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Buffering Capacity of Bacilli That Grow at Different pH Ranges

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Cytoplasmic buffering capacities and buffering by whole cells were examined in six bacterial species: *Bacillus acidocaldarius*, *Bacillus stearothermophilus*, *Escherichia coli*, *Bacillus subtilis*, *Bacillus alcalophilus*, and *Bacillus firmus* RAB. Acid-base titrations were conducted on whole cells and cells permeabilized with Triton X-100 or *n*-butanol. In all of the species examined, the buffering capacity of intact cells was generally a significant proportion of the total buffering capacity, but the magnitude of the buffering capacity varied from species to species. Over the entire range of pH values from 4 to 9.5, *B. subtilis* exhibited a cytoplasmic buffering capacity that was much higher than that of *B. stearothermophilus*, *B. acidocaldarius*, or *E. coli*. The latter three species had comparable cytoplasmic buffering capacities at pH 4 to 9.5, as long as optimal conditions for cell permeabilization were employed. All of the nonalkalophiles exhibited a decrease in cytoplasmic buffering capacity as the external pH increased from pH 5 to 7. At alkaline pH values, the two thermophiles in the study had particularly low cytoplasmic buffering capacities, and the two alkalophilic bacteria had appreciably higher cytoplasmic buffering capacities than any of the other species studied. Cytoplasmic buffering capacities as high as 1,100 nmol of H^+ per pH unit per mg of protein were observed in alkalophilic *B. firmus* RAB. Since previous studies have shown that immediate cytoplasmic alkalinization occurs upon loss of the active mechanisms for pH homeostasis in the alkalophiles, the very high buffering capacities apparently offer no global protection of internal pH. Perhaps, the high buffering capacities reflect protective mechanisms for specific macromolecules or processes rather than part of the mechanisms for bulk pH homeostasis.

Bacteria that grow at extreme pH values encounter a variety of biological and, specifically, bioenergetic challenges that derive from a central problem of pH homeostasis (6). Although investigation of these problems and the means by which the organisms solve them has intensified during the past decade, the understanding of the special adaptations of extreme acidophiles and alkalophiles is very incomplete. Specific properties which may contribute new insight are of considerable interest. Thus, the recent suggestion by Zychlinsky and Matin (15) that the cytoplasmic buffering power of *Thiobacillus acidophilus* might be part of the pH homeostatic mechanism of this acidophile focused our attention on the available data with respect to the buffering power of bacterial cells. Clearly, the values for internal buffering capacity (B_i) reported for *T. acidophilus*, 97 nmol of H^+ per pH unit per mg of protein for a shift from pH 6 to 5 and 260 nmol of H^+ per pH unit per mg of protein for a shift from pH 5 to 4, were higher than that found in the same study for *Escherichia coli*, i.e., 33 nmol of H^+ per pH unit per mg of protein for the shift from pH 6 to 5. However, relative to reports in the literature, mostly for gram-positive organisms, the values for *T. acidophilus* were not especially high, and that for *E. coli* was somewhat low. For example, Mitchell and colleagues (9, 10, 13), who outlined the conceptual groundwork and provided detailed technical approaches to such measurements, have found B_i values for *Micrococcus denitrificans* that increased from about 55 to 115 nmol of H^+ per pH unit per mg (dry weight) over a range of initial pH values from 8 to 6; Maloney (8), in a detailed study of *Streptococcus lactis* with an acid-pulse technique, has found B_i values from about 38 to 122 nmol of H^+ per pH unit per mg (dry weight), with the values increasing over a range of decreasing external pH values from 8 to 5; Collins and Hamilton (1), using ionophore-treated *Staphylococcus aureus*, have found B_i values from 76 to 130 nmol of H^+ per pH unit per mg (dry weight) over the pH range of 8 to 6; and Sanders and Slayman (11) have reported B_i values for *Neurospora crassa* that included approximately 85 mM H^+ per pH unit at pH 6.5. As an approximation, if one assumes that protein represents 37% of the dry weight, B_i ranges of 149 to 310, 103 to 329, and 205 to 351 nmol of H^+ per pH unit per mg of protein are calculated for *M. denitrificans*, *Streptococcus lactis*, and *Staphylococcus aureus*, respectively. Assuming the presence of 5 μl of cell water per mg of protein for *N. crassa*, a B_i of 425 nmol of H^+ per pH unit per mg of protein is calculated.

