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CONTROL OF MEMBRANE POTENTIAL BY EXTERNAL H^+ CONCENTRATION IN *BACILLUS SUBTILIS* AS DETERMINED BY AN ION-SELECTIVE ELECTRODE

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Summary

The membrane potential of intact bacteria was monitored by measuring the tetraphenylphosphonium ion distribution across the membrane using poly-(vinyl chloride) matrix-type electrode selective to tetraphenylphosphonium ion. It was found that the tetraphenylphosphonium ion was not countertransported against H^+ movement. The membrane potential of *Bacillus subtilis* was estimated to be 80—120 mV inside-negative at external pH 7. The effect of the external pH on the membrane potential was studied. It varied from 30 to 40 mV/decade change in the external $[H^+]$ in the pH region of greater than 6.5, increasing pH making it more inside-negative. The addition of carbonyl cyanide *m*-chlorophenylhydrazone depolarized the membrane, and the membrane potential approached the H^+ equilibrium potential. The addition of *N,N'*-dicyclohexylcarbodiimide did not abolish the pH dependence of the membrane potential. Increasing the external $[K^+]$ did not affect the pH dependence. CN^- partially depolarized the membrane. A parallel conductance model for membrane potential could explain the results qualitatively.

Introduction

Membrane potentials of bacteria are related to cellular activities via proton electrochemical gradients [1—4]. An artificially-imposed proton electrochemi-

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide.

cal gradient drives ATP synthesis in *Escherichia coli*, *Streptococcus lactis* and *Rhodospirillum rubrum* [5,6]. Transport of metabolites is also related to proton electrochemical gradients [7—12]. Bacteria can even be transported by an artificial proton electrochemical gradient [13,14]. However, little is known about the regulatory mechanisms of the membrane potential, which would be an important factor to establish the relationships between membrane potential and cellular activities. In this connection, a simple method for estimation of membrane potentials in various conditions was needed.

Skulachev et al. studied the translocation of synthetic, lipid-soluble cations and anions across mitochondrial membrane [15,16]. These ions were found to distribute across the membrane passively depending on the membrane potential. To estimate the membrane potential by monitoring tetraphenylphosphonium ion distribution across the membrane of bacteria, we used tetraphenylphosphonium ion-selective electrodes. The electrodes were constructed according to the methods of Davis et al. [17] and Shinbo et al. [18]. Ion-selective electrodes are specifically suitable for the estimation of membrane potential because they monitor free ion concentration which is responsive to the membrane potential rather than monitor the total concentration including non-ionic forms of precipitates.

In the present study, we show that the membrane potential of *Bacillus subtilis* estimated from tetraphenylphosphonium ion distribution was 80—120 mV inside-negative at pH 7, and that the membrane potential varied with external pH or pH gradient across the membrane. We attempt to explain the mechanism of this effect using a parallel conductance model.

Materials and Methods

Growth and preparation of bacteria. B. subtilis BC 26 (Phe 12, Arg A, Ery) was obtained from Dr. N.R. Cozzarelli (University of Chicago) through Dr. J. Shioi (Nagoya University). The bacteria were grown in a medium containing 1% Bacto-tryptone/0.5% NaCl/0.14 mM $CaCl_2$/0.2 mM $MgCl_2$/0.01 mM $MnCl_2$ [19] at 34°C to a late log-phase. The bacteria were centrifuged (6000 × *g*, 5 min) and washed twice in a basal solution containing 200 mM NaCl/10 mM potassium phosphate/0.1 mM EDTA/0.2 mM $MgCl_2$/0.14 mM $CaCl_2$/0.01 mM $MnCl_2$/0.5% glycerol (pH 6.8—7.0) (Ordal and Goldman [20], slightly adapted). These procedures were performed at room temperature (25°C). The bacterial suspension was then incubated at 34°C for 2 h, and recentrifuged (6000 × *g*, 5 min). The pellet of bacteria was suspended into an equal volume of a basal solution without glycerol, and stored on ice until measurement [21]. The measurements were performed in 10 ml basal solution without glycerol. Endogenous carbon source was sufficient for 2—3 h judging from oxygen consumption.

Chemicals. Tetraphenylphosphonium chloride was purchased from Merck, F.R.G.; sodium tetraphenylborate from Wako Pure Chemical Industries, Japan; and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) from Sigma Chemical Co., U.S.A. Other reagents were commercial products of analytical grade.

