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Influence of pH and Fluoride on Properties of an Oral Strain of *Lactobacillus casei* Grown in Continuous Culture

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A freshly isolated oral strain, *Lactobacillus casei* RB1014, was grown in continuous culture to compare the effects of pH and fluoride on growth and metabolism. The cells were grown at pH 7.0 to 3.2 in the absence of fluoride and from pH 7.0 to 5.4 with 20 mM NaF. Cell numbers varied from 3×10^9 to 30×10^9/ml on blood agar during alterations in the growth pH from 7.0 to 4.27. Only when the culture was stressed by lowering the pH to 3.2 were cell numbers drastically reduced. Cells growing at pH 7.0 without fluoride were unable to grow when plated on fluoride agar (10.5 mM) at pH 5.5; however, when the growth pH was allowed to decrease to 4.94, cells grew on the fluoride plates in numbers equal to those growing on blood agar. This fluoride tolerance trait appeared rapidly once pH control was removed and was lost when the culture was returned to pH 7.0. The addition of 20 mM NaF to the culture medium did not adversely affect growth, provided that the pH was maintained at 6.0 or above; cells tolerant to 10.5 and 16 mM NaF appeared on pH 5.5 plates during this phase. In cells removed from the chemostat throughout the experiment and incubated at the pH of growth in a pH stat, glycolytic activity was optimum at pH 5.5 in the absence of NaF. Fluoride stimulated glycolytic activity by cells incubated at pH 7.0 and by cells growing with 20 mM NaF, provided that the pH of growth remained at or above 6.0. A more detailed examination of the adaptation to fluoride tolerance during shifts to acidic pH values revealed that cells capable of growth on acidic fluoride agar plates appeared within 2 h of the start of the fall in pH of the chemostat culture. Estimation of the intracellular pH during the period of the initial pH fall revealed that the intracellular pH was identical to the extracellular pH (i.e., no pH gradient [ΔpH]), indicating that fluoride would not be transported into the cells to inhibit metabolism. However, once the pH of the medium was stabilized, ΔpHs were generated, with the ΔpH increasing as the pH declined. The inhibition of glycolysis by fluoride increased in proportion to the ΔpH. Cells grown at pH 5.5 generated larger ΔpHs than did cells grown at pH 7.0, although the values were normally small (~0.9 U). The data suggest that the inherent fluoride tolerance of *L. casei* RB1014 was associated with relatively small ΔpHs.

Considerable research has indicated a close association between the development of dental caries and the presence of *Streptococcus mutans* (15, 16, 23). However, various studies have suggested that in some cases other microorganisms, such as *Lactobacillus* and *Veillonella* species, may be involved in the demineralization process (5, 14, 16, 23, 26). Recently, we examined the microflora associated with incipient carious lesions in children living in an area with fluoridated water (R. M. Boyer and G. H. Bowden, Caries Res., in press). There was a significant association between the presence of *Lactobacillus* spp. and the progression of the lesion to a state needing restoration. *Lactobacillus* spp. were never isolated from static lesions or caries-free control sites. The presence of lactobacilli in overt carious lesions has been known for some time (9, 24), and this prompted earlier workers to regard members of this genus as important factors in the etiology of the disease (28). However, the association of lactobacilli with the progression of incipient lesions in a fluoridated area has not been previously reported.

A variety of epidemiological and clinical studies have demonstrated that fluoride is a highly effective anticaries agent (1, 7, 27), and part of this anticaries effect can be associated with the antimicrobial properties of this element (2, 11, 12). Fluoride is bound in plaque in much higher levels than those present in saliva, and bound fluoride can be liberated under acidic conditions (17). Furthermore, carious lesions accumulate acids (8) and fluoride (30); consequently, organisms growing in this habitat must be able to withstand a hostile environment, particularly since the inhibitory effect of fluoride increases as the pH decreases (3). It is not surprising, therefore, that oral *Lactobacillus* species have been found to be relatively resistant to the effects of fluoride compared with other oral bacteria (2, 22, 25, 26a).

Although there is little evidence to suggest that fluoride from drinking water eliminates bacteria from the plaque community, fluoride may give an ecological advantage to those bacteria, such as the lactobacilli, that are relatively resistant to the inhibitor by allowing them to compete with other bacteria in an acidic habitat such as a carious lesion (6). Once established, the lactobacilli would be able to grow and metabolize in the acidic environment in the presence of fluoride more readily than other acidogenic species and thus have a selective advantage in the ecosystem.

In a preliminary study (26a), we surveyed the effect of fluoride on the growth and metabolism of fresh and type strains of six *Lactobacillus* species. Generally, the fresh strains were more resistant to fluoride, and a significant number (25%) of the test strains were capable of growth at pH 4.5 in the presence of 5.3 mM NaF. Furthermore, glucose metabolism by washed cells of tolerant strains required 9.3 mM NaF to inhibit glycolysis completely at pH 5.0. The ability of these bacteria to function in such an acidic environment in the presence of this level of fluoride is unique among the bacteria that constitute the oral microflora (4, 11). Consequently, we examined in more detail the relationship

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between the aciduric and fluoride-resistant properties of one of the resistant strains, *L. casei* RB1014, isolated from a progressing, incipient carious lesion. The organism was grown in continuous culture to test the separate and combined effects of acidity and fluoride on cells growing in a controlled environment.