In view of our interest in bacilli that grow at both extremes of pH, we undertook a survey that would provide a more extensive data base for determinations of B_i in these organisms. We report here measurements of whole-cell buffering capacity (B_o) and B_i for the following: two obligately alkalophilic bacilli, *Bacillus alcalophilus* and *Bacillus firmus* RAB; two mesophilic neutralophiles, gram-positive *Bacillus subtilis* and gram-negative *E. coli*; one thermophilic neutralophile, *Bacillus stearothermophilus*; and the thermoacidophile *Bacillus acidocaldarius*. We have used the approach developed and used by others (1, 13, 15) in which the total buffering capacity (B_t) is determined by using suspensions of permeabilized cells.

MATERIALS AND METHODS

The organisms and growth conditions used in the study are listed in Table 1; all the bacteria were grown aerobically with shaking. For determinations of buffering capacity, cells were harvested in the logarithmic phase of growth. They were then washed with and resuspended in either deionized water or 200 mM KCl to a final concentration of 5 mg of cell protein per ml. Protein concentrations were determined by the method of Lowry et al. (7). Experiments were conducted on 5-ml samples of cell suspensions, which were stirred in a

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TABLE 1. Bacterial species used and their growth conditions

Organism	Source	Growth temp (°C)	Growth pH	Medium (reference)
B. acidocaldarius	ATCC 27009	50	3.5	Malate-salts (2)
B. alcalophilus	ATCC 27647	30	10.5	Malate-carbonate (3)
B. firmus RAB	Lab isolate	30	10.5	Malate-carbonate (3)
B. stearothermophilus	ATCC 7953	50	7.3	LG broth (4)
B. subtilis BD99	A. Garro	30	7.0	Spizizen salts-malate (14)
E. coli K-12	D. Calhoun	37	7.0	Penassay broth (12)

small beaker at 25°C. A Beckman ϕ71 pH meter and a Futura II combination electrode, model no. 39522, were employed. The B_o of the untreated cell suspensions was determined from the initial, rapid change in the pH of the medium upon the addition of small portions of HCl (usually 10 to 20 μl of 0.05 M HCl). For determinations of B_t, the cell suspensions were then treated with either 5 or 10% Triton X-100 (15) or with 5% *n*-butanol (13). The changes in pH_{out} caused by successive additions of acid, of the same size indicated above, were then recorded for the treated suspension. The value for B_i was calculated as the difference between B_t and B_o. B_o, B_t, and B_i values were determined for each small addition of acid or base in which the pH change was less than 0.1 pH unit. In addition, average values, encompassing pH changes of 1 pH unit, were calculated. For each cell suspension, the results of the following manipulations were examined several times each at various initial pH values: suspension of cells in deionized water or in KCl; use of 5 or 10% Triton or 5% *n*-butanol; inclusion or exclusion of carbonic anhydrase; upward titration with KOH over the same range in which the downward titrations with HCl were conducted; and determinations made at several cell protein concentrations. In the range of protein employed, the B_i values were independent of cell protein concentration and of the presence or absence of carbonic anhydrase. With few exceptions, they were also independent of the direction of the titration. In a small number of experiments, titrations with base (but not with acid) in the very alkaline range gave spuriously high values for B_i. Another partial exception related to titrations of *B. acidocaldarius* in an alkaline range of pH values. For such titrations, it was not possible to raise the pH to an initial alkaline pH by the addition of one large portion of base followed by downward titration with HCl; apparently, the large addition of base compromised cell integrity. Instead, gradual titrations upward were conducted. In only one experimental condition was there a difference between the suspensions in deionized water and those in KCl; cells of *B. subtilis*, at an initial pH of 8.5 only, seemed to lyse in KCl but not in water. Lysis of some of the other species, which is described below, was related to pH but not to the suspending medium. With all the species except *E. coli*, there was no difference in B_i values determined between experiments with cells permeabilized with Triton at either concentration and those in which butanol was used; with *E. coli*, as described in detail below, the agent used for permeabilizing the cells appeared to be important.

