Molecular Strategies of Creatures to Survive in Acidic Environments

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Invitation to the Acidic World

By

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PREFACE

It became generally accepted in the 19th century, perhaps even earlier, that living organisms were able to synthesize various compounds only when they were alive—such compounds were called organic compounds. At the end of the 19th century, Eduard Buchner found that alcohol can be synthesized in disrupted yeasts, demonstrating that life is not an essential factor for the production of organic compounds (Kohler 1971). This finding led to the discovery of enzyme-catalyzed chemical reactions. With this milestone discovery, a new research field, biochemistry, began, which sought to reveal the phenomena of living organisms based on chemical reactions. Research expanded into related areas—molecular biology, molecular genetics, cell biology—all of which are now considered branches of the life sciences.

The category of living organisms encompasses a huge number of species, ranging from microscopic viruses to humans. Researchers in the field have generally assumed that all living organisms have common mechanisms essential for supporting life and have focused their investigations on the discovery and analysis of such common mechanisms. Living organisms are diverse with their habitats covering almost all the areas of the earth. Different living things live under different conditions of temperature, pH, salinity, and hydrostatic pressure, etc., leading us to assume that living things have different systems to help them survive in different environments. It remains a challenge for researchers to successfully dissect the strategies that organisms use and the results of their research can help develop our understanding of life at the individual level.

It is a common idea that such strategies developed through genetic alteration over a long time span. There are two main theories. One is that genetic alteration occurred randomly and species acquired new and useful systems for survival coincidentally under particular environment conditions, which were then selected for, and species that failed to develop such systems became extinct—Darwin's theory of natural selection. The second theory is the neutral evolution theory, which proposes that genetic alterations occurred and accumulated in a certain direction independently of the environmental conditions (Charlesworth, Barton, and Charlesworth 2017). We have limited means to know how evolution progressed historically and verification has not been established experimentally; as such, we have only assumptions based on the analysis of fossils and genetic variations in present-day organisms. Genetic alterations may have been accumulated through the above two evolutional processes and occurred by turns or at the same time, leading to the evolution of modern organisms.

Living things are also impacted by changes in the conditions of their surroundings—from the womb to the tomb and through changes in climate and their travels. They may develop various strategies to overcome environmental changes. Unlike the evolution of living things, these strategies can be dissected experimentally. Among the conditions they encounter, changes in the pH of their habitat can often occur and threaten their continued existence. The main component of living things is protein, which consists of amino acids that have both acidic and alkaline residues; protonation and deprotonation of these residues occur within a narrow pH range. Since all enzyme activities mediating cellular reactions are pH-dependent, changes in pH dramatically affect cellular functions, often leading to death. We set ourselves, therefore, the challenge of trying to shed light on how living things can overcome changes in pH.

Over the past century, it has become well known that the habitats of microorganisms encompass a pH range from 1 to 11. A near neutral pH is favorable for all creatures, however, acidophilic bacteria inhabit extremely strong acidic environments and alkaliphilic bacteria can survive under strong alkaline conditions. Some neutrophilic bacteria can grow in a wide pH range, between 5 and 10, and maintain viability, without growth, below a pH of 4. Many researchers have tried to clarify bacterial strategies for living or maintaining viability in extreme pH conditions from both the biochemical and genetic points of view.

In higher animals, the body is covered by skin and environmental pH changes do not affect the pH inside the body. The human body contains 3-6 trillion cells and each cell is surrounded by a fluid whose pH is strictly kept at 7.4. Some diseased areas, however, can become acidified. In cancerous solid cell nests, the pH drops below 6. Even so, this change in pH is 1.5 pH units or fewer, which is very small compared to changes seen in the bacterial world. As such, for a long time now the strategies available to mammalian cells to deal with changes in pH have only occasionally been the focus of research. However, recent studies have shown that small pH changes can affect cellular metabolism, gene expression, and other functions, potentially posing a threat to life.