Tetraphenylphosphonium ion-selective electrode. The poly(vinyl chloride)

matrix-type electrode was constructed following the method for construction of the Ca^{2+}-selective electrode by Davis et al. [17]. Tetraphenylborate was used as ion exchanger for tetraphenylphosphonium [18]. Response of the electrodes to tetraphenylphosphonium was linear, at least up to $2 \cdot 10^{-7}$ M, and slopes of the electrode potential plotted against log [tetraphenylphosphonium ion] were 50—59 mV. One of the electrodes could measure up to $2 \cdot 10^{-8}$ M linearly, and could even detect the ion as low as $2 \cdot 10^{-9}$ M. No interference by inorganic ions such as Na^+ or K^+ (both at 1 M) was observed. There was a slight interference by H^+ in acidic solutions after the electrode was exposed to CCCP.

Measurement of electrode potential and external pH. Tetraphenylphosphonium ion-selective, pH and reference electrodes were immersed in 10-ml basal solution containing $1 \cdot 10^{-5}$ M tetraphenylphosphonium. Concentration of tetraphenylphosphonium and pH were monitored alternately by an electrometer under constant stirring at 25° C.

Calculation of membrane potential. To calculate membrane potentials from the corresponding tetraphenylphosphonium ion-selective electrode potentials, the following conversion formula was used:

$$\exp(-F\Delta\psi/RT) = (V/v)\ \{\exp(\alpha F\Delta E/RT) - 1\} \quad (1)$$

where $\Delta\psi$ is the membrane potential of bacteria measured from outside to inside; ΔE, the difference between the electrode potentials before and after the addition of bacteria; V, the volume of an external medium; v, the volume of bacteria added; R, the gas constant; T, the absolute temperature; α, the term describing deviation of the slope of the electrode potential from the Nernst slope (α was determined by the calibration curves for the tetraphenylphosphonium ion).

Results

Uptake and release of tetraphenylphosphonium ion by bacteria suspended in basal solution without buffer

Bacteria suspended in a basal solution were added to a basal solution without potassium phosphate. The pH of the solution was changed by addition of NaOH or HCl. After alkalinization, the pH of the solution gradually returned to near the level before alkalinization (Fig. 1), in spite of the fact that the buffering capacity of the solution itself was very limited. The concentration of phosphate incorporated with bacteria did not exceed 0.1 mM. Slow fall of the pH curve followed by fast rise indicates that the pH of the solution was regulated by H^+ transport of bacteria. In other words, alkalinization of the solution caused release of H^+ from (or accumulation of OH^- into) bacteria. The net change in the concentration of H^+ should be expressed by the difference of the two terms [22] as:

$$\Delta[H^+]_{obs}(t) = \Delta[H^+]_{alk} - \Delta[H^+]_{tr} \quad (2)$$

where $\Delta[H^+]_{obs}$ is the net change in the concentration of H^+; $\Delta[H^+]_{alk}$ the alkalinization by NaOH, and $\Delta[H^+]_{tr}$ the transport of H^+ by bacteria. It is clear that $\Delta[H^+]_{alk}$ is a much more rapid process than $\Delta[H^+]_{tr}$. A detailed

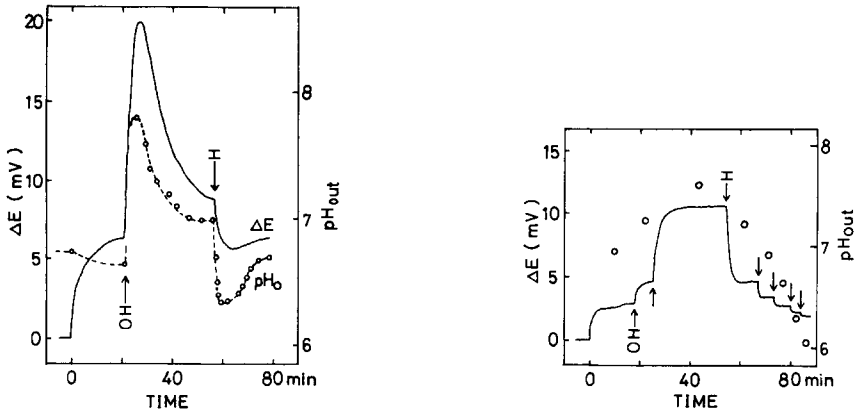

Fig. 1. Changes in the tetraphenylphosphonium ion-selective electrode potential and the external pH in a basal solution without buffer. At time zero, a suspension of bacterial pellet (50%, v/v; 150 μl) was added to 10-ml medium. At the times indicated by the first and second arrows, NaOH and HCl (arbitrary amounts) were added, respectively. An increase in the electrode potential difference (ΔE) indicates the corresponding decrease in external [tetraphenylphosphonium ion].

