Growth and Bioenergetics of Alkalophilic *Bacillus firmus* OF4 in Continuous Culture at High pH

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The effect of external pH on growth of alkalophilic *Bacillus firmus* OF4 was studied in steady-state, pH-controlled cultures at various pH values. Generation times of 54 and 38 min were observed at external pH values of 7.5 and 10.6, respectively. At more alkaline pH values, generation times increased, reaching 690 min at pH 11.4; this was approximately the upper limit of pH for growth with doubling times below 12 h. Decreasing growth rates above pH 11 correlated with an apparent decrease in the ability to tightly regulate cytoplasmic pH and with the appearance of chains of cells. Whereas the cytoplasmic pH was maintained at pH 8.3 or below up to external pH values of 10.8, there was an increase up to pH 8.9 and 9.6 as the growth pH was increased to 11.2 and 11.4, respectively. Both the transmembrane electrical potential and the phosphorylation potential (ΔGp) generally increased over the total pH range, except for a modest fall-off in the ΔGp at pH 11.4. The capacity for pH homeostasis rather than that for oxidative phosphorylation first appeared to become limiting for growth at the high edge of the pH range. No cytoplasmic or membrane-associated organelles were observed at any growth pH, confirming earlier conclusions that structural sequestration of oxidative phosphorylation was not used to resolve the discordance between the total electrochemical proton gradient (Δp) and the ΔGp as the external pH is raised. Were a strictly bulk chemiosmotic coupling mechanism to account for oxidative phosphorylation over the entire range, the ΔGp/Δp ratio (which would equal the H+/ATP ratio) would rise from about 3 at pH 7.5 to 13 at pH 11.2, dropping to 7 at pH 11.4 only because of the rise in cytoplasmic pH relative to other parameters. Moreover, the molar growth yields on malate were higher at pH 10.5 than at pH 7.5, indicating greater rather than lesser efficiency in the use of substrate at the more alkaline pH.

Alkalophilic *Bacillus firmus* OF4 grows at least as well at pH 10.5 as at pH 7.5 in batch and continuous culture on malate-containing medium (5). At the more alkaline pH values, the cytoplasmic pH is maintained below pH 8.5, a remarkable, Na⁺-dependent capacity for pH homeostasis in which secondary Na⁺/H⁺ antiporters apparently play a central role (13, 19). Having thus successfully confronted the primary biological challenge, the alkaliphile must further resolve a bioenergetic problem that results from this maintenance of a cytoplasmic pH (pHc) that is well below the external pH. Although a substantial transmembrane electrical potential (Δφ, positive out) is generated by respiration-dependent proton extrusion, the magnitude of the electrochemical proton gradient (Δp, acid and positive out) is reduced by the chemiosmotically adverse pH gradient (ΔpH). Extreme alkaliphiles do not use the much larger electrochemical gradient of sodium via a sodium-coupled synthase, probably because outward sodium movements must be coupled to proton accumulation for purposes of pH homeostasis, and a primary sodium cycle would subvert that major process (13). Since the F1,F0 ATP synthases of *B. firmus* OF4 (10) and at least one other extreme alkaliphile (11) are thus exclusively proton coupled, there are only two apparent ways to couple generation of a comparable phosphorylation potential (ΔGp) to bulk, delocalized chemiosmotic driving forces that are about threefold greater at pH 7.5 than at pH 10.5 (5). The oxidative phosphorylation machinery might be sequestered in organelles, but extensive investigation indicates no such arrangement (16) and, on the contrary, points to a disperse localization of the ATP synthase in a conventional cytoplasmic membrane (26).

Alternatively, the alkaliphilic synthase might use a variable H⁺/ATP stoichiometry. Since H⁺/ATP = ΔGp/Δp in a completely chemiosmotically coupled scenario, an increase in the coupling ratio would compensate for a decrease in the Δp at higher values of growth pH to allow generation of the same ΔGp. Hoffmann and Dimroth (12) have indeed argued, on the basis of measurements made on obligately alkalophilic *Bacillus alcalophilus* at pH 10, that the H⁺/ATP ratio would not have to be extremely high (only a little above 4) to account for the ATP/ADP ratios that they found. However, as noted by us (6, 13) and by Nicholls and Ferguson (22), studies at a single pH or even over a narrow range fail to assess just how large and variable the H⁺/ATP stoichiometry would have to be in order to account for the ATP synthesized. Also, the measurements of the ATP/ADP ratio and of the Δψ in the Hoffmann and Dimroth study (12) were apparently not made under the same conditions, which may have resulted in the recording of ATP/ADP ratios from cells in the original batch culture that had lower Δψ values than those aerated separately, in which the Δψ measurements were made. Impressively, their data still indicated that in *B. alcalophilus*, as in *B. firmus* OF4, the highest ATP/ADP ratios were not observed at the highest Δp values, as would be expected in a chemiosmotic coupling situation.