In addition to the basic systems existing in all forms of life, a better comprehension of how creatures respond to environmental acidification and life in the acidic world can not only increase our understanding of life, but also help us to acquire wisdom about how to improve people's lives. In this book, I wish to introduce the results of research into the acidic world at the molecular level. Of course, we can understand only a small part of this and a huge number of questions described in this book are left to future studies. However, their elucidation will help open up new ways to understand life.

WATER STRUCTURE AND PH

Organisms consist of a huge number of diverse cell types and each cell commonly contains water, which makes up approximately 70 % of the body. Cellular metabolisms are generally carried out in the aquatic phase. To achieve a better understanding of life, the structure of water should first be analyzed.

The detailed structure of water is still mysterious (Ball 2008). Molecules of water (H_2O) combine with each other using hydrogen bonds, as shown in Figure 1. The rate of movement of molecules decreases with a decrease in temperature, but no clear structural difference has been observed when water changes from its liquid form to ice and vice versa.

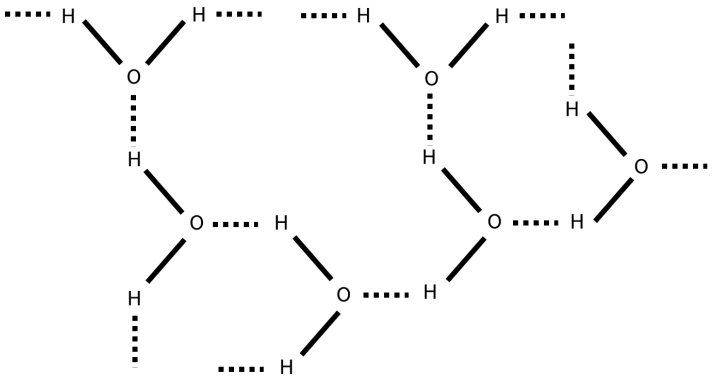

Figure 1. Structure of water. Solid line—covalent bond; dotted line—hydrogen bond. (Bond angles and lengths in this figure are not accurate.)

pH

pH is the negative value of the power of proton concentration, as follows.

$$pH = -\log[H^+]$$

H_2O dissociates to H^+ and OH^- ions and its dissociation constant is 10^{-14}. When $[H^+]$ is equal to $[OH^-]$, the pH value is 7. When $[H^+] > [OH^-]$, the pH value is less than 7 and we call this acidic. In contrast, when $[H^+] < [OH^-]$, the pH value is above 7 and we call this alkaline (Boyd 2000).

H^+ does not exist in this form—its actual form is H_3O^+ (hydronium). H_3O^+ and OH^- are bound to H_2O with a hydrogen bond (Ikeda et al. 2017). H_3O^+ ions can move in water, but this molecule itself does not move. Instead, a hydrogen bond moves through a changing electron cloud, as shown in Figure 2, and hence the speed of movement of H_3O^+ is very fast.

A bacterium is a small, single cell organism with a volume of $1\text{-}2 \times 10^{-15}$ liter. If bacterial cell volume is assumed to be 1×10^{-15} liter, with a pH of 9, then one bacterial cell contains 3.3×10^{10} ($(1{,}000/18) \times 6.02 \times 10^{23} \times 1 \times 10^{-15}$) H_2O molecules and the amount of H_3O^+ per cell is calculated as 0.6 ($10^{-9} \times 6.02 \times 10^{23} \times 1 \times 10^{-15}$). This means that one bacterial cell contains less than one H_3O^+ molecule. The first question that arises concerns the meaning of intracellular pH in bacteria. PH is a value averaged over time—H_3O^+ is present at a certain moment and there is a time period when the H_3O^+ molecule is not present. Bacterial cells are generally surrounded by an aquatic solution and H_3O^+ can move from the inside to the outside of bacterial cells and vice versa, although the rate of movement is slow. Therefore, the pH inside the cell is significant and the activity of intracellular enzymes can be affected by H_3O^+.

