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Induction of Pleomorphy and Calcium Ion Deficiency in *Lactobacillus bifidus*

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The induction of pleomorphism of *Lactobacillus bifidus* by NaCl was completely inhibited by $CaCl_2$. When the organism was cultivated in calcium-free medium, growth of the bifid form was exclusively observed. Supplementation of calcium ion in the medium caused bacilloid growth. Chemical analyses indicated that calcium content of the bifid form organisms was significantly less than that of the bacilloid form; i.e., in the former type, there was an approximately 30% decrease of calcium in the whole cells, and an 82% decrease in the cell wall, as compared with the respective content of the latter. These results indicate a suppressing role of calcium ion in the induction of pleomorphism of *L. bifidus*. Besides calcium content, sugar and amino acid compositions were shown to be different between the bifid and bacilloid forms. In the cell wall especially, the content of glucose in the bifid form was larger than that in the bacilloid form. Methionine and phenylalanine were present in the bifid form, but not in the bacilloid form. Cell walls of the bifid form organisms lacked a larger molecular weight peptidoglycan (7.5*S*) which was clearly detected in the bacilloid form. Evidence has been given for the relationships of calcium ion and cell wall components to the pleomorphism in *L. bifidus*.

In the previous report (5), it was described that pleomorphism of *Lactobacillus bifidus* was induced in a medium containing NaCl (0.35 M), and that such induction of pleomorphism was inhibited by adding $CaCl_2$ (0.01 to 0.02 M) to the medium. An antagonistic relationship between calcium and sodium ions regarding the pleomorphic response was thus suggested. The present paper deals with experiments performed to clarify the role of calcium ion in pleomorphism. Determinations of sugars, amino acids, and peptidoglycans in the cell wall were made with an aim to detect differences, if any, in chemical components of the bifid and bacilloid forms.

MATERIALS AND METHODS

Organism. *L. bifidus* was isolated from feces of an infant. The identification of the species was based on the descriptions of *Bergey's Manual* (7th ed., 1957).

Medium and culture condition. The basal culture medium consisted of 1% glucose and 0.04% cystine in nutrient broth enriched by addition of equal volume of beef liver infusion. After adjustment to *p*H 6.8, the medium was sterilized by autoclaving at 120 C for 15 min. The culture was incubated at 37 C under anaerobic conditions by the conventional alkali-pyrogallol method.

Preparation of cell wall. Cell wall preparations were made by a modification of a method of Cummins and Harris (2). Organisms were washed twice in distilled water and destroyed by a sonic vibrator (20 kc) at 0 C until no more intact cells could be observed. The sample was centrifuged at 120 × *g* for about 10 min, and the supernatant fluid was centrifuged at 9,000 × *g* for 30 min. The sediment from the second centrifugation was washed once in distilled water and then suspended in 0.05 M phosphate buffer (*p*H 7.6) containing 0.5 mg each of trypsin and ribonuclease per ml, and digested at 37 C for 2 hr. After a centrifugation at 9,000 × *g* for 30 min, the residue was washed twice in distilled water, and digested at 37 C for 18 hr with 0.02 N HCl containing 1 mg of pepsin per ml. The residual material obtained by centrifugation was washed several times in distilled water and used as the cell wall preparation.

Quantitative analyses. Contents of calcium ion in medium, intact cells, and cell wall preparations were estimated by the method of Tyner (9). The samples were heated at 450 C for 30 min in a muffle furnace, and the ash was dissolved in 4 N HCl. After HCl was driven off in an oven, the residue was dissolved in small amounts of 0.1 N acetic acid. After being warmed for a few minutes, 4 ml of distilled water and 2 ml of 0.1% chloranilic acid were added to 1 ml of the solution. The mixture was allowed to stand for 30 hr at room temperature in the dark. Calcium content was estimated spectrophotometrically at 500 nm. Calcium ions in cell wall preparations were determined, because of their small yields from cells, by use of an atomic absorption spectrophotometer (Hitachi type 207) at

422.7 nm. Preliminary experiments indicated that values obtained by the two methods were practically identical.

Sugars and amino acids in the cell wall preparations were additionally determined. Estimation of sugars was carried out by a modification of the method of Cummins and Harris (2). The preparation was hydrolyzed in 2 N H_2SO_4 in a sealed tube at 100 C for 2 hr. After filtration, the hydrolysate was neutralized with solid $Ba(OH)_2$. The supernatant fluid was evaporated in vacuo to dryness. The final product was redissolved in 0.5 ml of distilled water and partitioned by paper chromatography by using Toyo Roshi no. 51 A papers. A solvent system consisting of 120 parts (in volume) ethyl acetate, 50 parts pyridine, and 40 parts water was used. The developed papers were dipped into a mixture containing 2.0 ml of aniline, 3.3 g of phthalic acid, and 95 ml of acetone, and dried at 105 C for 5 to 10 min. The colored spots were examined by a densitometer (Toyo Kagaku DM 6). In parallel, the same spots were subjected to quantitative determination of sugars by the method of Somogyi-Nelson (8).