MATERIALS AND METHODS

Bacteriology. *L. casei* RB1014 was isolated as described elsewhere (Boyar and Bowden, in press). Before use in continuous culture experiments, the organisms were transferred in vitro seven times after isolation from human dental plaque. *Streptococcus mutans* was isolated as previously described (6).

Growth conditions. Continuous growth was achieved in a New Brunswick chemostat (model C30; New Brunswick Scientific Co., Inc., Edison, N.J.) with a working volume of 375 ml (12). The chemostat was modified to include flow through a variable-speed peristaltic pump (Watson-Marlowe, Falmouth, England). The medium inlet into the growth chamber passed through a heated tube held at 45°C with three 50-Ω resistors coupled to a 25-W transformer and a rheostat to prevent growth in the medium inlet tube. The medium (C-1) contained the following (per liter): tryptone, 10 g; yeast extract, 5 g; KH_2PO_4, 1.33 g; K_2HPO_4, 2.66 g; $MgSO_4 \cdot 7H_2O$, 10 mg; $FeCl_2$, 10 mg; $FeCl_2$, 10 mg; $MnSO_4 \cdot 4H_2O$, 10 mg; and NaCl, 10 mg. Glucose was at 2 mg/ml, and with this amount of glucose, the growth of the organism in the chemostat was limited by the carbon source.

General experimental design. The basic design of the continuous culture experiment was a modification of that reported previously (12). Stage 1 was an initial phase of steady-state growth at pH 7.0. Stage 2 was continuous growth between pH 7.0 and 3.2. Stage 3 was reestablishment of growth at pH 7.0. At stage 4, NaF was added to the growth medium to give a final concentration of 20 mM, and the pH was adjusted to 5.4. The dilution rate was 0.11 h^{-1} (mean generation time, 6.3 h), and the experiment was performed in the same chemostat with the same culture over a 30-day period.

Each experiment was designed to monitor the following: (i) total viable cell counts and cell counts on fluoride-containing plates; (ii) routine maintenance of the chemostat, including optical density, dry weight, and pH readings of the culture; and (iii) the glycolytic activity of washed cells obtained from the chemostat at each stage measured after incubation with glucose in a pH stat in the presence and absence of NaF.

Viable cell counts. Counts of the total numbers of viable cells in the medium in the chemostat chamber were made throughout the experiment. Dilutions of the medium up to 10^{-10} were made in reduced transport fluid (24). Counts were made with a spiral plater (Spiral System Inc., Cincinnati, Ohio) onto blood agar plates (blood agar base no. 2, CM 271; Oxoid Canada, Inc., Toronto, Canada) with 5% (vol/vol) sheep blood (Atlas Laboratories, Winnipeg, Canada) and onto a basal medium (6) at pH 6.5, 6.0, and 5.5 with fluoride at concentrations up to 16 mM. Duplicate plates were incubated at 35°C in 15% H_2–75% N_2–10% CO_2 for 16 h. Counts were made on plates viewed under a stereomicroscope on samples before and after sonication; the counts after sonication exceeded the counts on nonsonicated samples by a factor of 1:3 to 1:1.46, and the sonicated counts were recorded.

Routine maintenance of the chemostat. Daily routine maintenance included optical density readings in a Klett-Summerson colorimeter with a red filter (640 to 700 nm) and dry weight measurements. Cell dry weight determinations were carried out by filtering three 2- to 3-ml culture samples through prewashed, preweighed polycarbonate filters (0.4-μm pore size; Nuclepore Corp., Pleasanton, Calif.). The pH of the culture and the dilution rate were determined at least once a day. The glucose concentration in each batch of medium and in the culture filtrates was determined by the glucose oxidase method of Kingsley and Getchell (21).

Assay for glycolytic activity. Glycolytic activity was measured with washed cells and with cells collected and assayed directly from the growth chamber of the chemostat. Cells for washed cell experiments were collected at 0°C from the overflow for short periods (1 to 5 h). The cells were centrifuged (10,000 × *g* for 10 min), washed in saline (0.85%), and suspended at a concentration of 25 mg/ml. The cell density was determined from a Klett unit versus dry weight standard curve, which had been constructed for the test strain, as well as by dry weight determinations by the filtration technique. The glycolytic rate of this cell suspension was determined in an anaerobic pH stat at constant pH (7.0) as previously described (13), except that the reaction mixture contained tryptone (10 g/liter) and yeast extract (5 g/liter) in 10 mM sodium potassium phosphate buffer (pH 7.0), to which was added 5 mg (dry weight) of cells in a total volume of 4.9 ml. The cell suspension was constantly mixed by a magnetic stirring bar in a stream of nitrogen gas, and the pH was kept constant with standardized 0.1 M KOH added by means of a Radiometer Autoburette (model ABL 1a). The cell mixture was preincubated for sufficient time (usually 10 min) to run down the traces of carbohydrate present in the tryptone-yeast extract buffer mixture; the cells contained no endogenous carbohydrate reserves. The glycolytic rate was then determined after the addition of 100 μl of 0.5 M glucose (final concentration, 12 mM).