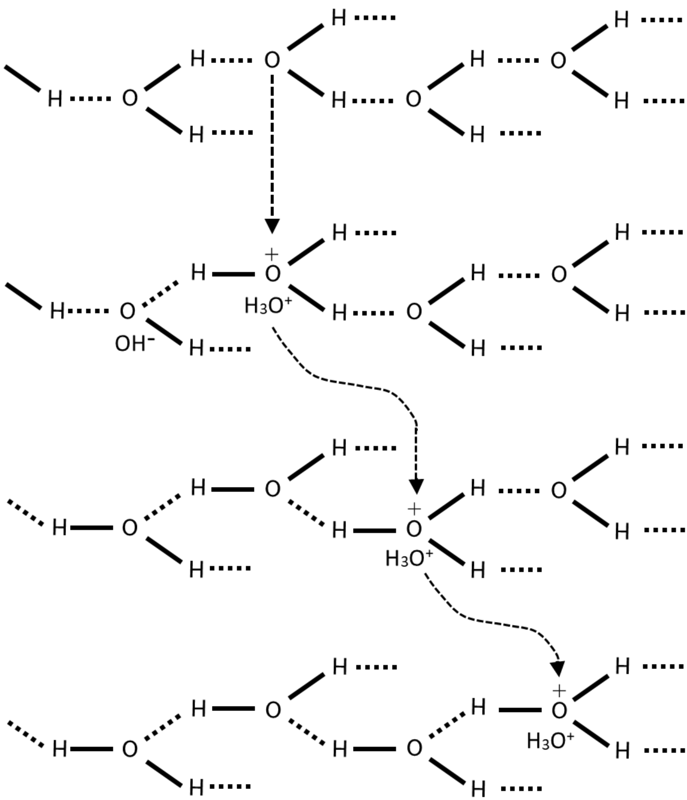

Figure 2. Movement of H_3O^+. H_3O^+ moves through the change in a covalent bond into a hydrogen bond and a hydrogen bond into a covalent bond and this is mediated by the movement of the electron cloud. OH^- moves independently to H_3O^+. (Bond angles and lengths in this figure are not accurate.)

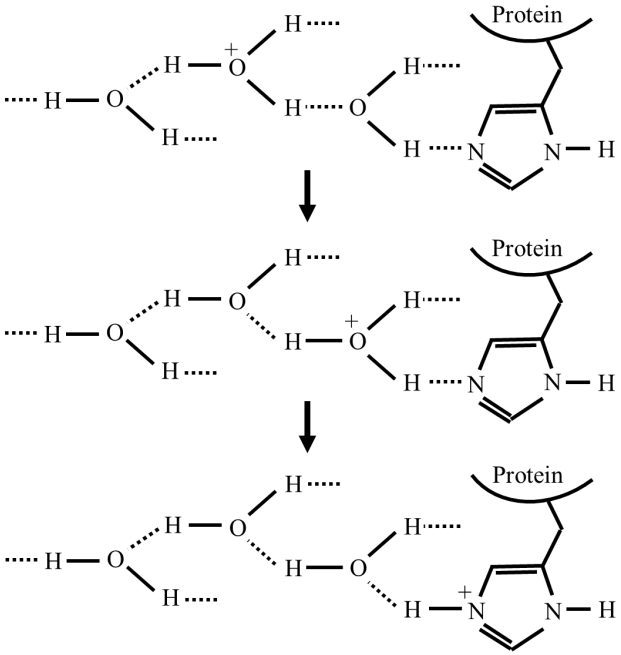

Figure 3. Movement of H_3O^+ and protonation of a protein's histidine residue. After H_3O^+ appears, it can move very rapidly through the movement of the electron cloud and a histidine residue can be protonated in a short period of time. (Bond angles and lengths in this figure are not accurate.)

The molarity of water is 55.6 (1000/18) and the ratio of H_3O^+ to H_2O is $1.8x10^{-9}$ at pH 7. The second question then is whether negatively charged amino acid residues meet H_3O^+ for protonation in the intracellular space when the ratio of H_3O^+ to H_2O is very low. Protonation and deprotonation occur with histidine residues of proteins and phosphate residues of nucleic acids in a pH range of 6 to 8. As described above, H_3O^+ can move very quickly via the rapid movement of electron density and thus protonation and

deprotonation can easily occur, as illustrated in Figure 3, even if the amount of H_3O^+ is very low. When averaging over time, the ratio of protonated to deprotonated residues is close to that found in an aquatic solution of large volume.

