bacteria were lyophilized and lipid was extracted from 5-mg samples with chloroform-methanol by a modification of the method of Vorbeck and Marinetti (9). Methanol (8 ml) was added to the sample and the mixture was heated in a water bath at 65 C for 5 min. After cooling, 16 ml of chloroform was added and the suspension was

shaken at room temperature. The residue was removed by filtration. Water (4 ml) was added to 20 ml of the filtrate and the mixture was gently shaken. The chloroform phase was evaporated to dryness under nitrogen, suspended in a small volume of chloroform, and kept at −15 C under nitrogen. Fatty acids were methylated with methanolic hydrochloride (6). The composition of methyl esters of the fatty acids was determined by gas-liquid chromatography by using a Packard gas chromatograph equipped with a hydrogen-flame ionization detector. The column contained ethylene glycol succinate on a stationary phase on 100/120 mesh Chromabsorb P as a support. The percent composition was calculated from the areas of each component in the chromatogram. Only fatty acids present at levels greater than 1% at any stage of growth were recorded.

Chain length and saturation of fatty acids were assigned by relative retention times. The fatty acid labeled C_{19} has not been identified further, but comparison with other studies on the *S. faecalis* bacteria would suggest that the C_{19} is a cyclopropane fatty acid, lactobacillic acid.

RESULTS

Table 1 shows the percent fatty acid composition of the L-forms and parent bacteria. T_{531} cultured in BHI without sucrose had higher $C_{18:1}$ and lower C_{18} than L-forms T_{53} and T_9 cultured in media containing 0.5 M sucrose in both log and stationary cultures. In the stationary phase of growth, the percentage composition of C_{19} was higher in T_9 and T_{53} than T_{531}. Both these changes were similar to those seen in the parent bacteria cultured in the two types of media except that in the exponential cultures the differences in C_{18} and $C_{18:1}$ were not significant.

The fatty acid composition of the L-forms was influenced by the stage of growth, as has been shown previously with many bacteria. These changes were more marked in $C_{18:1}$ and C_{19}, with an increase in C_{19} associated with a decrease in $C_{18:1}$. These changes were most marked in T_{53} and T_9 with little change occurring in T_{531}.

The total percentage of C_{16} fatty acids in L-form membranes was less than the total percentage of C_{18} fatty acids in both log and stationary phase of growth. This contrasted with fatty acids from *S. faecalis* bacteria, where total C_{16} fatty acids were greater than the C_{18} fatty acids. These changes are similar to those reported by Panos et al. (7) in *S. pyogenes* and its derived L-form.

DISCUSSION

The present results suggest that *S. faecalis* L-forms and bacteria vary in the amount of C_{18} fatty acids and C_{19} lactobacillic fatty acid in the membrane dependent on the sucrose content of the medium. Without added sucrose in the medium there was an increase in the unsaturated fatty acid 18:1 and a decrease in C_{18} and C_{19} cyclopropane fatty acid. Because sucrose is not metabolized by *S. faecalis*, the sucrose effect is probably osmotic, although other osmotic stabilizers have not yet been studied.

Previous studies have shown that L-forms of *S. faecalis* may grow in BHI without an osmotic stabilizer and that this adaptation to lower osmolalities was associated with a reduction in the total internal cation concentration (5). The association of the two observations, a reduction in the total internal cation concentration and changes in C_{18}, $C_{18:1}$, and C_{19} fatty acids, raises the possibility that osmoregulation in the *S. faecalis* L-forms (and bacteria) may be taking place by altering fatty acid composition of the membrane. The nature of fatty acids in phosphatides may alter membrane function. Increasing unsaturation of long-chain fatty acids

TABLE 1. *Percent fatty acid composition of S. faecalis L-forms and bacteria*[a]

S. faecalis	Media	Log culture						Stationary culture					
		C_{14}	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	C_{19}	C_{14}	C_{16}	$C_{16:1}$	C_{18}	$C_{18:}$	C_{19}
L-forms													
T531	BHI	4.0	31.4	7.3	8.8	48.4	Tr	2.3	27.3	6.3	8.3	53.2	2.7
T53	BHISUC[b]	1.9	34.5	4.9	15.8	43.3	Tr	1.8	35.2	2.8	17.2	32.5	10.8
T9	BHISUC	3.6	34.7	6.2	15.9	39.7	Tr	2.0	27.5	4.4	15.1	36.3	14.7
Bacteria													
S. faecalis	BHI	5.6	41.2	7.6	6.9	38.9		5.9	40.3	7.9	7.6	34.7	3.8
S. faecalis	BHISUC	5.3	43.3	7.5	8.9	35.0		6.0	38.7	8.2	10.4	25.4	11.4

[a] This table does not include a peak that appeared variably in the extract of the bacterial cells which was at the position of a C_{23} fatty acid and came from the BHI media which has a high concentration of this substance.

[b] BHI with 0.5 M sucrose.

has been shown to increase permeability in artificial membranes (2). Razin et al. (8) found that oleic acid and other unsaturated long-chain fatty acids added to the medium (and found to be incorporated into the membrane) increased the resistance of *Mycoplasma laidlawii* to osmotic lysis. Others have shown that the degree of unsaturation of the fatty acids of erythrocytes correlated with the resistance of the red cells to hemolysis (10). The observations on changes in permeability with changes in unsaturation of fatty acids (3) have related to permeability of non-electrolytes but may also relate to anions and cations. DeGier et al. (2) found that valinomycin-induced permeation of liposomes by $^{86}Rb^{+}$ was enhanced by the degree of unsaturation of the fatty acids in the bilayers.

These observations, together with the present study of *S. faecalis* L-forms, raise the possibility that osmoregulation may be taking place in many organisms by the altering of fatty acid composition of the membrane.

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