Fig. 2. Changes in the tetraphenylphosphonium ion electrode potential in a buffered basal solution. At time zero, a suspension of bacterial pellet (50%, v/v; 120 μl) was added to 10-ml medium. At the times indicated by the arrows pointing upwards and downwards, 10-μl 1 M NaOH and HCl were added, respectively. ΔE (———); pH_{out} (○)

analysis of the process after alkalinization as shown in Fig. 1 revealed that the apparent half relaxation time of $\Delta[H^+]_{tr}$ was about 6 min. If tetraphenylphosphonium ions are countertransported against the movement of H^+, tetraphenylphosphonium ions must be taken up by a similar process to $\Delta[H^+]_{tr}$. Fig. 1 shows that tetraphenylphosphonium ions were released during the slow process of H^+ release. The countertransport mechanism for tetraphenylphosphonium ion uptake or release is impossible. It will be more reasonable to assume that tetraphenylphosphonium ions are transported down the membrane potential and that in steady states tetraphenylphosphonium ions are distributed across the membrane according to the membrane potential. This assumption, in turn, leads to the possibility that the membrane potential of bacteria is controlled by external pH.

Uptake and release of tetraphenylphosphonium ions by bacteria suspended in a buffered basal solution

To characterize further the dependence of tetraphenylphosphonium ion transport upon external pH, the experiments hereinafter were performed with the buffered basal solution described in Materials and Methods. The buffer in the solution reduced the pH change caused by H^+ transport of bacteria. As shown in Fig. 2, alkalinization of the solution made tetraphenylphosphonium ions enter the interior space of bacteria, but in this case the ions did not emerge until the external pH was lowered. This indicates, as is expected, that the membrane potential is controlled by the external pH or the pH gradient across the membrane. When the external pH was raised from about 7 to 8 by NaOH and was then decreased by HCl, the tetraphenylphosphonium ion-selective electrode potential showed hysteresis as can be seen in Fig. 2. On the

other hand, when the external pH was decreased to about 6 and then increased, the hysteresis of the tetraphenylphosphonium ion-selective electrode potential was very slight or was not observed at all, as is shown in Fig. 3.

In Fig. 3, the electrode potential was converted to the membrane potential of bacteria according to Eqn. 1. In the conversion formula, the amount of tetraphenylphosphonium ions adsorbed to the membrane of bacteria was neglected. For estimation of the amount adsorbed, bacteria treated with toluene and/or heated (80°C, 10 min) were used instead of intact bacteria [18]. The resultant change in the tetraphenylphosphonium ion-selective electrode potential was 0.5 mV when 150 μl of the treated bacteria were added. This change was about that expected by dilution with 150 μl basal solution only, i.e., 0.36 mV. Therefore, binding of the tetraphenylphosphonium ion to bacterial membrane is negligibly small compared with the amount taken up into the interior space of bacteria.

For calculation of $\Delta\psi$, it was postulated that the internal space of bacteria is about 50% of the pellet volume. The actual internal space of bacteria will not be very different to this value, considering that the inulin space of *E. coli* was 38—45% of the pellet [23] and that the dimensions of *B. subtilis* and *E. coli* are similar under microscopic observation. Values of $\Delta\psi$ for intact *B. subtilis* at pH 7, fell within −80 to −120 mV. Although the accuracy of the absolute values of $\Delta\psi$ depends on the accuracy of the volume of bacteria added, accurate changes in $\Delta\psi$ can be calculated without the knowledge of V/v in Eqn. 1 [18]. Because $\partial(\Delta\psi)/\partial(\Delta E)$ does not depend on V/v, but only on ΔE according to the conversion formula, i.e., $\partial(\Delta\psi)/\partial(\Delta E) = \alpha \exp(\alpha F\Delta E/RT)/\{1 - \exp(\alpha F\Delta E/RT)\}$. Fig. 3 is an example of $\Delta\psi$ plotted vs. external pH. The slope of $\Delta\psi$/decade change in $[H^+]$ was 30—40 mV in the pH region from about 6.5 to 8.