Amino acids in the cell wall fractions were determined by using an amino acid autoanalyzer (Shibata Rikagaku AA-600) after hydrolysis in 6 N HCl in a sealed tube at 100 C for 10 hr.

Peptidoglycans of cell wall. Peptidoglycans of the cell wall were isolated by a modification of the method described by Westphal et al. (10). The cell wall preparations were added to 90% (w/w) phenol and were permitted to stand at room temperature for 24 hr. The insoluble substance, collected by centrifugation at 9,000 × *g* for 30 min, was washed four times in distilled water, resuspended in a small amount of distilled water, and heated in a sealed tube at 100 C for 1 hr. After centrifugation at 9,000 × *g* for 30 min, the supernatant fluid, free from any flocculent material, was added into 3 to 5 volumes of absolute alcohol containing sodium acetate (final concentration, 1%), and the mixture was stored at 0 C for 24 hr. The precipitate was collected by centrifugation at 9,000 × *g* for 30 min and redissolved in a small amount of distilled water. The solution was dialyzed against distilled water at 0 C for 24 hr. After dialysis, 5 volumes of absolute alcohol was added to the dialysate, and the mixture was stored at 0 C for 24 hr. The material was then centrifuged at 9,000 × *g* for 30 min. The precipitate was washed once in distilled water and twice in acetone, and the final product was dried in vacuo.

The peptidoglycan preparation thus obtained was dissolved in 0.1 N NaCl to give a final concentration of 1%, and the sedimentation coefficient was determined by an ultracentrifugation analyzer (Hitachi UCA-1).

RESULTS

Calcium ion contents in bifid and bacilloid forms. Amounts of calcium ion in the medium, intact cells, and cell wall preparations were compared under the conditions of bacilloid and bifid growth. The results obtained are shown in Table 1.

It is clear that calcium ion contents in intact cells of bifid form are significantly smaller than those of bacilloid form. The difference is much more marked in the cell wall preparations. The sum of the calcium ion concentration in culture filtrates and intact cells were practically equal to that in basal medium.

Effect of chelating agents. By additions of chelating agents, such as oxalic acid, chloranilic acid, ethylenediaminetetraacetic acid, and glycoletherdiaminetetraacetic acid to the culture medium, bifid forms were induced. When calcium chloride (0.02 to 0.06 M) was added to the culture medium deprived of calcium by addition of oxalic acid, reversal of the bifid to bacilloid forms occurred. These results are clearly indicative of the essential role of calcium ions in the bacilloid form.

Sugars and amino acids in cell wall. Examples of determination of sugars and amino acids in cell wall preparations from bifid and bacilloid forms are tabulated in Tables 2 and 3.

Glucose and total sugars were more abundant in the bifid form than in the bacilloid form. In the present studies, more amino acids were detected both in bifid and bacilloid forms of *L. bifidus* than those reported by previous investigators (3). It is notable that methionine and phenylalanine are present in the bifid form, whereas both are undetectable in the bacilloid form.

TABLE 1. *Calcium ion content[a] in cultures of bifid and bacilloid forms of Lactobacillus bifidus*

Sample	Bifid form	Bacilloid form
	μg	μg
Preincubated basal medium (per ml)	28.5 ± 0.53	28.5 ± 0.53
After incubation for 22 hr		
Culture filtrate (per ml)	26.6 ± 0.37	23.3 ± 0.24
Intact cell (per mg, dry wt)	2.9 ± 0.08	4.1 ± 0.09
Cell wall (per mg, dry wt)	0.075 ± 0.0034	0.41 ± 0.028

[a] The values given are means of determinations on five samples and the standard deviations of the means.

TABLE 2. *Sugar composition in the cell walls of Lactobacillus bifidus*

Sugar	Bifid form[a]	Bacilloid form[a]
Rhamnose	0.747	0.790
Galactose	0.366	0.293
Glucose	0.545	0.379
Mannose	Trace	Trace
Glucosamine	Trace	Trace
Galactosamine	ND[a]	ND[b]
Undetermined	+	+

[a] Expressed as micromoles per milligram (dry weight).
[b] Not detectable.