CHEMIOSMOTIC THEORY AND H^+-ATPASE

In the process of oxidative phosphorylation in mitochondria, ATP synthesis is coupled with electron transfer through the respiratory chain. The first hypothesis proposed to account for the mechanism of this complex reaction was that the change in the redox state of one or more hypothetical proteins synthesize ATP directly; however, such a protein has not yet been identified. An astonishing hypothesis on the mechanism of ATP synthesis is the chemiosmotic theory proposed by Peter Mitchell (Mitchell 1961; 2011, Figure 4).

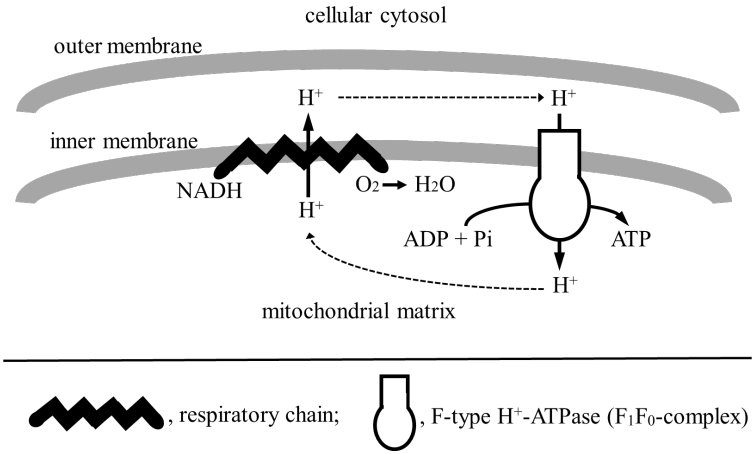

Figure 4. Chemiosmotic theory for oxidative phosphorylation.

The respiratory chain transports protons across the inner membranes of the mitochondria, resulting in the generation of an electrochemical gradient of protons between the spaces on both sides of the inner membranes. The electrochemical potential of protons is called the proton-motive force (PMF) and is expressed by the following equation:

$$PMF\ (mV) = \Delta\varphi + RT/F \times \ln([H^+]in/[H^+]out) = \Delta\varphi - Z\Delta pH,$$

where Z = 61.5 at 37 ℃ and $\Delta pH = pH_{outside} - pH_{inside}$. Δφ is the membrane potential and the external potential is set to zero. PMF is generally 'inside negative'.

The F-type H^+-ATPase (F_1F_0-complex), consisting of multiple subunits, synthesizes ATP using energy released from the energetically downward movement of protons through the mechanism (Collinson et al. 1996). Aerobic bacteria also use oxidative phosphorylation, with a similar mechanism to that of mitochondria.

The identification and purification of the protein complex catalyzing ATP synthesis in oxidative phosphorylation has been performed for both bacteria and mitochondria. The complex is now simply called F-type H^+-ATPase or F-ATPase. This enzyme consists of two parts: the peripheral part F_1 and the membrane embedded part F_0. The F_1 part was isolated in *Escherichia coli* (*E. coli*) (Evans 1970). The whole enzyme, F_1F_0-complex, was first isolated in mitochondria in the early 1970s (Collinson et al. 1996) and then in *E. coli* (Foster et al. 1980). The F_1 and F_0 parts contain 5 (α, β, γ, δ, and ε) and 3 (a, b, and c) subunits, respectively. There are additional proteins associated with F-ATPase, but, except for a few proteins, their functions remain unclear.

The reaction mediated by F-ATPase is reversible. The isolation of this enzyme was carried out using ATP hydrolysis and the ability for ATP synthesis coupled with the proton movement was proved using a purified enzyme.

Some anaerobic bacteria have an anaerobic respiratory chain whose terminal enzymes use various electron acceptors, such as nitrate (NO_3^-), sulphate (SO_4^{2-}) or carbon dioxide (CO_2), but not oxygen (Roediger 2008). The chain generates the proton-motive force and F-ATPase synthesizes ATP using this proton-motive force (Richardson 2000).