This system was also used to test for the effect of NaF on the glycolytic rate at pH 7.0. Once the rate was established in the absence of NaF, 20- to 50-μl samples of NaF (1 M) were added to the same reaction mixture in consecutive additions at suitable time intervals (usually 3 to 5 min), and the amount of KOH required to maintain the pH at 7.0 was recorded for a specified time at each fluoride concentration. The fluoride concentration was calculated after considering the change in volume of the reaction mixture.

The glycolytic activity by in vivo cells collected directly from the chemostat was estimated in conjunction with measurements of the internal pH (pH_i) of the cells growing at different external pH (pH_e) values in the chemostat. Culture (6 ml) was removed from the chamber and incubated in the pH stat as described for the washed cell experiments. After a 3-min incubation at 37°C, glucose was added to give a final concentration of 6 mM. The glycolytic rate was recorded for 6 min, at which time NaF was added and the rate was recorded as previously described. Cell dry weight determinations were carried out for each direct cell sample. The maximum amount of sample collected from the chamber at any one time was normally 10 ml, and removal of this volume periodically did not perturb the culture, since it represented less than 3% of the total culture volume. In all cases, the assay was completed within 20 min of the cells being removed from the chemostat.

Measurements of pH in growing cells. The pH_i of growing cells of *L. casei* RB1014 was assayed with [^{14}C]salicylate (500 Ci/mol) (18) under the conditions described above for the assay of glycolytic activity by direct cells. The assay

consisted of three phases: (i) the initial phase consisting of unaltered cells, which represented the pH_i of the growing in vivo cells; (ii) the pH_i after the addition of glucose; and (iii) the pH_i after the addition of 12.5 mM NaF. During each period, duplicate 0.5-ml samples were removed and the cells were separated from the medium by centrifugation through a silicone oil mixture (50% fluid 550 and 50% fluid 556 [vol/vol]; Dow Corning Corp., Midland, Mich.) as described by Kashket and Baker (18). After removal of the aqueous phase and oil, the tubes were cut above the cellular pellet and counted in 5 ml of Aquasol (New England Nuclear Canada Ltd., Montreal, Canada) in a liquid scintillation counter.

Cells growing at pHs 7.0 and 5.5 were tested for their ability to generate pH gradients (ΔpH) to determine whether growth pH was a factor. In these experiments, the cells were removed from the chemostat and the pH was adjusted to values between 4 and 7 with either Tris crystals or HCl. After an incubation period of 15 min in a pH stat at the desired pH, the pH_i was determined as previously described.

Intracellular water volume. The intracellular and the extracellular water volumes were determined with the same cells employed for the pH_i measurements. Cells were incubated with ^3H_2O and [^{3}H]polyethylene glycol for 5 min and centrifuged through silicone oil as previously described (18, 20). The intracellular water volume values for *L. casei* RB1014 grown in the chemostat with limiting glucose varied with growth pH from 2.43 to 1.49 μl/mg (dry weight) of cells between pHs of 7.0 and 4.1, respectively. Similar measurements were carried out with chemostat cultures of *S. mutans* 2452 and gave an average value of 2.01 μl/mg (dry weight) of cells at pH 5.5.

Chemicals. Radioactive salicylate and polyethylene glycol were purchased from New England Nuclear, Montreal, and tritiated water was purchased from Amersham, Oakville, Canada. All other reagents were of analytical grade.

RESULTS

Chemostat studies. Earlier work had established that *L. casei* RB1014 would grow at pH 5.0 in broth in the presence of 5 mM NaF (26a). To further study the effects of pH and fluoride on the growth characteristics of *L. casei* RB1014, we employed a four-stage growth protocol in a chemostat similar to that previously described for *S. mutans*. and *S. mitior* (12). Stages 1 and 3 established steady-state growth at pH 7.0 with a constant supply of limiting glucose (2.0 mg/ml) at a dilution rate of 0.11 h^{-1} (mean generation time, 6.3 h). Stage 2 represented growth at lower pHs to a minimum of 3.2, whereas growth in stage 4 occurred at pHs as low as 5.4 in the presence of 20 mM NaF. Thus, cells were always in a controlled anaerobic environment in either fluoride-free or fluoride-containing medium where the pH of the culture was the only growth variable.

As shown in Fig. 1, the initial phase (stage 1) established growth at pH 7.0 such that at steady state, cell numbers of 3.2×10^9/ml were obtained. On day 9, the pH control system on the chemostat was turned off, initiating stage 2, and within 2 days the pH of growth had stabilized at 4.95. During growth at this pH, cell numbers rose to 9.04×10^{10} on blood agar plates. Surprisingly, during the same period almost equivalent cell numbers appeared for the first time on agar plates containing 10.5 mM NaF at pH 5.5, which had been incubated for 16 h. These cells had had no prior exposure to fluoride.

The 16-h incubation period for all plate counts was selected to avoid, as far as was possible, counting cells that had grown through adaptation while on the agar plate (12).