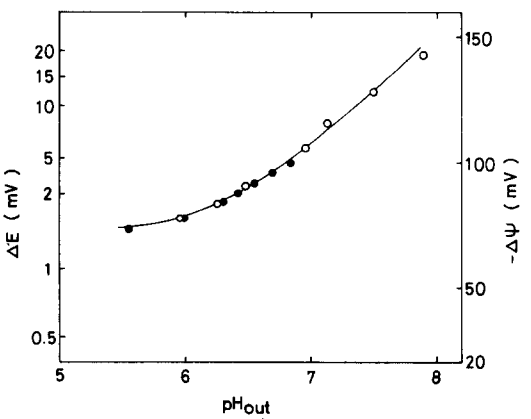

Fig. 3. Influence of external pH on ΔE and $\Delta\psi$ (the membrane potential of bacteria). The pH was first decreased (●), and then increased (○). Axis of the ordinate is proportional to $\Delta\psi$.

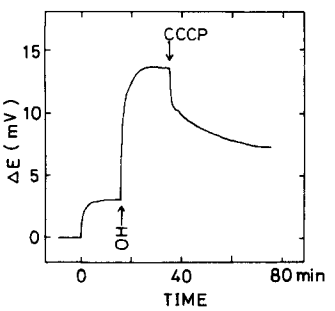

Fig. 4. Influence of carbonyl cyanide *m*-chlorophenylhydrazone CCCP on ΔE in buffered basal solution. At time zero, a suspension of bacterial pellet (50%, v/v; 120 μl) was added to 10-ml medium. At the times indicated by the first and second arrows, NaOH (arbitrary amount) and CCCP (2 μM, final concentration) were added, respectively.

Effect of CCCP on membrane potential

The above result may indicate that the membrane of *B. subtilis* is permeable to H^+ and that $\Delta\psi$ is determined by the H^+ passive diffusion. To know whether this is true or not, a proton conductor, CCCP, was applied to the bacterial suspension, and the resulting changes in the tetraphenylphosphonium ion distribution across the membrane of bacteria were observed. Fig. 4 shows the change in the tetraphenylphosphonium ion distribution when $2 \cdot 10^{-6}$ M CCCP was added after the membrane potential was made more negative by NaOH addition. In order to know the change in $\Delta\psi$, the interference of CCCP on the tetraphenylphosphonium ion-selective electrode potential must be determined. The selectivity of this type of electrode among lipid-soluble organic ions is, in general, poor. $2 \cdot 10^{-6}$ CCCP was applied to basal solutions (pH 7) supplemented, respectively, with 150 μl basal solution only, 150 μl suspension of toluene-treated and heated bacteria, and 150 μl suspension of intact bacteria. The corresponding ΔE values were 5.5, 5.5 and 5.8 mV, respectively. Hence, $\Delta\psi$ at pH 7 in the presence of CCCP must be zero or positive. From an estimation using 5,5-dimethyl-2,4-oxazolidinedione distribution (equilibrium dialysis method), the internal pH of *B. subtilis* was 7.3—7.4 under an external pH of 7 (unpublished data). Distribution of salicylic acid also indicated that the internal pH of the bacteria was about 7.4 under an external pH between 6 and 8 (Shioi, J., personal communication). The internal pH of *B. subtilis* was similar to that of *E. coli* [23]. These results indicate that $\Delta\psi$ of *B. subtilis* upon addition of CCCP approaches the H^+ equilibrium potential which is supposed to be inside-positive at pH 7.

Effect of N,N'-dicyclohexylcarbodiimide (DCCD) on pH dependence of membrane potential

DCCD is known to inhibit membrane-bound H^+-translocating ATPase [3]. If H^+-translocating ATPase is attributable to the pH dependence of the membrane potential, DCCD will inhibit this effect. The effect of DCCD (111 μM) on membrane potential is shown in Fig. 5. Clearly, DCCD did not inhibit the pH effect. When NaOH was added (the external pH became about 7.5) after the addition of DCCD, the membrane potential difference increased more than that of the control. When DCCD was added after NaOH addition, the membrane potential difference was also increased, though slowly in this case (this means that the action of DCCD occurred in a slow process). These results should be regarded as qualitative, since the tetraphenylphosphonium ion-selective electrode potential without bacteria began to drift slowly, upon addition of DCCD, in the direction opposite to that in Fig. 5 for unknown reasons.