Living organisms use various enzymes to hydrolyze ATP. The enzyme catalyzing ATP hydrolysis was first identified by Skou in 1957 (Skou 1957) and was named Na^+/K^+-ATPase. It participates in the regulation of Na^+ and K^+ levels in mammalian cells. The second enzyme to hydrolyze ATP was found in *Streptococcus faecalis* (now classified as *Enterococcus hirae*) by Abrams and his coworkers in 1960 (Abrams, McNamara, and Johnson 1960). The purified enzyme contains two kinds of subunit, which were later revealed to be a part of F-type H^+-ATPase. This bacterium has no respiratory chain and hence no system for oxidative phosphorylation operates. The physiological function of F-type H^+-ATPase in this bacterium, without oxidative phosphorylation, was demonstrated in the 1980s, as described on pages 17-25. In addition to these enzymes, a large number of ion-translocating ATPases have been identified and characterized, as described in the following chapters.

From an evolutionary point of view, ion transport systems using energy released from ATP hydrolysis evolved first and an H^+ translocating ATPase,

F-ATPase, was most likely selected as an ATP synthesizing enzyme once organisms developed the capacity for oxidative phosphorylation after the surface of the globe became filled with oxygen.

Beyond the dissection of the oxidative phosphorylation mechanism, the chemiosmotic theory proposed by Peter Mitchell propounded the importance of proton concentration and its movement for our understanding of life, resulting in the development of new fields of the life sciences based on electrochemistry.

MICROORGANISMS IN THE ACIDIC WORLD

Bacteria have been used as model organisms for research in biochemistry, molecular biology, and molecular genetics. Unlike higher organisms, microorganisms inhabit almost all areas of the globe's surface, including both poles, the deep sea, under ice sheets, and in hot springs. The pH values of their habitats range from 1 to 11 and they may have special strategies for surviving under such extreme pH conditions compared to higher organisms. Bacterial strategies to survive in extremely acidic conditions have been a focus of research and it has been found that bacteria have developed two ways for growing and maintaining viability at an acidic pH. These strategies involve the maintenance of a neutral cytoplasm to resist decreases in the surrounding pH and the utilization of enzymes working at an acidic pH when cytoplasmic pH drops below the range of intracellular pH control.

1. Bacterial structure

Bacteria come in two structural types. Gram-negative bacteria are covered by two membrane layers with both outer and inner membranes. The inner membrane is often called the cytoplasmic membrane. Bacteria are also covered by a cell wall. The cell wall of gram-positive bacteria is thicker than that of gram-negative bacteria. *E. coli* and *Enterococcus hirae* are, respectively, gram-negative and gram-positive. *E. coli* has porins in the outer membranes through which small molecules with a size of less than

600 Daltons can pass (Novikova and Solovyeva 2009). Large molecules, including proteins, can move freely through the cell wall, but they cannot move across the inner and outer membranes. The inner and outer membranes have specific transport systems that mediate the movement of specific molecules, including proteins, across them. Many proteins exist in the space between the inner and outer membranes, called the periplasmic space, in gram-negative bacteria. Such proteins find it difficult to escape cells even if they are not bound to the membranes. In contrast, proteins working in the cell surface of gram-positive bacteria are bound to the cytoplasmic membrane to prevent their release because no membrane barrier exists outside the cytoplasmic membrane.

