Buffering capacity and membrane $H^+$ conductance of protease producing facultative alkaliphilic bacterium *Bacillus flexus* from mangrove soil

P Kanna, S Ignacimuthu* and M Gabriel Paulraj

Entomology Research Institute, Loyola College, Chennai 600 034, India

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A facultative alkaliphilic protease-producing gram-positive rod-shaped bacteria (EMGA 5) was isolated from mangrove soil and confirmed as *Bacillus flexus* by the 16S rDNA sequence. Buffering capacity and membrane $H^+$ conductance of this alkaliphilic isolate were investigated for the cells grown at $pH$ 7.2 and 10.5 using acid pulse technique. Suspensions of *B. flexus* cells grown in poly peptone yeast glucose medium at $pH$ 10.5 exhibited higher cytoplasmic membrane buffering capacity values (70 µmol $H^+$/mg protein at $pH$ 9.9) than the cells grown at $pH$ 7.2 (41 µmol $H^+$/mg protein at $pH$ 9.9). *B. flexus* grown aerobically at $pH$ 7.2 showed higher $H^+$ conductance values than the cells grown at $pH$ 10.5 (0.032 µmol $H^+$/s/mg protein at $pH$ 9.9 and 0.028 µmol $H^+$/s/mg protein at $pH$ 9.8, respectively). The present study revealed that the buffering capacity and membrane $H^+$ conductance of the *B. flexus* isolates were influenced by $pH$ of the medium.

**Keywords:** *Bacillus flexus*, Buffering capacity, Facultative alkaliphile, Membrane $H^+$ conductance, Protease

Most microorganisms are neutrophiles as they survive at $pH$ 5-8.5 with maximum growth rates at $pH$ 7.4. Bacteria that grow at extreme $pH$ values encounter a variety of biological, in particular bioenergetic challenges, posed by $pH$ homeostasis. Alkaliphiles, mostly bacilli grow both in neutral as well as highly alkaline $pH$ ranges. Some alkaliphiles which grow in $pH$ 10-11 have ecological and industrial importance. Amino acid uptake by alkaliphilic bacteria is controlled by Na$^+$ in a $pH$ range 7-10. Therefore, these cells have to extrude Na$^+$ from the cytosol to generate sodium motive force. Some marine bacteria exhibit a respiration-driven primary Na$^+$ pump sensitive to protonophores and a proton pump. However, no evidence for such Na$^+$ pump has been reported in the alkaliphiles. Investigations on these issues including the means adopted by the organisms to solve them have intensified during the past decade.

In the present study, we have investigated the buffering capacity and membrane $H^+$ conductance of protease producing facultative alkaliphilic gram-positive rod *Bacillus flexus* EMGA 5 grown at $pH$ 7.2 and 10.5.

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*Corresponding author:*

Telefax: +91 44 28174644; E-mail: entolc@hotmail.com

Currently at Department of Environmental Engineering, Konkuk University, 1-Hwayang-Dong, Gwangjin-Gu, Seoul 143-701, South Korea

**Materials and Methods**

**Sampling and isolation of protease-producing bacteria**

Soil samples collected from mangrove region of Kakinada district, Andhra Pradesh, India were brought to the laboratory and stored at 4°C before processing. One g soil was used for isolation of alkaliphiles using polypeptone yeast glucose (PPYG) medium at $pH$ 10.5 and incubated at 30°C for 48 h. Selected isolates were maintained as 20% glycerol stocks at −70°C and screened for protease production on a skimmed milk agar medium containing 2% of skimmed milk powder and 1% of tryptone (w/v). Colonies that showed a clear zone of proteolytic activity were selected; the protease production and its activity were confirmed with different $pH$ (6.0-12.0) and temperature ranges 15-40°C.

**Taxonomic identification**

The selected isolate was designated as EMGA 5 and characterized using standard biochemical tests. The 16S rDNA was amplified using the forward primer 27f (5’AGA GTT GAT CMT GGC TCA G 3’) and the reverse primer 1492r (5’TAC GGY TAC CTT GGT ACG ACT T 3’). The primers were purchased from MWG, Bangalore, India. Each PCR was performed in a total volume of 25 µl reaction in 200 µl PCR tubes. Each reaction consisted of 1X Taq buffer XT-20 containing 1.5 mM MgCl$_2$ (Genei, India), 0.75 U
of Taq polymerase XT-20 (Genei, India), 0.1 mM of dNTPs (Eppendorf, Germany) and 7 pico moles of each primer (MWG, India) and 25 to 50 ng of genomic DNA.

Amplifications were carried out in a thermocycler (Mastercycler Personal, Eppendorf, Germany) with initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 90 s and final extension at 72°C for 10 min and stored at 4°C. It was cloned into TA cloning vector (MBI Fermentas) and the cloned plasmid DNA was purified and sequenced with universal primers M13f, M13r and 341f (5′ CCT ACG GGA GGC AGC AG 3′). The homologies of the resulting sequences were searched using the BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The 16S rDNA sequence of strain EMGA 5 was deposited in GenBank.