RESULTS

Titrations of the untreated and permeabilized whole cells of the six bacterial species in the study immediately showed that both the B_o and the B_t values were quite different from species to species. Three of the organisms exhibited B_t values up to about 700 nmol of H^+ per pH unit per mg of protein, whereas the three others exhibited values at least three times higher. The two sets of data are shown in Fig. 1 and 2, in which the data points from at least two independent titrations of the B_o and B_t of each species are presented; these data are representative of a larger number of titrations for each species. With all four of the nonalkalophilic species,

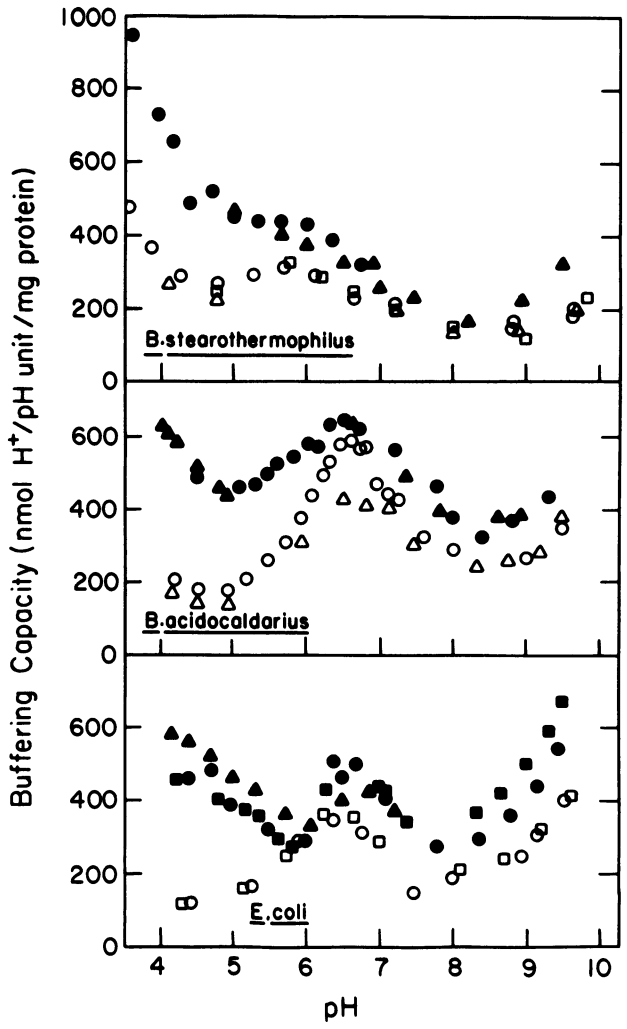

FIG. 1. B_o and B_t values for *B. acidocaldarius*, *B. stearothermophilus*, and *E. coli* over a range of external pH values. B_o (open symbols) and B_t (closed symbols) values were determined from titrations with successive additions of small amounts of acid or base as described in the text; the titrations of B_t shown were conducted on cells that were permeabilized with Triton, except for those of *E. coli*, for which the data shown are from titrations in which *n*-butanol was used. Symbols of different shapes represent data from independent experiments.