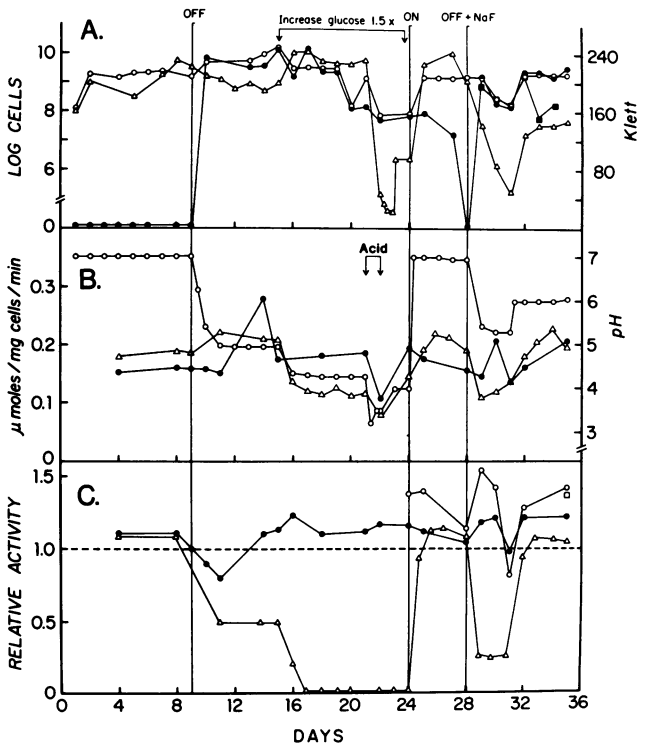

FIG. 1. Response of *L. casei* RB1014 to changes in pH during growth in a chemostat in the presence and absence of NaF at D = 0.11 h^{-1}. pH control was turned off on day 9, on again on day 24, and off on day 28, when fluoride was added to the medium (final concentration, 20 mM). The glucose concentration was increased 1.5 times between days 15 and 23, and acid was added on days 21 and 22 to reduce the pH. (A) Total cell counts. Blood agar (○), agar (pH 5.5) with 10.5 (●) and 16 (■) mM NaF, Klett reading (△). (B) The pH (○) and glycolytic rate. Direct cells at the pH of growth (△) and washed cells at pH 7.0 (●). (C) Relative glycolytic activity. Direct cells at the pH of growth plus 5 mM NaF (△) and washed cells (pH 7.0) plus 10.5 (○), 24 (●), and 120 (□) mM NaF.

Whereas the bacteria may have adapted to the fluoride in a relatively short time (Fig. 1), this would not have been evident on plate cultures for which the colonies of the organisms were only easily visible after 16 h of incubation. Preliminary evidence had indicated that adaptation on plates did not occur to an appreciable extent within the first 24 h, since cells grown in the chemostat at pH 7.0 and unable to grow on the fluoride plates at pH 5.5 within 16 h only began to appear on plates after incubation for 72 h; the number of cells that did adapt and grow to visible colonies after 72 h, however, was far fewer (about 10^4/ml) than the total viable counts (3.2×10^9/ml).

On day 15, a 1.5-fold higher concentration of glucose was added to the medium, resulting in a further reduction of the pH to 4.27; the culture remained glucose limited. This level was maintained for a 6-day period and caused a slight drop in cell numbers to 4.7×10^9/ml on blood agar as well as a concomitant drop in the cell numbers on the 10.5 mM NaF (pH 5.5) agar plates. Only when acid was added to the system to reduce the pH to 3.2 on day 21 was there a drastic alteration in the cell numbers. The fall in cell concentration over the next 36 h was monitored frequently with Klett readings, and "wash out" of the culture would have occurred had the pH not been adjusted to 3.5 and then to 4.0. On day 22, when the culture pH was 3.5, cell numbers had

TABLE 1. Effect of growth pH on cell numbers and glycolytic activity when calculated at a constant amount of biomass

Stage	F concn in medium (mM)	pH_e	Growth	Glycolytic activity	
			CFU × 10^{10}/mg of cells	nmol/mg per min	nmol/min per CFU × 10^{10}
1	0	7.0	19.6 ± 6.7	164	8.37
2	0	4.95	142.5 ± 3.5	218	1.53
	0	4.25	64.4 ± 11.9	115	1.79
	0	3.5	2.57 ± 0.08	65	25.3
3	0	7.0	25.1 ± 1.3	213	8.49
4	20	5.4	16.0[a]	113	7.06
	20	6.0	56.6[a]	207	3.66

[a] Single samples.

fallen to 8.5×10^7/ml. On day 24, the pH was reestablished at 7.0 (stage 3), and cell numbers on blood agar returned to normal values (1.8×10^9/ml) on day 25. However, over the 4-day period at pH 7.0, the counts on the 10.5 mM NaF (pH 5.5) agar plates fell to zero, confirming an association of fluoride tolerance with growth in an acidic environment.