Both the Hoffmann and Dimroth studies on *B. alcalophilus* (12) and earlier studies on *B. firmus* OF4 (5) were conducted in batch cultures, with no attempt made to rigorously establish an upper limit of pH for growth and thus ascertain how big the bioenergetic dilemma might be. In fact, no studies on alkalophilic *Bacillus* species in which growth was shown while the measured pH was at 11 or more have been reported. Accord-
ingly, the factor(s) ultimately limiting growth is completely unclear. Therefore, in this study, we sought to determine the upper limit for rapid growth of facultatively alkaliphilic *B. firmus* OF4 in continuous pH-controlled cultures and to study, for the first time, the bioenergetic parameters at various pH values in such cultures. By using malate-limited continuous cultures, the molar growth yields at pH 7.5 and pH 10.5 were also to be determined, to see whether differences would be in a direction supporting a mechanism for oxidative phosphorylation employing a variable H⁺/ATP, i.e., greater yields at lower pH. It was anticipated that these studies would further clarify the magnitude of the bioenergetic challenge to extreme alkaliphiles with respect to oxidative phosphorylation and would also indicate whether it was this capacity, problems of pH homeostasis, or some other factor that limits growth at the upper edge of the pH range.

**MATERIALS AND METHODS**

**Growth conditions.** *B. firmus* OF4, isolated in our laboratory (4), was used for all studies. Growth in batch culture, for inocula and characterization of variants, was in the highly buffered malate-containing medium previously described (5) at the pH values indicated at 30°C.

For continuous culture experiments, *B. firmus* OF4 was grown at 30°C with vigorous aeration in a New Brunswick Bioflow fermentor with a working volume of 350 ml. The same carbonate-buffered medium used in the batch cultures was used for cultures grown at highly alkaline levels, whereas a phosphate-buffered medium that was otherwise of the same composition was used for cultures at pH 8.5 or lower. The continuous culture system was operated at constant pH by the addition of the medium in the reservoir to the target pH value for a given run and by the addition of NaOH or HCl as needed during growth at particular pH values. An immersed pH electrode which acted as sensor for base or acid addition was linked to a pump that delivered the required amount to readjust the pH to the target level upon detection of deviation. The values detected by the immersed electrode were checked for accuracy against a calibrated external electrode whose accuracy had been verified against prepared buffers. The cultures were maintained at steady state at *A*₆₀₀ in the range of 0.2 to 0.4. Continuous culture runs were conducted in at least duplicate for every condition reported, and multiple samplings were taken at each steady state.

**Generation times.** Generation times (*t*ₜ) were calculated from the flow rates that allowed the turbidity to remain within the 0.2 to 0.4 target range under any given condition. This was determined empirically by adjusting the rate of addition of fresh medium and monitoring *A*₆₀₀. The most rapid rate of medium addition into the culture vessel that allowed maintenance of a steady *A*₆₀₀ in the target range was determined over long time frames (>4 h). Increasing the flow rate by 10% from such values typically resulted in washout. The flow rates were converted to dilution rates by dividing by the fixed volume of the fermentor, and the dilution rates were used as an index of growth rate (μ) (2). The *t*ₜ was calculated from the relationship *t*ₜ = In 2/μ (2).

**Bioenergetic measurements.** Samples for determination of Δp and ΔGₚ were withdrawn from steady-state cultures of *B. firmus* OF4 growing continuously at the indicated pH value. Measurements of all parameters were conducted on the same samples, in the growth medium, at the same cell concentrations and assay conditions. ATP and ADP concentrations were measured by luminometry as described previously (6), and phosphate concentrations were determined by the colorimetric method of Lebel et al. (20). The values for ΔGₚ were calculated by using values of ΔG° reported for the measured cytoplasmic pH (27). The Δp component of the Δp was determined from the distribution of weak acids or weak bases as described by others (32), and the transmembrane electrical potential was similarly determined as recently described in detail (19) from the distribution of ³H-tetraphenylphosphonium. Motility was assessed by phase-contrast microscopy.

**Electron microscopy.** For transmission electron microscopy, glutaraldehyde-fixed cells were postfixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin (Epon; Electron Microscopy Sciences, Fort Washington, Pa.). Thick sections were stained with methylene blue and azure II. Smaller areas were then selected for ultrastructural study. Thin sections were stained with uranyl acetate-lead citrate. They were examined in a JEM 100cx electron microscope (JEOL Ltd., Tokyo, Japan). The methods were as described by Hayat (6).