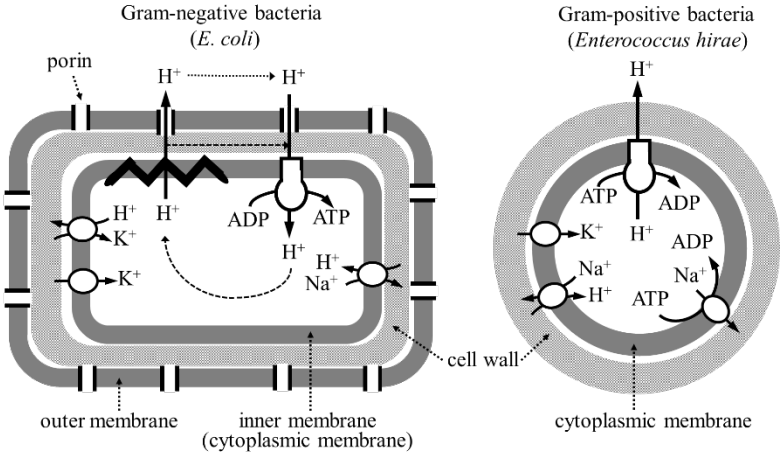

Figure 5. Structures of gram-negative and gram-positive bacteria. Ion transport systems in *E. coli* and *Enterococcus hirae* are illustrated. (For details, see text.)

E. coli has both a respiratory chain and F-ATPase, which mediates oxidative phosphorylation under aerobic conditions. Enterococci and their close relations have F-ATPase, but lack a respiratory chain, whereas many gram-positive bacteria have both systems. Enterococcal F-ATPase extrudes protons in a reverse reaction to regulate cytoplasmic pH, as described on pages 17-25.

2. Cytoplasmic pH regulation in *E. coli*

Adaptation strategies for acidic environments have been a subject of research since the 1980s. *E. coli* can grow in a medium with a pH range of 5 to 9, maintaining an internal pH of 7.5 in a medium pH range of 6 to 8. The cytoplasmic pH gradually decreases at a pH below 6 (Figure 6).

Biological membranes have a hydrophobic layer and charged molecules find it difficult to move across them. Hydrogen bonds are created between the H atom of H_3O^+ and the O or N atoms of proteins, phospholipids, and some glycolipids in the membranes. As a result, protons can move across the membranes through these hydrogen bonds at a rather high rate, resulting in cytosolic acidification in an acidic medium. Biochemical investigations have revealed that all enzymes show pH-dependent activity, because proteins consist of bipolar amino acids that are protonated or deprotonated with changes in pH. Intracellular acidification reduces those cellular metabolisms mediated by pH-dependent enzyme activity. Therefore, in acidic surroundings, it is essential to suppress any drop in pH in the cytoplasm to keep cytoplasmic metabolism active via the extrusion of protons that have invaded from the surroundings.

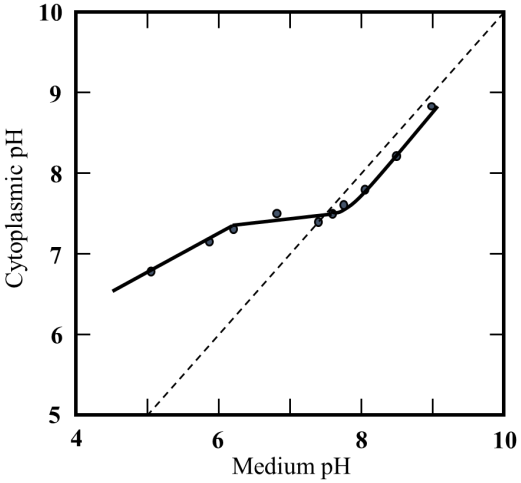

Figure 6. Cytoplasmic pH values in *E. coli*. The broken line represents the cytoplasmic pH equal to the medium pH. (For original data, see Mugikura et al. 1990.)

How does *E. coli* minimize cytoplasmic pH change in an acidic medium? It can be supposed that one or more proton transport systems regulate cytoplasmic pH in *E. coli*. Three kinds of transporters have been identified in this bacterium: Na^+/H^+ and K^+/H^+ antiporters and the respiratory chain (Figure 5). Na^+/H^+ antiporters mediate the exchange of Na^+ with H^+ and the direction of movement depends on the sum of the concentration gradients of both ions when the exchange ratio is one. The electrical potential participates in this movement only when the exchange ratio is not one. *E. coli* generally maintains cytoplasmic sodium ions at a lower level compared to the external level and it has been proposed that the sodium ion gradient

drives Na^+/H^+ antiporters to extrude protons for cytoplasmic pH regulation (Padan et al. 2005). *E. coli* has three Na^+/H^+ antiporters. A mutant form of the bacterium deficient in all Na^+/H^+ antiporters was found to be sensitive to a high level of sodium ions in the external medium; when the level of sodium ions in the medium was low, the mutant was able to grow at the same rate as that of the wild *E. coli* type at any pH, indicating that Na^+/H^+ antiporters do not participate in cytoplasmic pH regulation and their main physiological role is to maintain cytoplasmic sodium ions at a low level (Ohyama, Igarashi, and Kobayashi 1994).