On day 28, 20 mM NaF was added to the growth medium, the pH control was turned off, and the pH decreased to 5.4. The cells were stressed by this procedure, since the counts on blood agar fell from 1.7×10^9 to 0.2×10^9/ml over a 3-day period, a fact more readily seen with the corresponding Klett readings. Establishment of a constant pH of 6.0 on day 31 stabilized cell numbers at 3.1×10^9/ml. Of interest again was the reappearance on day 30 of high levels of cells (1.4×10^9/ml) able to tolerate 10.5 mM NaF (pH 5.5). Furthermore, there also appeared for the first time cells (7.3×10^8/ml) able to grow in the presence of 16 mM NaF (pH 5.5).

A further observation that was made during the experiment was a change in the numbers of viable cells that contributed to 1 mg dry wt of biomass (Table 1). During stage 2, when the pH was 4.95, there was a sevenfold increase (142×10^{10}) in viable cell numbers for 1 mg (dry weight) of biomass when compared with cells growing at pH 7.0 (19.6×10^{10}). This suggested that there had been a reduction in cell size as the pH was lowered. However, the further reduction in pH during stage 2 to 3.5 reversed this trend, since the number of viable cells at pH 3.5 (2.57×10^{10}/mg [dry weight] of biomass) was almost eight times less than that at pH 7.0, suggesting that the cells had substantially increased in size. Reestablishment of the pH at 7.0 during stage 3 resulted in a return of the numbers of cells per milligram of biomass to a figure close to that of cells growing at pH 7.0 in stage 1.

Glycolytic activity. As a measure of the effect of environmental stress on the culture, cells were removed periodically from the chemostat and assayed for glycolytic activity either directly from the chemostat at the pH of growth or as a washed cell suspension at pH 7.0. The latter was included to indicate whether inherent changes had occurred in the cells after the various changes in the environment. As seen in Fig. 1B, the glycolytic activity of unaltered or direct cells increased slightly in phase 2 as the pH decreased from 7.0 to 4.95. However, a further reduction to pH 4.27 on day 15 resulted in almost a 50% reduction in activity (i.e., 0.115 versus 0.218 μmol/mg per min), and growth at pH 4.0 further reduced activity. The return to pH 7.0 in stage 3 resulted in higher activity than in stage 1 for the first 2 days (days 25 and 26) before a return to a value typical of cells at pH 7.0. Growth at pH 5.4 in the presence of fluoride (stage 4) produced cells with glycolytic activity similar to that of cells grown near pH 4.0; however, adjustment of the culture to pH 6.0 resulted in activity typical of cells growing at pH 7.0 without fluoride.

The activity of washed cells collected periodically throughout the experiment and incubated with glucose at pH 7.0 mimicked to some extent the results with the direct cells. An increase in specific activity was seen on day 14 when the pH was 4.95; however, this may have been an aberration, since activity on day 15 was lower. The pH optimum for glycolysis was obviously lower than 7.0, since cells grown at 4.25 possessed 20% more activity (0.183 versus 0.156 μmol/mg per min) than did cells grown at pH 7.0. A noticeable drop in activity was observed during the brief period when cells were stressed at pH 3.5 in stage 2. Activity did return to normal when the pH was reestablished at pH 7, and it remained near that level even at pH 5.4 with fluoride in stage 4.

An interesting comparison can be made between the cells growing at each stage by calculating the glycolytic activity as a function of CFUs (Table 1). As the growth pH was lowered to 4.95 and 4.25 in phase 2, each cell unit possessed only 20% of the activity of each cell at pH 7.0. However, the generation of large cells at pH 3.5 resulted in cell units with three-fold higher activity than cells at pH 7.0. Cells growing with fluoride in stage 4 at pHs 5.4 and 6.0 had 83 and 43% of the activity, respectively, of the activity at pH 7.0 in stage 3.

The effect of NaF on glycolysis by direct and washed cells throughout the experiment can be seen in Fig. 1C. The effect of 5 mM NaF on cells removed directly from the chemostat and incubated with glucose at the pH of growth varied with the pH. Fluoride was stimulatory at pH 7.0 but inhibited glycolysis by 50% at pH 4.95 and by 100% at pH 4.25 and below. In stage 4, the cells received 5 mM NaF in addition to the 20 mM NaF present in the medium, and consequently the cells at pH 5.4 were stressed by this level of NaF (25 mM). Surprisingly, when the pH was raised to 6.0 glycolysis was stimulated by this level of fluoride.

In the washed cell experiments, the addition of 10.5 mM NaF at pH 7.0 stimulated glycolysis except for a brief period at the onset of the acid environment at the beginning of stage 2. Maximum inhibition during the 3-day period from days 9 to 12 was only 20%, whereas a maximum stimulation of 24% was observed at this level of fluoride during other periods of the experiment. Surprisingly, the addition of 24 mM NaF to metabolizing cells collected after day 24 resulted in a further stimulation of glycolytic activity by as much as 50%. Only on day 31, when cells were stressed by both the acid environment and fluoride, was inhibition (20%) observed. Furthermore, when the culture was stabilized at pH 6.0, the addition of 120 mM NaF to fermenting cells stimulated glycolysis by 35%.