**Molar growth yields.** Molar growth yields on D,L-malate-containing media at either pH 7.5 or 10.5 were determined by using continuous cultures at each of the two pH values that contained 0, 2.5, and 5.0 mM D,L-malate in the reservoir instead of the standard concentration of 50 mM D,L-malate, but otherwise with the same medium as in the other experiments; the cultures grown in the absence of malate exhibited some growth that was attributable to the presence of 0.1% yeast extract in the complete medium. Malate consumption was determined from measurements of both D-malate and L-malate in culture samples from which cells had been removed by centrifugation. Total consumption was calculated by subtracting the malate concentration in the medium in the culture vessel from that measured in the reservoir. The isomers of malate were assayed with stereospecific malate dehydrogenases (Boehringer Mannheim) by monitoring NADH consumption spectrophotometrically.

For determinations of the dry weight, a calibration curve relating *A*₆₀₀ to dry weight was established by weighing tared samples of cultures that were prepared in the same medium at various *A*₆₀₀ values. Cell samples were washed with distilled water and then dried to constant weight in an oven. Dry weights of cells growing in continuous cultures were calculated from measured *A*₆₀₀ values, using the calibration curve. All growth yield measurements were performed in triplicate on independent experiments. The values for malate consumption and biomass production obtained for growth on 0 mM malate were used to correct the values at 2.5 mM malate. In each experiment, the value obtained for the 2.5 and 5.0 mM malate pair was the same as that calculated from the 0 and 2.5 mM malate pair.

**RESULTS**

**Growth and bioenergetic properties at various pH values.** At pH 7.5, *B. firmus* OF4 grew with a *t*ₜ of 54 min; the *tg* decreased to 37 min at pH values from 8.5 to 10.6 (Fig. 1). The *pHₘₙ* was 7.5 to 7.7 at values of external pH up to 9.5 and then increased to pH 8.2 or 8.3 at external pH values of 10.6 to 10.8. The *tg* increased to 94 min at pH 10.8, and upon a further increase in the external pH to 11.2 and 11.4, marked increases in *tg* to 290 and 690 min, respectively, were observed. These latter increases in *tg* accompanied a significant rise in *pHₘₙ* to 8.9 and 9.6, the pattern suggesting a correlation between the increase in *tg* and a decreasing ability to maintain a low *pHₘₙ*. Indeed, although data are not shown, variant cells were readily isolated from the continuous culture at pH 11.1, with higher Na⁺/H⁺ antiport activity and a faster initial growth at pH 11 than for the wild-type parent but no change in the cytochrome
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FIG. 1. Generation times, motility, and bioenergetic parameters of *B. firmus* OF4 growing in continuous cultures at different, constant values of external pH. *B. firmus* OF4 was grown in continuous culture at various values of external pH (*pH*<sub>out</sub>) as described in Materials and Methods. The *r*<sub>v</sub> values and various bioenergetic parameters were measured as indicated in the text, and observations of motility were made (and are qualitatively reported at the top).

levels or ∆ψ. As shown in Fig. 2, those pH values at which the slowest growth rates and the pronounced rise in *pH*<sub>in</sub> were found also corresponded to conditions in which cells often grew in chains; nonetheless, at all pH values, a preponderance of the cells were single or exhibited a single septum. Moreover, except for the chain formation, the fine structural features observable in thin-section preparations were unchanged over the very broad pH range studied, as shown for representative cells from cultures at pH 7.5 and 11.2 in Fig. 2. In particular, no intracellular or lens-like intramembrane structures (29) that might sequester the oxidative phosphorylation machinery were observed.

*B. firmus* OF4 generated substantial ∆ψ values over the entire pH range, with a gradual increase from −140 mV at pH 7.5 to −182 mV at pH 10.6, followed by a peak values of −200 mV at pH 10.8 before a return to the range of −182 to −185 mV at pH 11.2 and 11.4 (Fig. 1). The ∆p declined significantly between values of the growth pH of 7.5 and 10.6, because of the increasing acidification of the cytoplasm relative to the medium pH. As found previously (e.g., reference 6) for cells

