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Lowering of Cytoplasmic pH Is Essential for Growth of *Streptococcus faecalis* at High pH

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The growth of *Streptococcus faecalis* at high pH was significantly stimulated by carbonate. In the absence of added carbonate the cells were unable to grow at a pH above 9.5, but in media containing 50 mM HCO_3^- they grew even at pH 10.5. Both rate and yield of growth at pH 9.5 were significantly stimulated by as little as 5 mM carbonate. The cytoplasmic pH in growing cells was maintained at about 7.8 to 8.2, whereas the medium pH ranged from 8.4 to 9.5. Nigericin and gramicidin D, ionophores which conduct protons, blocked growth at pH 9.5 but not at pH 7.5. These results indicate that lowering of the cytoplasmic pH is essential for the growth of this organism at high pH.

There is now substantial evidence from studies on a variety of bacteria that the cytoplasmic pH (pH_{in}) is maintained within a relatively narrow range. The cytoplasmic pH range of acidophiles is about 6.5 to 7.0, that of neutrophiles is 7.5 to 8.0, and that of alkalophiles is 8.5 to 9.0 (2). It is generally assumed that pH_{in} is regulated by various cation transport systems. In *Escherichia coli* grown at low pH, pH_{in} is increased by a combination of proton expulsion, via the respiratory chain, and electrogenic potassium influx. At high pH, pH_{in} is lowered by the influx of protons via the Na^+/H^+ or K^+/H^+ antiporter (2). Similar mechanisms have been proposed for *Bacillus alcalophilus* (12) and *Vibrio alginolyticus* (13). On the other hand, it was proposed that in *Streptococcus faecalis* ATCC 9790 pH_{in} is regulated at 7.5 to 7.7 solely by increasing the pH (9). In *S. faecalis*, which has no respiratory chain, pH_{in} is increased by a combination of proton extrusion, via a proton-translocating ATPase, and electrogenic potassium influx (10). When the medium pH rises above 8.0, however, the cytoplasmic pH of *S. faecalis* increases as medium pH increases. Moreover, in a previous study the growth rate of *S. faecalis* decreased markedly at a medium pH above 8.0, and no growth was observed at a pH above 9.0 (9). These observations all indicate that *S. faecalis* has no system to lower the pH_{in}.

On the other hand, the ability to grow in high-pH media (pH 9.6) is one of the characteristics by which *S. faecalis* and *S. faecium* are identified (4). Is *S. faecalis* ATCC 9790 exceptionally alkaline sensitive? In the present study I examined the growth of ATCC 9790 at high pH and found that it grew well at pH 9.6 provided that carbonate was present in the medium.

All of the experiments in the present study were conducted with either *S. faecalis* (*faecium*) ATCC 9790, which was generously supplied by F. M. Harold, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo., or mutant AS25, derived from this strain. AS25, which is defective in proton extrusion and H^+-ATPase activity (9), was kindly supplied by H. Kobayashi, Chiba University, Chiba, Japan. Cells were grown at 37°C in a complex medium containing the following components per liter: 10 g of tryptone (Difco Laboratories), 5 g of yeast extract (Difco), 10 g of glucose, and 6.9 g of K_2CO_3 (KTY medium). A solution of K_2CO_3 at four times the final concentration was autoclaved separately and added aseptically to the medium. K_2CO_3 was replaced with $KHCO_3$ (at pH 7.5) or K^+-2-[*N*-cyclohexylamino]ethanesulfonic acid (K^+-CHES) (pH 9.5), but, in all cases, the given concentration of K^+ was adjusted to 100 mM by the addition of KCl or KOH. Finally, the medium contained approximately 110 mM K^+ ions and 10 mM Na^+ ions.

The growth of cells was monitored by measuring the optical density at 650 nm with a Perkin-Elmer spectrophotometer (model 35). The growth rates were determined between the optical density of 0.1 and 0.2. There was no significant change in the medium pH during this period. The stated value of medium pH represents the initial pH.