Determination of the yield coefficient (grams of cells per mole of glucose) for *L. casei* RB1014 growing in continuous culture between pHs 3.5 and 7.5 with limiting glucose in the absence of fluoride revealed that the maximum yield was between pHs 5.0 and 6.5 (Fig. 2). Optimum glycolytic activity was between pHs 5.5 and 6.5 (data not shown), confirming the observations in Fig. 1.

Adaptation to fluoride resistance. The appearance of fluoride-resistant cells on day 10 in Fig. 1 in response to a change in the pH of the medium was examined in more detail in a separate experiment. Cells were grown under the same conditions as in Fig. 1, except that the glucose concentration was doubled to 4 mg/ml, and cells were removed and plated

on fluoride-containing agar at more frequent intervals after the pH control system was shut off. During the same period, the pH_i of the cells was determined with labeled salicylate. The total cell counts on blood agar over a period of 144 h (6 days) was not significantly affected by a decline in the pH of the medium (pH_e) from 7.0 to 4.1 (Fig. 3). Within 2 h of the start of the pH drop, cells began to appear in significant numbers on agar plates containing 2.6 mM NaF (pH 5.0) and 10.5 mM NaF (pH 5.5). By 24 h, cell numbers on these plates and those on 16 mM NaF (pH 5.5) were equivalent to the control plates (Fig. 2A). During this period, the pH_e had fallen to 4.1, and the pH_i had fallen to just below 5.0.

The return of the pH_e to 7.0 resulted in immediate equilibration of the pH_i with the external medium so that there was virtually no ΔpH. The rapid shift in pH from 4.1 to 7.0 resulted in a mild perturbation of the culture such that the total viable cell count fell from 4×10^8 to 0.8×10^8/ml within 7 h of the pH change. This is seen more dramatically with the counts on 2.6 mM NaF (pH 5.0) agar plates. Cells are normally able to grow at this fluoride and pH level, albeit at a slightly lower count. Thus, the change in pH from 4.1 to 7.0 resulted in a significant but transitory change in the culture.

Relationship of pH_i to fluoride. Casual observation of Fig. 3B indicates that for a significant portion of the pH fall in the initial 6 h, the pH_e and pH_i were very similar; i.e., there was virtually no ΔpH. This is more clearly seen in Fig. 4, which plots the ΔpH as a function of the pH_e for this period. Cells of *L. casei* RB1014 did not generate a significant ΔpH when growing in a environment in which the pH was falling from 7.0 to 5.8, although ΔpH increased rapidly from pH 5.5 to 4.1. The profile for *S. mutans* 2452 under the same conditions, on the other hand, exhibited a negative linear relationship throughout the region from pH 4.5 to pH 7.0.

Subsequently, it was shown that the absence of a ΔpH in *L. casei* was a unique feature of cells growing in an increasingly acidic environment, since cells stabilized at pH values below 7.0 would establish a ΔpH, the magnitude of which was a function of the pH_e. In this experiment, cells were grown continuously at pHs 7.0 and 5.5 and removed directly from the chemostat, and the pH_i was determined at pHs between 7.0 and 4.0 in a pH stat after a preincubation period of 15 min. Cells grown at both pHs 7.0 and 5.5 exhibited ΔpHs at all incubation pHs below 7.0 (Table 2). What was

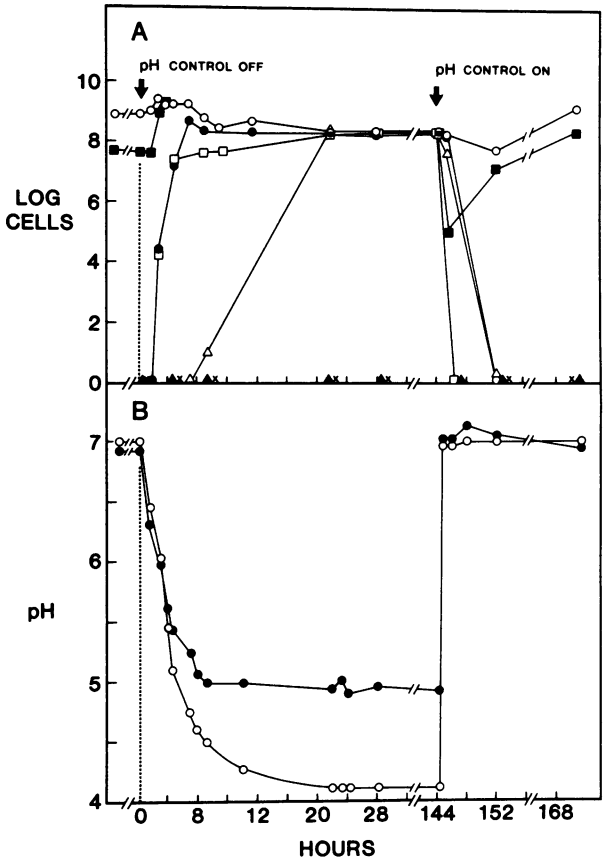

FIG. 3. Effect of a drop in growth pH (pH_e) on the pH_i of *L. casei* RB1014 and the ability to grow on fluoride-containing agar. (A) Total cell counts on agar containing 0 (○), 10.5 (●), 16 (△), and 21 (▲) mM NaF at pH 5.5 and 2.6 (■), 5.3 (□), and 10.5 (×) mM NaF at pH 5.0. (B) pH_e (○) and pH_i (●).