Effect of pH on membrane potential in KCl solution

Another possibility for the pH dependence of the membrane potential is that the external pH affected the K^+ permeability of the membrane. To test this possibility, we measured the membrane potential in a basal solution supplemented with 200 mM KCl in place of NaCl. Since the K^+ equilibrium potential in KCl solution is different from that in NaCl solution, the pH effect is expected to differ between these solutions if the K^+ permeability varies with the external pH. Fig. 6 shows that the pH effects in KCl and NaCl solu-

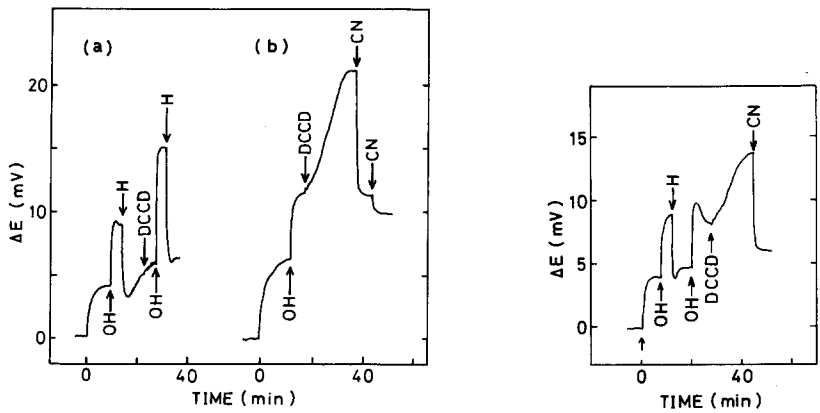

Fig. 5. Influence of *N,N'*-dicyclohexylcarbodiimide (DCCD) and CN^- on the pH effect in buffered basal solution. At time zero, (a) 120 µl and (b) 150 µl suspensions of bacterial pellet (50%, v/v) were added to 10-ml medium. NaOH and HCl, 35 µl of 0.5 M solutions; DCCD, 111 µM final concentration; KCN, 0.4 and 0.8 mM final concentrations.

Fig. 6. Influence of external pH, DCCD and CN^- in buffered KCl solution. At time zero, a suspension of bacterial pellet (50%, v/v; 120 µl) was added to 10-ml medium. NaOH and HCl, 35 µl of 0.5 M solutions; DCCD, 111 µM final concentration; KCN, 0.4 mM final concentration.

tions were similar. This indicates that the K^+ permeability was low and did not increase with the external pH.

Effect of CN^- on membrane potential

The pH effect may indicate that the change in the membrane potential caused by external pH is a consequence of the respiratory electrogenic H^+ pump. To determine whether or not this is true, the effect of CN^- was examined. KCN (0.4 and 0.8 mM, final concentrations) was added to the solution after the membrane was hyperpolarized by addition of NaOH in the presence of DCCD (Fig. 5). The pH increases of the solution on addition of 0.4 and 0.8 mM KCN, were about 0.2 and 0.4, respectively. The pH increase of about 0.4 corresponds to 15 mV hyperpolarization of the bacterial membrane, but a rapid depolarization (though partial) of the bacterial membrane was observed on addition of KCN. Oxygen electrode measurements indicate that 0.4 and 0.8 mM KCN inhibited 70—80% and 90% of respiration, respectively. It is clear that the electrogenic H^+ pump contributed to generation of the membrane potential; however, from oxygen electrode measurements, the respiration rate did not increase with increasing external pH and was approximately constant at external pH values from 6 to 8 (unpublished data). Therefore, the possibility that the pH effect is controlled by respiration was excluded.