Generation of a pH gradient (low internal pH) by growing *S. faecalis* cells was determined at 25°C on the basis of the equilibrium distribution of [^{14}C]methylamine and [^{14}C]benzylamine (2, 10). When the turbidity of the culture reached an optical density of 0.2 at 650 nm, [^{14}C]methylamine (10 μM; 4 μCi/μmol) or [^{14}C]benzylamine (10 μM; 4 μCi/μmol) was added to 10 ml of culture. The accumulation of these radioactive probes was determined by filtering culture fluid through a Whatman GF/C glass filter and measuring the counts per minute on the filters. By the same procedure, generation of membrane potential (inside negative) and ΔpH (high internal pH) by growing cells was measured with [^{3}H]tetraphenylphosphonium (9.3 μM; 5 μCi/μmol) and [^{14}C]acetylsalicylic acid (9.8 μM; 3.5 μCi/μmol), respectively (10). Nonspecific binding of these probes to cells was determined with boiled cells or cells treated with *n*-butanol (8). Before filtration of cultures, the turbidity was measured at 650 nm, and the dry weight of cells was calculated from the relationship of turbidity to cell dry weight. The volume of cytoplasmic water space was determined with [^{3}H]sorbitol; 2 μl was used per mg (dry weight) of cell (1).

Kobayashi (9) reported that the optimal medium pH range for growth of *S. faecalis* ATCC 9790 is 6.5 to 7.8. He used Tris as a buffer for media at high pH and found that the growth rate dropped sharply above pH 8.0 (9). Since *S. faecalis* grows at pH 9.6 (4), however, the effect of a high medium pH on the growth rate of *S. faecalis* ATCC 9790 was examined (Fig. 1A). It has been reported that the growth of enterococci at high pH is stimulated in media buffered with carbonate (3). In the present study, the growth rate in media containing 50 mM carbonate or bicarbonate was 2.1 h^{-1} (corresponding to a doubling time of 29 min) at pH 7.0 to 8.4. Strain ATCC 9790 still grew even at pH 10.5 at the growth rate of 0.7 h^{-1} (doubling time, 86 min). In the absence of

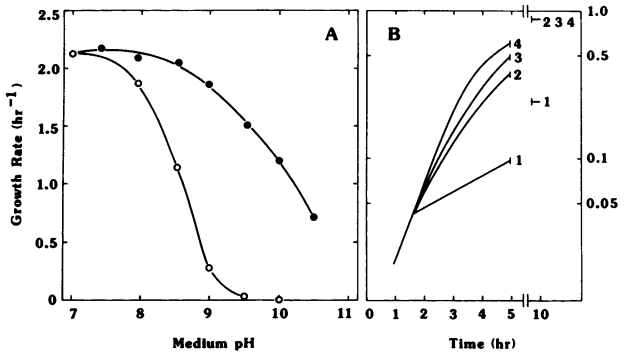

FIG. 1. Effect of bicarbonate on the growth of *S. faecalis* ATCC 9790. (A) Growth rates of ATCC 9790 at high pHs. Cells were grown at 37°C on the complex medium, and growth rates were determined as described in Materials and Methods. Symbols: ○, growth in media containing no added HCO_3^-; ●, growth in media containing 50 mM HCO_3^-. (B) Effect of carbonate concentration on the growth of ATCC 9790. Cells were grown on the complex medium, which was buffered with 100 mM K^+-CHES (pH 9.2), containing various concentrations of carbonate. The growth of cells was monitored by measuring the turbidity at 650 nm. Curves: 1, no carbonate added; 2, 5 mM carbonate added; 3, 10 mM carbonate added; 4, 20 mM carbonate added.

carbonate or bicarbonate, the cells grew at the optimal rate at pH 7.0 to 7.5 but failed to grow at pH 9.5. Neither CHES (Fig. 1B) nor 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) added to the medium could duplicate the effect of carbonate. These results indicated that, although ATCC 9790 has a marked tolerance to high pH, carbonate is required for growth of *S. faecalis* at high pH. The dependence of cell growth at pH 9.5 on the concentration of carbonate is shown in Fig. 1B. In media without added carbonate, a biphasic growth curve was obtained. Cells grew well initially, but after one doubling the growth slowed markedly. Both rate and yield of growth were substantially stimulated by carbonate. Carbonate at a concentration of as little as 5 mM decreased the doubling time from 170 min to 50 min. The growth yield in the presence of 5 mM carbonate was similar to that in the presence of a high concentration of carbonate. These results indicate that carbonate (actually HCO_3^- or CO_2) is required for growth at high pH.