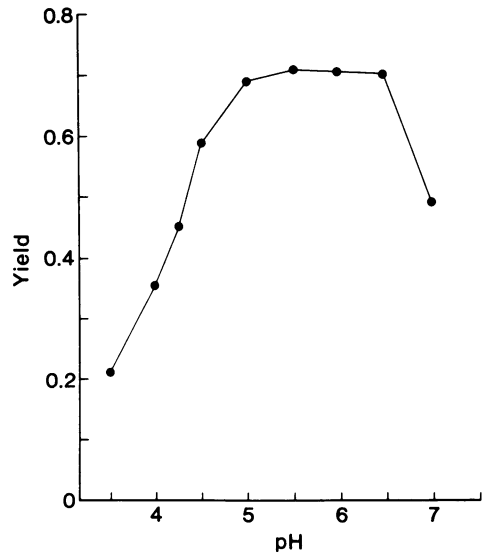

FIG. 2. Effect of growth pH on the yield of *L. casei* RB1014 grown in continuous culture (D = 0.1 h^{-1}) with glucose limitation (Yield = gram [dry weight] of cells per mole of glucose).

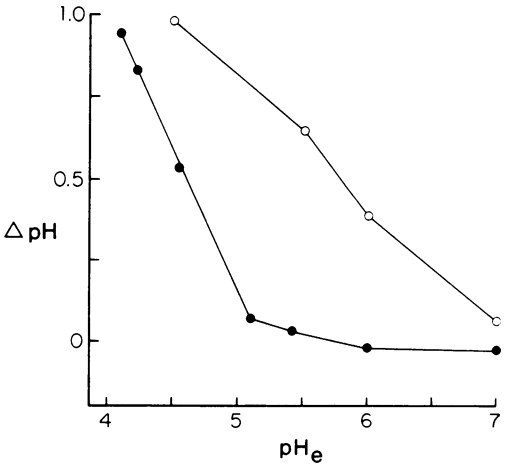

FIG. 4. Comparison of the ΔpHs generated by *L. casei* RB1014 (●) and *S. mutans* 2452 (○) growing at pHs between 4.1 and 7.0 in continuous culture with glucose limitation at D = 0.11 h^{-1}.

TABLE 2. Effect of growth pH on the ability of chemostat-grown cells to generate ΔpHs when stabilized at pHs between 4 and 7 in a pH stat

Growth pH	ΔpH at pH_e of:						
	7.0	6.5	6.0	5.5	5.0	4.5	4.0
7.0	0.02	0.12	0.18	0.23	0.27	0.32	0.42
5.5	0.0	0.12	0.29	0.37	0.52	0.60	0.71

unexpected in this experiment was the observation that cells grown at the lower pH were capable of larger ΔpHs than were cells grown at pH 7.0. Thus, it would appear that some form of adaptation had occurred which resulted in increased ΔpH-generating activity.

We reasoned that these pH profiles would have a significant effect on the ability of these cells to tolerate fluoride, since a ΔpH is essential for fluoride transport (30). To test for this, the pH_i of *L. casei* cells incubated with glucose was determined in the presence and absence of NaF when exposed to pH_es of 7.0, 5.5, and 4.1. The uptake of fluoride would be expected to acidify the cytoplasm through the transport of fluoride into the cells as HF and its subsequent dissociation to H^+ and F^- (10, 30).The glycolytic rate of cells incubated at a pH_e of 7.0 was unaffected by 13 mM NaF, whereas that of cells at pH_e 5.5 was inhibited by 28% by 1.5 mM NaF, and that of pH_e 4.1 cells was completely inhibited by 0.5 mM NaF (Table 3). The ΔpH for the cells incubated at pH 5.5 was 0.36, and the addition of 1.5 mM NaF reduced the ΔpH to 0.25, indicating a small degree of acidification of the cytoplasm. With the cells incubated at pH 4.1, the ΔpH was 0.83 without fluoride and 0.76 in the presence of 0.5 mM NaF. In both cases, it is difficult to see how these relatively minor changes in the pH_i were responsible for the degree of inhibition observed.

DISCUSSION

The data from the experiments reported here show that *L. casei* RB1014 was inhibited by fluoride with the effect increasing as the pH was decreased in the classical fashion (3). However, the results do indicate that higher concentrations of fluoride are required to inhibit metabolism by the organism than are required to inhibit other bacteria at low pH (6, 11, 12, 26a).

One of the more interesting observations was the appearance of cells able to grow on pH 5.5 agar containing 10.5 mM NaF within several hours of the initiation of a fall in pH from 7.0 (Fig. 1 [phase 2] and Fig. 2). Clearly, the only change the cells were subjected to during this period was the decreasing pH of the medium as the result of self-generated acid endproducts. Furthermore, this fluoride tolerance trait was lost rapidly when the culture was returned to pH 7.0 (Fig. 1 and 2). Obviously, the change in cell physiology was a phenotypic one, relying on preexisting properties of the cell.