**Growth conditions**

The facultative alkaliphilic *B. flexus* EMGA 5 was grown on PPYG broth at pH 7.2 for 18 h at 30°C and 150 rpm. One percent inoculum (0.8 OD at A600) was introduced into 100 ml PPYG medium with two different pH (pH 7.2 and 10.5)12. The broth was incubated at 30°C and grown aerobically at 150 rpm. The cultures were harvested in the mid log phase (12-16 h).

**Bacterial cell preparation**

The bacterial cells (100 ml) were centrifuged at 10,000 rpm for 10 min at 4°C; pellet was resuspended in half the volume of 300 mM KCl and centrifuged. This was repeated twice and the cells were resuspended in 7 ml of 300 mM KCl to the final concentrations of 20-25 mg of cell protein per ml and stored at 4°C13.

Protein concentration was determined by Bradford method14 using bovine serum albumin (Sigma) as the standard.

**Buffering capacity and membrane H⁺ conductance**

The buffering capacity and membrane H⁺ conductance of the bacteria were measured by an acid pulse technique13,15-18. Experiments were conducted in 5 ml of cell suspension containing 5 mg whole cell protein which was stirred magnetically in a 25 ml beaker at 28°C and the initial pH was noted. The cell suspension was adjusted to pH 7.0 by adding 50 µl of 100 mM HCl or 100 mM KOH and pre-incubated for 2 h. After preincubation, valinomycin (Sigma) was added to the suspension, and the final concentration was 10 µM. The stock was prepared in small volume of <0.1% acetone. The cells were allowed to equilibrate for about 2 h with intermittent mixing and 230 µl of freshly prepared carbonic anhydrase (Sigma) (4.5 mg in 230 µl of 300 mM KCl) solution was added just prior to the assay. Vigorous mixing was performed after insertion of the pH electrode and pH was noted. After 10 min, an alkali (5 to 10 µl aliquots of 100 mM KOH in 300 mM KCl) was added and changes in external pH were recorded for 3 min.

After pre-incubation period, during which protons attained an equilibrium distribution across the membrane, a small quantity of acid or base was added, deflecting external pH value. If the membrane permeability to H⁺ was relatively low, the initial acidification or alkalization recorded a titration of only outer buffering capacity (Bₒ). With the continued incubation, as protons finally crossed the membrane, the final equilibrium pH reflected titration of the total buffering power of the cell (Bᵢ). The cytoplasmic membrane buffering capacity (Bᵢ) was obtained as the difference between Bᵢ and Bₒ, i.e. Bᵢ = Bᵢ-Bₒ. The membrane H⁺ conductance was calculated using the measurements of buffering capacity and rate at which pH approached final equilibrium19 with the following formula.

\[ C_m^{H^+} = \frac{\{(B_o \times B_i)/B_i\} \times \ln 2}{T_{1/2}} \]

where \( C_m^{H^+} \) represents estimated conductivity to H⁺ and \( T_{1/2} \) is the half-time with which external pH approaches final equilibrium. Each experiment was repeated thrice independently and the data were analyzed using polynomial graph13,15-18. At 30 s intervals, the difference between the measured pH and final equilibrium pH was plotted on a logarithmic scale against time. Because of the mixing artifact, and half-time for response of the pH recording system was 2-5 s, pH changes during the first 15 s were not used in these plots.

**Results and Discussion**

The morphological and biochemical tests revealed that the isolate, was a gram-positive rod-shaped spore-forming, facultative alkaliphilic strain. It was confirmed as *B. flexus* (EMGA 5) using 16S rDNA sequence, submitted in GenBank under the accession no. EU602312. It produced protease that hydrolyzed casein at pH 7.2-10.5 at 30°C. The enzyme production
and activity was higher in alkaline pH and was within the range of other alkaline proteases\(^3\).

The studies on buffering capacity and proton conductance of biological membranes in relation to the growth condition are lacking\(^20\). The results on the buffering capacity and membrane H\(^+\) conductance values for B. flexus grown on PPYG medium at pH 7.2 and 10.5 showed that the cells grown at pH 10.5 had higher buffering capacity than those grown at pH 7.2 (Fig. 1A & B). Cytoplasmic membrane buffering capacity (B\(_i\)) values are shown in Fig. 2. The cells grown at pH 7.2 exhibited lower B\(_t\) and B\(_i\) values than at pH 10.5 (Table 1). The buffering capacity values B\(_t\) and B\(_o\) were obtained as a function of pH for bacterium grown at different pH values. The method employed in this study provided reproducible measurements of these parameters over a wide range of external pH. This was in agreement with the B. subtilis results, wherein the bacterial cells maintained a constant ion permeability of their membrane with the external pH\(^20\).