Kobayashi (9) pointed out that the maintenance of cytoplasmic pH within the range 7.5 to 7.7 is essential for optimal growth of *S. faecalis*. He also suggested that *S. faecalis* has no system to lower the cytoplasmic pH (9). Since his experiments were performed without carbonate, it is important to examine the cytoplasmic pH at high medium pH in the presence of carbonate. The cytoplasmic pH values of growing cells as a function of medium pH are shown in Fig. 2. At pH 7.0, pH_{in} determined with acetylsalicylic acid was calculated to be 7.5, which corresponds to a proton potential of −30 mV. On the other hand, at pH 8.4 pH_{in} with benzylamine or methylamine was found to be 7.8, which corresponds to a proton potential of +35 mV. Moreover, pH_{in} at pH 9.5 was calculated to be 8.2 (a proton potential of +77 mV). These results clearly indicate that growing cells maintain the cytoplasmic pH at about 7.8 to 8.2, even when growing in medium with a pH as high as 9.5. At pH 10.0, however, pH_{in} increased to 8.8. At pH 8.5, no accumulation of acetylsalicylic acid was detected. No methylamine or benzylamine accumulated in the cells at pH 7.5. Accumulation of methylamine and benzylamine at pH 9.5 was blocked by addition of the membrane-permeable weak base ethanolamine (50 mM). More importantly, it was blocked with 5 μg of nigericin or gramicidin D per ml (data not shown). These results suggest that growing *S. faecalis* cells lower the pH_{in} when grown in alkaline medium. The growth and ability of this organism to lower the pH_{in} when grown in alkaline medium were also observed in the other complex medium (NaTY medium), which contained approximately 115 mM Na^+ ions and 10 mM K^+ ions.

Harold and Van Brunt (6) demonstrated that the circulation of H^+ and K^+ is not obligatory for *S. faecalis* to grow, provided that the cells are grown on a rich medium with a high concentration of K^+ and a slightly high pH (7.5 to 7.7). The effect of nigericin on the growth of *S. faecalis* at pH 7.5 and 9.5 is shown in Fig. 3. Nigericin (5 μg/ml) did not inhibit growth at pH 7.5. At pH 9.5, however, cell growth was completely stopped by addition of the ionophore, which blocked the generation of the reversed ΔpH at pH 9.5 (see above). Cell growth at pH 9.5 was also inhibited by the addition of gramicidin D (5 μg/ml) and by the addition of the membrane-permeable base ethanolamine (50 mM). In contrast, inhibition of growth by ionophores at pH 9.5 was reversed by a pH shift from 9.5 to 7.5. These results suggest that the lowering of cytoplasmic pH is essential to cell growth at high pH.

The cytoplasmic pH in *S. faecalis* is regulated by H^+-ATPase at low pH (9, 10). To determine whether the H^+-ATPase participates in the lowering of cytoplasmic pH under alkaline conditions, the growth of AS25, which is an *S. faecalis* mutant defective in H^+-ATPase (11), was examined at high pH. In media containing carbonate, the cells grew at pH 9.6. The growth rate of AS25 was 2.0 h^{-1} (doubling time, 30 min) at pH 8.0 and 1.4 h^{-1} (doubling time, 43 min) at pH 9.6. Also, the pH gradient (ΔpH) of growing AS25 cells was