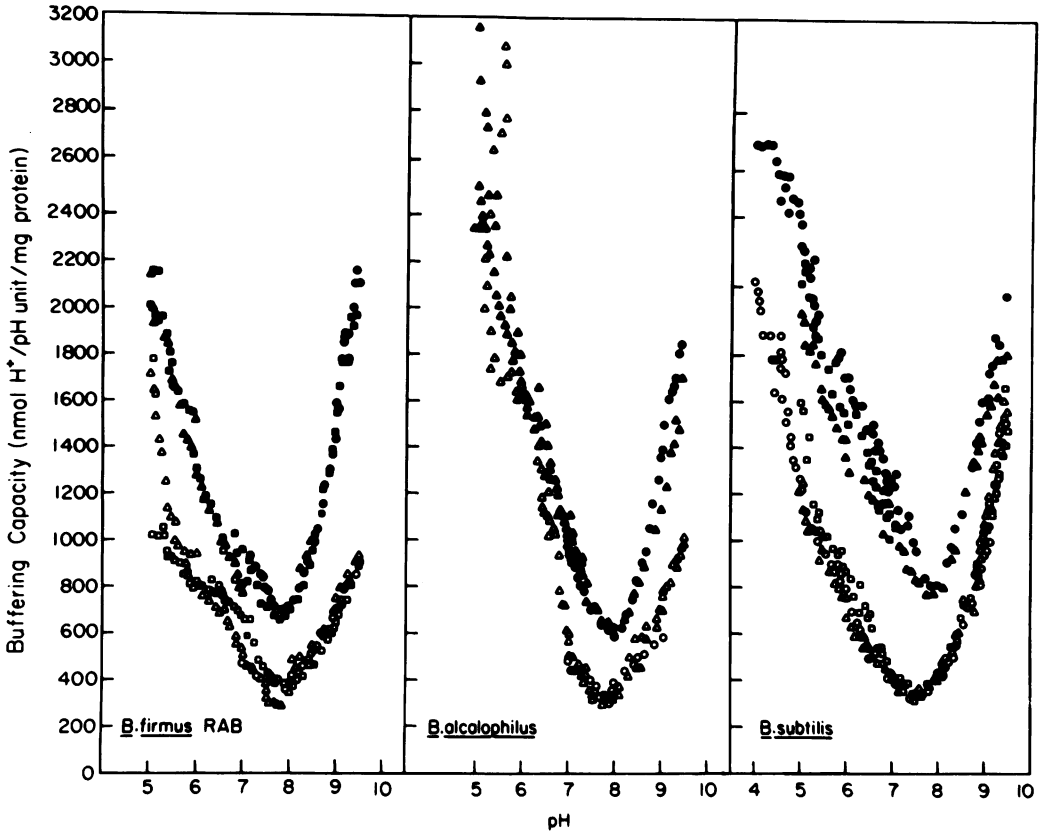

FIG. 2. B_o and B_t values for *B. alcalophilus*, *B. firmus* RAB, and *B. subtilis* over a range of external pH values (see legend to Fig. 1). All of the data shown for B_t in this figure were taken from titrations in which the cells were permeabilized with Triton.

the B_o values were appreciable and varied in some reproducible manner over the broad range of external pH values examined (Fig. 1 and 2). In these species, there was no apparent compromise of cell integrity, which might have caused the B_o values to increase to the level of the B_t, except in occasional determinations at rather extreme pH values. By contrast, the two alkalophilic species exhibited reasonable B_o values only in the neutral and alkaline ranges of pH values. Below pH 6.5, the apparent B_o approached and often reached the total buffering capacity (Fig. 2); this was presumed to reflect a loss of cell integrity.

Values for the cytoplasmic buffering capacity (B_i) were calculated from experimental data of the type shown in Fig. 1 and 2 and are shown in Fig. 3. Clearly, the B_i of *B. subtilis* was far greater than those of the other species over the range of pH values from 4 to 8. All four nonalkalophilic species, however, exhibited a decline in the B_i as the pH was increased from 5 to 6.5 or 7. Interestingly, the B_i of *B. acidocaldarius* was not significantly higher than any of the species examined in the most acidic range of pH, although the B_i of *E. coli* and of *B. stearothermophilus* were somewhat lower than that of the acidophile between pH 5.5 and 6.5. It should be noted that the data presented for *E. coli* in Fig. 1 and 3 are those obtained with cells that were permeabilized with *n*-butanol. Treatment with Triton was apparently much less effective in achieving total access of the B_i to titration (Table 2).

At highly alkaline external pH values, the B_i of *B. subtilis* was still higher than those of the other nonalkalophilic species but was now exceeded by those of the two alkalophilic species. Indeed, the B_i of *B. firmus* RAB at pH 9.5 was the highest value found in the study. Also of interest was the finding that both thermophiles, acidophilic *B. acidocaldarius* and neutralophilic *B. stearothermophilus*, exhibited rather low B_i values in the alkaline range of pH.

DISCUSSION

There are several general notions about the buffering capacity of bacterial cells that emerge from this study, some of which are confirmatory of earlier work by others. (i) B_i values as well as the cell surface buffering capacities of bacteria vary markedly between one species and another. It is interesting that mesophilic, neutralophilic *B. subtilis* should possess a rather dramatically high buffering capacity, especially in the acid range. In that range, there was no apparent relationship between the natural habitat of the organism with respect to pH and either the B_o or the B_i. (ii) B_i generally decreased with increasing pH in the acid range of pH values; this confirms the findings of others (8, 11, 13, 15) and is expected. (iii) In the six species studied here as well as in most reports by other investigators (e.g., Maloney [8] and Scholes and Mitchell [11]), the B_o was a significant proportion of the B_t; thus, for example, in any of the species studied here, changes in the pH_{out} of a dense cell suspension would not be completely reflected in changes in the cytoplasmic pH because of the buffering capacity of the cell surface.