The explanation for the rapid appearance of fluoride tolerance in these experiments is not clear. Fluoride has been shown to have various specific effects on bacteria. Glycolysis is inhibited by fluoride at the enolase step (3, 11), and the resultant reduction in the supply of phosphoenolpyruvate inhibits sugar transport via the phosphoenolpyruvate-phosphotransferase system (11). Fluoride is also known to exist in bound and free forms within cells (19), and the inhibitor is generally thought to be transported into cells as HF, probably by passive diffusion (30). The latter process has been shown to require a ΔpH with the uptake of fluoride occurring in proportion to the size of the ΔpH (10, 30). This observation is consistent with the notion that fluoride is more inhibitory at low pH_es, since it is generally believed that the pH_i of neutrophilic bacteria is close to neutrality. Consequently, the growth of these bacteria in acidic environments would create large ΔpHs between external and internal aqueous phases. A significant portion of the fluoride in acidic environments would be in the form of HF, which is readily transported into the more alkaline cytoplasm, where it dissociates into F^- and H^+, the latter contributing to a reduction in the cytoplasmic pH (10). This, in turn, would reduce metabolic activity, since cellular enzymes would be exposed to suboptimal pH values.

In ascribing a mechanism for the appearance of fluoride tolerance in *L. casei* RB1014, it is perhaps useful to reflect on the properties exhibited by the organism in these experiments. (i) Cells growing at pH 7.0 when transferred to fluoride (10.5 mM) agar plates at pH 5.5 generated small ΔpHs (Table 3). (ii) Cells were devoid of ΔpHs during periods of rapidly falling pH_e (Fig. 2). (iii) When fluoride tolerance appeared, inhibition of glycolysis increased as the pH decreased (Fig. 1, Table 4). (iv) The acidification of the cytoplasm by fluoride, particularly at low pH, was minimal. (v) Maximum growth and metabolism occurred between pHs 5.0 and 6.5. (vi) Cells increased their capacity to generate a ΔpH when shifted from growth at pH 7.0 to pH 5.5 (Table 3). Connecting all of these observations in a single hypothetical mechanism may not be possible.

In reviewing the facts, one is nevertheless struck by the fact that *L. casei* RB1014, like other members of the genus *Lactobacillus*, grows well in acid environments in which the pH_i is not near neutrality (Tables 3 and 4). For example, cells growing at pH 5.5 would have pH_is near 5.9; consequently, one must assume that the enzymes of glycolysis possess lower pH optima than less aciduric bacteria. As a consequence of this, cells transferred from pH 7.0 to pH 5.5 (Fig. 1 and 2) would be moving to a more favorable environment.

The relatively small ΔpHs in *L. casei* in acidic environments also mean that less HF is transported into the cells (30). This was confirmed by the relatively small decreases in the pH_i on the addition of fluoride at pH 5.5 and below (Table 4).

A possible explanation for the rapid appearance of the fluoride tolerance seen in Fig. 1 and 2 is as follows. Cells growing at pH 7.0 (Fig. 1, stage 1) were growing suboptimally and were inhibited when shifted to pH 5.5 plates containing 10.5 mM NaF, because a small ΔpH was generated and fluoride was transported into the cells. When pH control in the chemostat was removed and the pH began to fall, the cells were not particularly adept at generating ΔpHs

TABLE 3. Effect of fluoride on the pH_i and the rate of glycolysis of cells of *L. casei* RB1014 grown in continuous culture with glucose limitation at pH_e 7.0, 5.5, and 4.1

pH_e	pH_i	Rate of glycolysis[a]	Inhibition of glycolysis (%)
Control			
4.1	4.93	125	
5.5	5.86	193	
7.0	7.01	199	
With fluoride (mM)			
4.1 (0.5)	4.86	0	100
5.5 (1.5)	5.75	138	28
7.0 (13)	7.02	194	3

[a] Nanomoles of acid neutralized per milligram of cells per minute.

(Fig. 2 and Table 3), and they were plated on pH 5.5 agar (with 10.5 mM NaF) devoid of a ΔpH. Initially, fluoride was not transported, and since the cells were in a more favorable growing environment, they began to grow and divide rapidly, and although they rapidly acquired the ability to generate ΔpHs and would, as a consequence, take up fluoride, growth had been established. One cannot forget the fact that the cells growing on plates with fluoride were growing in an environment different from that in broth. Furthermore, it is clear that the colonies on pH 5.5 plates growing in the presence of NaF were smaller, which is indicative of some inhibition by fluoride. However, the cell counting procedure did not discriminate between large and small colonies, and consequently the data in Fig. 1 and 2 may appear more absolute than is really the case.

One can conclude that *L. casei* RB1014 is inhibited by fluoride, but higher concentrations of the inhibitor are required to completely inhibit metabolism. We suggest that small ΔpHs generated by these cells in acidic environments resulted in lower uptake of fluoride into the cells with the resultant smaller inhibitory effect.

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