TABLE 3. *Amino acid composition in the cell walls of Lactobacillus bifidus*

Amino acid	Bifid form[a]	Bacilloid form[a]
Lysine	0.330	0.267
Histidine	0.004	0.003
Arginine	0.005	0.006
Aspartic acid	0.015	0.019
Threonine	0.311	0.262
Serine	0.322	0.274
Glutamic acid	0.329	0.295
Proline	ND[b]	ND
Glycine	0.036	0.030
Alanine	1.225	0.951
Cysteine	ND	ND
Valine	0.013	0.013
Methionine	0.004	ND
Isoleucine	0.006	0.007
Leucine	0.011	0.015
Tyrosine	0.234	0.156
Phenylalanine	0.006	ND
Undetermined	+	+

[a] Expressed as micromoles per milligram (dry weight).
[b] Not detectable.

Schlieren patterns of cell wall peptidoglycan fractions. Typical examples are shown in Fig. 1. The patterns of the preparations from the bifid form are different from those of the bacilloid form; i.e., the peptidoglycans from the former organisms are apparently lacking in certain larger components.

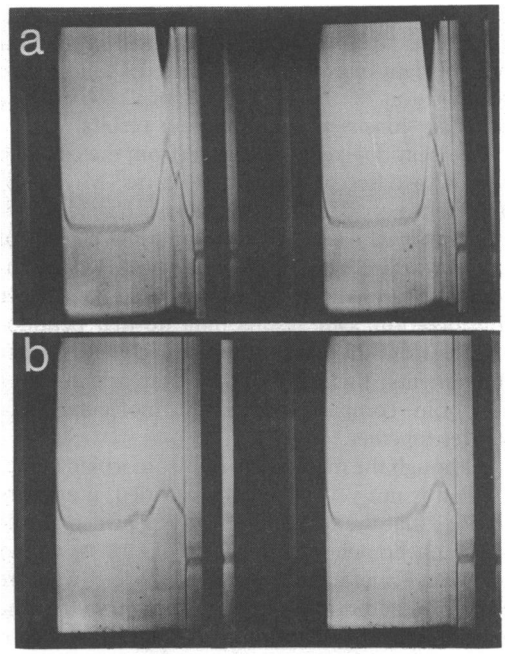

FIG. 1. *Ultracentrifugation of the peptidoglycan fractions extracted from the cell wall of Lactobacillus bifidus. Sedimentation is from right to left; photographs of the schlieren patterns were taken at 33 and 51 min, respectively, after reaching a speed of 55,430 rev/min at 15 C. (a) Bifid form, 1.7S and 3.2S; (b) bacilloid form, 2.2S, 3.2S, and 7.5S; values are sedimentation coefficients of the components.*

DISCUSSION

It is well known that *L. bifidus* shows branched forms under various culture conditions. We previously reported the induction of pleomorphism by univalent alkali metallic ions (5). The induction was completely inhibited in a medium containing 0.35 M NaCl plus 0.02 M $CaCl_2$. The results suggested that the pleomorphism in the organism depended on the availability of calcium ions. The present study has actually shown that the calcium content in intact cells and cell walls is significantly lower in the bifid form than in the bacilloid form (Table 1). The fact that the bifid form is induced by addition of chelating agents gives additional supporting evidence. Grula et al. (4) reported fragmentation of filaments in *Erwinia* cells by addition of divalent cations such as Zn^{2+}, Ca^{2+}, or Mn^{2+}. We also observed that the bifid form of *L. bifidus* fragmented into bacilli when it was transferred into the basal medium. In higher plants, it is considered that calcium ion is effective in binding the cell wall substances and is related to the wall plasticity [e.g., Bennet-Clark (1)]. From this point of view, the role of calcium ion in inhibiting the random branching in bacteria cell may be explained analogously by the results in higher plants.

Besides calcium ion contents, compositions of sugars and amino acids in the cell wall were different in the bifid and bacilloid forms. Pine and Boone (7) found that conversion of the morphology of *Actinomyces israelii* from intertwined mycelial hyphae to small diphtheroid

cells was accompanied by a change in the ratio of aspartic acid to alanine in the cell wall. Our present data indicating differences of amino acid composition of the two forms of *L. bifidus* are probably consistent with previous results.

The peptidoglycans extracted from the cell wall were also different in the two forms. Miyaji (6) observed no difference in polysaccharides obtained from the cell wall of S- and R-type cells of *L. acidophilus*. This discrepancy between the investigations of these two groups deserves further consideration. Our results indicate that peptidoglycans from the bifid forms are lacking in larger components; this may suggest that calcium ions may join comparatively small molecular substances together.

Although the mechanism of pleomorphic inducduction is not yet thoroughly clarified, it appears obvious that the calcium ion plays a principal role in the phenomenon. Change(s) of sugars, amino acids, and related substances of the cell wall are probably additional important factors in the morphological alterations. Apparent stoichiometric relationships between calcium and peptidoglycans in the cell wall are indicated.

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