The most dramatic finding with respect to bacteria that grow in different pH ranges was the observation of significantly higher B_i values in the alkalophilic bacteria at high external pH values than in any of the other species, including the generally well-buffered *B. subtilis*. It will be of

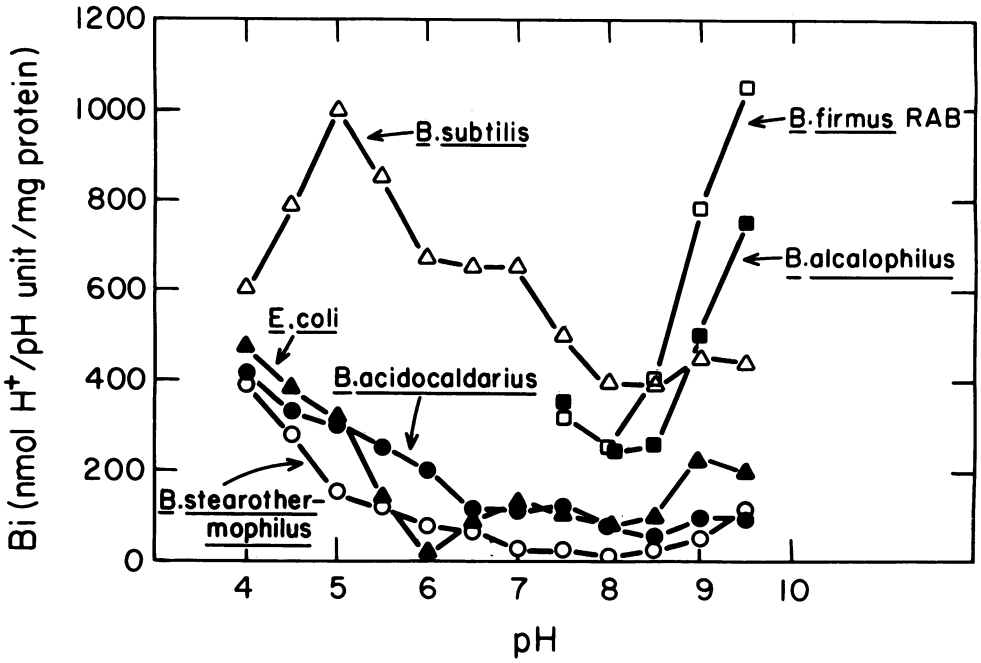

FIG. 3. B_i as a function of the external pH. B_i values calculated from experiments such as those presented in Fig. 1 and 2 are plotted as a function of pH_{out} for each of the species in the study.

interest to examine whether there are particularly high levels of basic proteins or polyamines or both in the alkalophile cells. It is important to note that whatever the nature of the buffering compounds at alkaline pH, they do not protect the alkalophile when the capacity for active maintenance of the transmembrane pH gradient is compromised. Previous work in our laboratory (5) has shown that upon inactivation of the Na^+/H^+ antiporter that functions in acidification of the cytoplasm of *B. firmus* RAB, the cytoplasmic pH reaches the external pH of 10.5 as quickly as we can measure experimentally. Thus, even a B_i as high as 1,100 nmol of H^+ per pH unit per mg of protein has no observable impact upon a failure of active pH homeostasis in the alkalophile. What, then, might be the significance and role of the high B_i found here? Perhaps basic molecules are present in specific locations for the protection of particular macromolecules (e.g., nucleic acids) or processes (e.g., protein synthesis). Alternatively, the B_i might protect the cell in the face of minor perturbations in external pH.

Finally, there are several technical problems in connection with measurements of buffering capacity by this approach that have been underscored by the current study. First, when a wide pH range is employed, there are problems of cell integrity which interfere with measurements of the B_o for some cells at some pH values. Also of importance is the finding that for at least one gram-negative species, the method of permeabilizing the cells strongly influences whether the entire B_i is rendered titratable. In the *E. coli* cells that were permeabilized with Triton, low B_i values were obtained that were completely comparable to the values found by Zychlinsky and Matin (15), using the same method. However, when *n*-butanol was used, significantly higher values were obtained. It will be of interest to study at least some of the same organisms by a method in which the decay of an acid pulse is used for determination of both the buffering capacity and the passive proton permeability. Preliminary experiments on *B. acidocaldarius* by the method of Maloney (8) indicate that both of these parameters are in the range found for other bacteria.

TABLE 2. Use of *n*-butanol to permeabilize *E. coli* cells results in higher B_i values than use of Triton X-100

Solvent used to permeabilize cells	B_i values over unit pH ranges			
	5–4	6–5	7–6	9.5–8.5
5% *n*-butanol	369 ± 45	115 ± 23	133 ± 12	234 ± 20
10% Triton X-100	128 ± 32	35 ± 30	0	181 ± 40

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