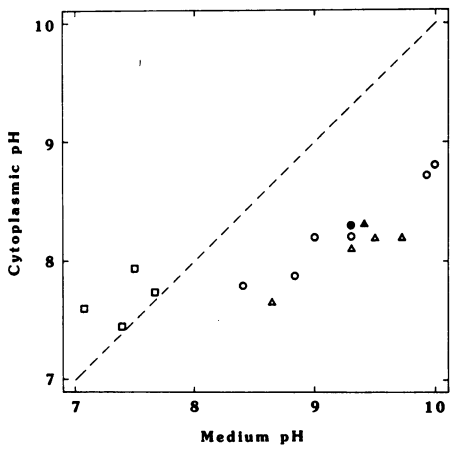

FIG. 2. Cytoplasmic pH in growing *S. faecalis* ATCC 9790 cells at high pHs. Cells were grown on the complex medium containing 50 mM $KHCO_3$ or K_2CO_3 (open symbols) or on the complex medium containing 100 mM K^+-CHES (pH 9.5) (closed symbols). At A_{650} = 0.2, [^{14}C]methylamine (10 μM; 4 μCi/μmol), [^{14}C]benzylamine (10 μM, 4 μCi/μmol), or [^{14}C]acetylsalicylic acid (9.8 μM, 3.5 μCi/μmol) was added to the culture. Cytoplasmic pH was determined by analysis of equilibrium distribution of these probes. Symbols: ○ and ●, methylamine; △ and ▲, benzylamine; □, acetylsalicylic acid. Nonspecific binding of these probes to cells was equivalent to approximately 3,000 cpm/mg of cells. At pH 9.5, accumulation of [^{14}C]methylamine in growing cells was equivalent to approximately 6,000 cpm/mg of cells, which corresponds to a ΔpH of 1.3 pH units (low internal pH). The broken line represents the cytoplasmic pH which is equal to the medium pH.

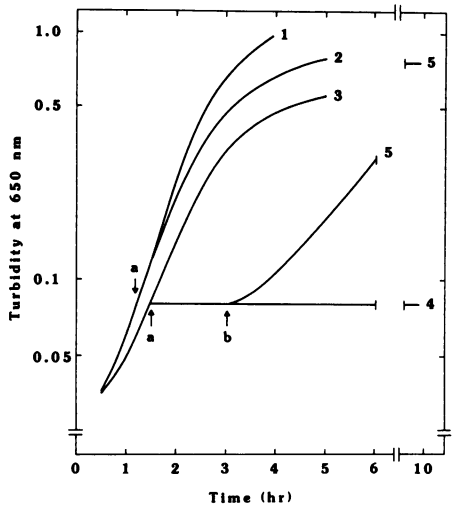

FIG. 3. Effect of nigericin on the growth of ATCC 9790 at high pH. Cells were grown at 37°C on the complex medium at either pH 7.5 (curves 1 and 2) or pH 9.5 (curves 3, 4, and 5). The arrow at a indicates the addition of 5 μg of nigericin per ml to tubes 2, 4, and 5. The arrow at b indicates the addition of HCl to tube 5 to decrease the medium pH to 7.5.

calculated to be 1.4 pH units (low internal pH) at pH 9.5 (data not shown). These results suggest that, at high pH, proton extrusion via the H^+-ATPase is not involved in lowering of cytoplasmic pH. Membrane potential ($\Delta\psi$) (inside negative) in growing cells was examined by using [^{3}H]tetraphenylphosphonium ion as a probe. $\Delta\psi$ was estimated to be almost zero at pH 8.5 and 9.5. Consequently, the magnitude of proton motive force (Δp) was +35 mV at pH 8.5 and +77 mV at pH 9.5. Thus, surprisingly, Δp of growing *S. faecalis* cells is reversed at high pH. The reversed Δp cannot be the driving force for proton influxes via a Na^+/H^+ antiporter (7). In media containing as much as 110 mM K^+, the K^+ concentration gradient across the membrane is less than 5, which corresponds to approximately 40 mV of a chemical potential of K^+. There is not evidence indicating the existence of a K^+/H^+ antiporter in this organism. Proton influx via a hypothetical secondary K^+/H^+ antiporter is not sufficient to account for the reversed ΔpH of 1.3 pH units at pH 9.5. There must be another new mechanism (a primary proton-linked pump?) to lower the cytoplasmic pH at high environmental pH. It is conceivable that HCO_3^- or CO_2 participates in lowering cytoplasmic pH. However, the reversed ΔpH was generated in cells growing on alkaline media buffered with CHES instead of with carbonate (Fig. 2). Therefore, carbonate is required for the growth of this organism at high pH but probably not for lowering of cytoplasmic pH. It has been reported that the fermentation type of streptococci is heterofermentative at high pH (5). HCO_3^- may be essential for catabolism to form fermentation products at high pH. Experiments on mechanisms of lowering pH_{in} and on carbonate (HCO_3^- or CO_2) metabolism are now in progress.

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