FIG. 2. Thin sections of cells of *B. firmus* OF4 grown in continuous culture at pH 7.5 or 11.2. Representative fields of cells from cultures at the two pH values are shown in panels A and B, and a single, representative cell from each culture is shown at higher magnification in panels C and D. (A and C) pH 7.5; (B and D) pH 11.2.
incubated in the pH range around 10.5, the observation of a higher Δψ in a particular sample set, e.g., −200 mV at pH 10.8, was accompanied by a correspondingly lower pH_{in}, presumably because the electrogenic antiporters that account for intracellular proton accumulation are energized by the Δψ. As a result, the total Δp was steady at about −50 mV between pH 10.6 and 11.2, increasing at pH 11.4 only because of the rise in pH_{in}. By contrast, the ΔGp increased over almost the entire pH range, decreasing only between pH 11.2 and 11.4. The ΔGp/Δp ratio is shown as a function of growth pH in Fig. 3. At pH 7.5, the ratio was approximately 3, rising precipitously until the highest value of 13 was reached at pH 11.2. The parameter that changed the most in these calculations was the external pH, which was set over a range of almost four full pH units, whereas the pH_{in} varied in a range of only slightly over two pH units, and changes in the ΔGp and Δψ were comparatively more modest. Also, interestingly, the ΔGp/Δp ratio and putative H^{+}/ATP ratio decreased to 7 at pH 11.4, partly because of a reduction in the ΔGp but largely because of the increase in the calculated Δp that resulted entirely from an apparent failure of pH homeostasis.

A second energetic process in <i>B. firmus</i> OF4 that is coupled to the Δψ, but via Na^{+} rather than protons, is motility (3a, 17). As shown qualitatively at the top of Fig. 1, motility, as observed by phase-contrast microscopy, was greatest at pH 10.6 and 10.8. No motility was observed at pH 7.5, and it is not known whether <i>B. firmus</i> OF4 synthesizes flagella at that pH.

**Molar growth yields on malate.** The molar growth yield on malate for <i>B. firmus</i> OF4 cultured continuously at pH 7.5 was determined to be 53 mg (dry weight)/mmol of malate consumed (Table 1). At pH 10.5, a value of 82 mg (dry weight)/mmol of malate was found. These values are higher than reported previously for batch cultures, and the difference between pH 7.5- and pH 10.5-grown cells is greater (5). In those earlier studies, cells were growth limited by the malate concentrations, but the actual malate consumption was not determined. In the current experiments, the higher efficiency of malate utilization at pH 10.5, 66%, compared with 43% at pH 7.5, accompanied an enhanced ability to use d-malate at the higher pH, as indicated by the lower preference ratio of 3:1 for d-malate versus l-malate at the higher pH (Table 1).

**DISCUSSION**

Alkaliphilic <i>B. firmus</i> OF4 is clearly capable of robust nonfermentative growth at pH values above 11. Even the slowest rates of growth observed in this study were well within the range for many conventional neutralophiles. Moreover, it is likely that obligately alkaliphilic strains grow even better than facultatively alkaliphilic <i>B. firmus</i> OF4, since the membrane lipids of obligately <i>B. firmus</i> RAB, for example, are apparently better optimized for growth in the very alkaline range of pH at the expense of a capacity for growth in the near neutral range (3). At the upper edge of the pH range that was compatible for rapid growth of <i>B. firmus</i> OF4 in the current study, the most likely limiting factor was the capacity for pH homeostasis. In an average of several determinations, there was a small initial increase in t_{c} at pH 10.8 that preceded observations of markedly increased values of cytoplasmic pH. However, the conditions under which the t_{c} values were calculated may have been at slight variance from those at which the bioenergetic parameters were measured. The t_{c} was calculated directly from cells growing in the primary culture vessel, while it was necessary to remove samples from this vessel in order to measure the bioenergetic parameters. With that small initial exception, the cytoplasmic pH was the parameter whose change best correlated with increasing t_{c} in the most alkaline pH range. A physiological process that appeared to be compromised concomitantly was normal septation; formation of chains has similarly been observed during growth of anaerobic, alkaliphilic <i>Clostridium paradoxum</i> at the upper end of its pH range (21). There may be some step in septation that is generally sensitive in bacilli as the cytoplasmic pH rises, or an SOS-like response that includes an effect on division takes place at the alkaline edge of cytoplasmic pH, as has been reported for Enterobacteria coli (28).

Above pH 11.0, <i>B. firmus</i> OF4 exhibited no decline in the Δψ relative to that at lower pH values. The capacity for oxidative phosphorylation, as indicated by the magnitude of the ΔGp, was even higher at pH 11.2 than at lower pH values, falling off slightly only after the external pH was raised further so that pH homeostasis was compromised markedly. Thus, it is unlikely that either the primary generation of a Δp or the synthesis of ATP was the major limiting factor in the rate of growth at the upper edge of the pH range. Oxidative phosphorylation by the extreme alkaliphilic was remarkably resistant to a very alkaline medium pH and to cytoplasmic pH values above 9; this observation was consistent with earlier findings in experiments with starved cells that were poised at different pH values (6). That the capacity for pH homeostasis was indeed the limiting factor above pH 11 was supported by the finding of variants with increased Na^{+}/H^{+} antiport activity.