The passive H\(^+\) conductance of the B. flexus (data obtained from smooth curves that described the behavior of passive proton conductance) and the buffering capacity were sensitive to the proton concentration at the external surface over the pH range studied. B. flexus grown in pH 7.2 showed slightly higher H\(^+\) conductance than the cells grown at pH 10.5 (Fig. 3A-C). Membrane H\(^+\) conductance corresponds to the rate at which protons leak inward from the surrounding medium. The parameters, such as proton motive gradient and rate of outward pumping determine the pH tolerance of a bacterium.

This study revealed that B. flexus EMGA 5 grown aerobically had membrane H\(^+\) conductance values different from other neutrophiles\(^16,17,21,22\). The proton conductance was drastically reduced between pH 7 and 8, but steadily increased at pH 10. Since permeability of the membrane depends on physico-chemical characteristics such as the temperature and lipid packing, the proton pump may not be active near the neutral pH. Also, the proton permeability of the membrane at high temperatures (while increasing the pH from 7 to 8) could be compensated by an elevated rate of proton extrusion in the environment\(^23\).

![Fig. 1](image1.png)

![Fig. 2](image2.png)

Table 1—Buffering capacity and membrane H\(^+\) conductance of Bacillus flexus EMGA 5

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Growth medium pH</th>
<th>Maximum buffering capacity (µmol of H(^+/)pH unit/mg protein)</th>
<th>Maximum H(^+) conductance (µmol of H(^+/)s/pH unit/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus flexus EMGA 5</td>
<td>7.2</td>
<td>B(_t): 70 ± 5.3 (9.9) B(_o): 29 ± 3.4 (9.9) B(_i): 41 ± 5.1 (9.9)</td>
<td>0.032 ± 0.004 (9.9)</td>
</tr>
<tr>
<td></td>
<td>10.5</td>
<td>92 ± 19.1 (9.9) B(_o): 22 ± 14.4 (9.9) B(_i): 70 ± 9 (9.9)</td>
<td>0.028 ± 0.003 (9.8)</td>
</tr>
</tbody>
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EMGA, internal label; B\(_t\), total buffering capacity; B\(_o\), outer membrane buffering capacity; B\(_i\), cytoplasmic membrane buffering capacity.
Under the influence of different pH conditions, imposition of valinomycin-mediated potassium diffusion potential of membrane energizes the ATP synthesis. The H⁺ translocation involved in ATP synthesis could increase the buffering capacity values. In order to rule out this possibility, the cells were allowed to equilibrate for about 2 h with intermittent mixing after the addition of valinomycin. In addition, the buffering capacity and membrane H⁺ conductance for protons vary from one strain to another, as has been shown for Staphylococcus and Bacillus.

Interestingly, the range of external pH at which we could measure the buffering capacity and membrane H⁺ conductance of B. flexus EMGA 5 was lower than the facultative alkaliphilic Bacillus sp. EMGA 29 grown at pH 10.5. The buffering capacity and membrane H⁺ conductance of type strains B. amyloliquefaciens MTCC 1482 and S. aureus ATCC 25923 were influenced by pH of the medium (unpublished). Certain enzymatic activities could be induced by a change in external pH, and the surface characteristics of gram-positive bacteria depend on the pH of the cultures and the energized state of the membrane.

The cell wall of Bacillus lentus C-125, an alkaliphilic bacterium grown at pH 10.0 was found to be 20% thicker and showed about three-times the negative charge density than those grown at pH 7.0. Differences in the proton motive force between respiring and fermenting cells were reported for gram-positive and gram-negative bacteria. Aerobically growing cells had a greater proton motive force than the cells growing anaerobically, possibly due to the pH of the medium. In another study, the cell wall of B. subtilis showed less negative charge due to the carbon metabolism creating a proton motive force.

In addition to peptidoglycon, alkaliphilic Bacillus spp. contain certain acidic polymers, such as galacturonic, gluconic, glutamic, aspartic and phosphoric acids. The negative charge in the acidic non-peptidoglycon components may give the cell surface its ability to absorb sodium and hydronium ions and repulse hydroxide ions and allow the cells to grow in alkaline environments. The maintenance of pH homeostasis is an important factor in industrial production of enzymes, especially in extremozymes. Generally, extremozymes possess the same activity and stability, similar to their homologous mesophilic enzymes, but they can function in extreme conditions. The alkaliphiles use proton translocating ATP synthase to generate ATP in order to escape from high pH and low chemiosmotic driving force, thus keep their internal pH near neutral.

The high buffering capacity and H⁺ conductance of the protease producing facultative alkaliphile, B. flexus EMGA 5 could be useful for the leather industry, as it may survive and produce enzyme in extreme pH conditions during fermentation. Further studies are warranted in the regulatory mechanisms involved in homeo-proton permeability adaptation of the cytoplasmic membrane.

References
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