Discussion

Studies on the energetics of mitochondria, chloroplasts, and bacteria in the framework of the theory of chemiosmosis by Mitchell, have been performed with great success [1—3]. No doubt H^+ flux plays a key role in the energy conversion. In the present paper, we studied the effect of external pH on the

membrane potential of *B. subtilis*. It was found that the membrane potential was controlled by external pH. The experiments were aimed at revealing the cause of the pH dependence of the membrane potential ($\Delta\psi$). The results are summarized as follows. Addition of CCCP depolarized the membrane. This means that $\Delta\psi$ is not equal to the H^+ equilibrium potential. Addition of DCCD did not abolish the pH dependence of $\Delta\psi$; therefore, pH dependence is not caused by membrane-bound H^+-translocating ATPase. In basal solutions containing 200 mM NaCl and 200 mM KCl in place of NaCl, the pH dependence of $\Delta\psi$ was similar. This excludes the possibility that K^+ permeability increased in alkaline medium (increase of K^+ permeability hyperpolarizes the membrane in NaCl solution as a consequence of the generation of a K^+ diffusion potential). CN^- depolarized the membrane. This result showed that electrogenic H^+ extrusion by respiration contributed to the generation of $\Delta\psi$. However, the pH dependence of $\Delta\psi$ cannot be explained by changes in the electrogenic pump activity, since the rate of respiration was rather constant at pH 6—8.

In order to explain these results, we used a parallel conductance model for membrane potential [24]. The model could explain the results summarized above, at least qualitatively. The basic postulate is that the passive current of an ion species is the product of the conductance for the ion and the motive force of the ion. Then the total ionic current should be described as follows:

$$I = g_H(\Delta\psi - E_H) + \Sigma g_i(\Delta\psi - E_i) + J_H + \Sigma J_i \tag{3}$$

where g_H is the conductance for H^+; E_H, the H^+ equilibrium potential; g_i, the conductance for the ith ion species; E_i, the equilibrium potential of the ith ion species; J_H, the electrogenic current of H^+; J_i, the electrogenic current of the ith ion species; and the summation is made excluding H^+. Under steady-state conditions the total current, I, must be zero. Solving for $\Delta\psi$ under steady-state conditions, we can obtain the following expression:

$$\Delta\psi = \frac{g_H}{g_H + \Sigma g_i} E_H + \frac{\Sigma g_i E_i}{g_H + \Sigma g_i} - \frac{J_H + \Sigma J_i}{g_H + \Sigma g_i} \tag{4}$$

With the relationship between E_H and ΔpH ($= pH_{out} - pH_{in}$) the expression is rewritten as:

$$\Delta\psi = -2.3\frac{RT}{F}\frac{g_H}{g_H + \Sigma g_i}\Delta pH + \frac{\Sigma g_i E_i}{g_H + \Sigma g_i} - \frac{J_H + \Sigma J_i}{g_H + \Sigma g_i} \tag{5}$$

pH_{in} is considered to be constant (about 7.4) with respect to pH_{out} (Shioi, J., personal communication), and J_H seems to be constant from the measurement of the respiration rate (unpublished observation). If we furhter postulate that $(g_H)/(g_H + \Sigma g_i)$ is equal to about 1/2 and that it does not vary with pH_{out}, the slope of $\Delta\psi$ as a function of pH_{out} becomes about 30 mV. The effect of CCCP is explained by an increase in g_H as follows. Since ΔpH becomes zero at pH_{out} 7.4, $\Delta\psi$ can be written as:

$$\Delta\psi = \frac{\Sigma g_i E_i}{g_H + \Sigma g_i} - \frac{J_H + \Sigma J_i}{g_H + \Sigma g_i} \tag{6}$$

Evidently, an increase in g_H makes $\Delta\psi$ approach to zero, i.e., depolarization.

On the other hand, a part of g_H corresponding to H^+-translocating ATPase must become smaller on addition of DCCD. The expected change in $\Delta\psi$ at pH_{out} about 7.4 is hyperpolarization, as observed in Figs. 5 and 6. CN^--induced partial depolarization is also expected from Eqn. 6 if J_H becomes smaller on addition of CN^-.

In membrane vesicles of *E. coli*, $\Delta\psi$ did not vary with external pH [25,26]. In terms of the present model, this might be the consequence of small g_H or large g_i values. In any event, establishing the validity of the present model requires more detailed comparisons of the model with measurements. Measurements of $\Delta\psi$ under various conditions would enable an evaluation of the conductances. Leak conductance of H^+ would be small according to the chemiosmotic theory. We hope that the parallel conductance model will lead to a deeper understanding of bacterial energetics.

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