There was a large change in the ΔGp/Δp over the range of growth pH of <i>B. firmus</i> OF4. The values found at pH 7.5 and 10.5 correlated very well with the values reported earlier for batch cultures (5), but the broader range of pH used here revealed a much greater variation in the ratio with pH than

**TABLE 1. Molar growth yields of <i>B. firmus</i> OF4 on malate at pH 7.5 and 10.5**

<table>
<thead>
<tr>
<th>pH_{out}</th>
<th>Molar growth yield^a (mg [dry wt]/mmol of malate consumed)</th>
<th>Efficiency^a (%)</th>
<th>Preference ratio (mmol of L-malate consumed/mmol of D-malate consumed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>53</td>
<td>43</td>
<td>5:1</td>
</tr>
<tr>
<td>10.5</td>
<td>82</td>
<td>66</td>
<td>3:1</td>
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</tbody>
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^a Determined as described in Materials and Methods.

^b Determined as milligrams (dry weight) produced per milligram of malate consumed.
found before. Moreover, it was apparent under these carefully controlled growth conditions that the parameter that contributed most substantially to the change in ΔΔpH/ΔpH was the external pH. This parameter was set, controlled, and monitored with both the internal electrode and regular periodic measurements with a calibrated external electrode during each experiment. Numerous controls and standardizations indicate no pH-dependent loss of accuracy in these measurements, nor in the less direct determinations of the bioenergetic parameters that exhibited smaller fluctuations (13, 19).

Again as in earlier studies (16, 26), there was no indication of cytoplasmic or membrane-associated organelles that could sequester the machinery for oxidative phosphorylation from the transcytoplasmic membrane gradients that diminish with increasing growth pH. If a purely chemiosmotic mechanism, with complete coupling of oxidative phosphorylation to the bulk delocalized ΔpH, obtained over the whole pH range, the H+/ATP ratio would therefore rise from about 3 at pH 7.5 up to 13 at pH 11.2. The H+/ATP coupling ratio would then putatively fall to 7 at pH 11.4. That unlikely conclusion derives from a calculation that was affected primarily by the failure of pH homeostasis rather than anything directly related to oxidative phosphorylation. Marked differences in the H+/ATP stoichiometry of a given H+−coupled synthase have been reported in reconstituted systems in which the lipid was varied (31), and Krenn et al. (18) have recently suggested a correlation between the H+/ATP stoichiometry of a given ATP synthase and the content and pattern of charged residues in the primary structures of F0 subunits c and a. The current experiments do not exclude the use of an enormously variable H+/ATP ratio in the alkaliphile. However, several points should be noted. First, there are no significant differences between the membrane lipid composition of pH 7.5- and pH 10.5-grown cells of B. firmus OF4 (1). Second, both molecular biological and biochemical data indicate that B. firmus OF4 possesses a single ATP synthase species, which is the product of a unique apn operon and is assembled in an invariant subunit stoichiometry and membrane concentration (12a, 14). Finally, were the H+/ATP ratio to increase from 3 to 13, there would have to be some combination of an increasing H+/O and a reduced molar growth yield to accommodate the changing H+/ATP ratio. In fact, there are high pH-dependent increases in several respiratory chain components (24), with the increase in the cadA-type terminal oxidase having been shown to occur at the transcriptional level (25). However, there is no evidence for a progressive increase in the possible proton-translocating complexes over a broad range of pH values. Thus, to accommodate an increasing H+/ATP, at least some diminution in the molar growth yield would be expected. Just the opposite was observed, with a substantially greater molar growth yield on malate found at pH 10.5 than at pH 7.5. The molar growth yields at both pH values, but especially at pH 10.5, were in the high end of the range of values reported for aerobic growth of bacteria on Krebs cycle intermediates (23), e.g., 61.8 mg (dry weight)/mmol of citrate consumed for Aerobacter aerogenes (7) and 34.8 mg (dry weight)/mmol of malate consumed for Pseudomonas fluorescens (9). Other models for the energization of oxidative phosphorylation in the alkaliphile at pH values between 10 and 11.4 must in any event take account of several qualitative observations that are also difficult to reconcile with a strictly chemiosmotic model. Alternative models, including delocalized proton movements on the membrane surface, e.g., as proposed by others (15, 30), or direct proton transfers between a respiratory chain complex and the ATP synthase (6), are under examination.

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