K^+/H^+ antiporters mediate the exchange of K^+ with H^+ and the direction of movement depends on the sum of the concentration gradients of both ions and the electrical potential, as with Na^+/H^+ antiporters. *E. coli* always maintains potassium ions at a higher level in the cytoplasm than the level in the surrounding medium. If K^+/H^+ antiporters could take up potassium ions with proton extrusion, the cytoplasm would be regulated at a near neutral pH under acidic conditions. This would be the best way to isolate a mutant deficient in K^+/H^+ antiporters for examining how pH regulation is mediated by them. Multiple K^+/H^+ antiporters have been proposed (Brey, Rosen, and Sorensen 1980), but their genes have not yet been identified.

E. coli has a respiratory chain and F-ATPase and ATP can be synthesized via oxidative phosphorylation under aerobic conditions (Figure 5). The respiratory chain may be involved in pH regulation. If proton extrusion through the respiratory chain regulates cytoplasmic pH in a medium with a pH below 7.5, a mutant deficient in respiratory activity would be unable to grow in acidic conditions. The respiratory chain is a complex system

consisting of multiple pathways for electron transfer and proton extrusion. For this reason, multiple mutations may be required to achieve deficiency in respiratory chain activity and, up to now, no such mutant has been isolated. The respiratory chain has a central role in energy metabolism and hence *E. coli* may be unable to survive without it.

The cytoplasmic pH is maintained within a narrow range of 7.4 to 7.6 with external pH values of 6 to 8 (Figure 6). If the respiratory chain is the main regulator of cytoplasmic pH, its activity should be at its maximum at a medium pH of 6 and decline at a medium pH of around 7.5; however, activity remains almost constant in a medium pH of 6 to 8 (Padan, Zilberstein, and Rottenberg 1976). The proton-motive force has a similar value of around -200 mV within this medium pH range and the cytoplasmic pH remains near constant. Therefore, ΔpH is 1.5 (-92.3mV) and Δφ is -100 mV at a medium pH of 6. ΔpH decreases to near zero and Δφ decreases to -200 mV with an increase in the medium pH to 8. This suggests the possibility that the maintenance of a constant cytoplasmic pH is achieved by pH-dependent interconversion of the proton-motive force from Δφ to ΔpH, which occurs at a pH of less than 7.5, until the cytoplasmic pH reaches a value of 7.5 (Bakker and Mangerich 1981).

Since the capacitance of the *E. coli* inner membrane is 0.6-0.7 μF/cm^2 (Bai, Zhao, and Asami 2006), -100 mV of Δφ is generated by the movement of a very small number of protons (thousands of protons per cell), which generates ΔpH of fewer than 0.001 pH units, based on experimental data that suggests the buffering capacity of bacterial cytoplasm is 4x10^7 protons/pH/cell (Suzuki and Kobayashi 1989). Therefore, the generation of

ΔpH requires the discharge of Δφ and interconversion of Δφ into ΔpH. *E. coli* has multiple K^+ transport systems (Figure 5) and it is possible that one or more electrogenic K^+ transporters mediate the interconversion of Δφ into ΔpH. For pH regulation by this system, the activity of the interconversion has to be active at an acidic pH and to decline at pH 7.4 and above. No K^+ transport system has shown such pH-dependent activity, ruling out the possibility that a K^+ transporter is a key regulator of pH regulation, although K^+ uptake may be involved in pH regulation via interconversion. It therefore remains unclear how the cytoplasmic pH is regulated at around 7.4 in *E. coli*. One or more unidentified systems may participate in cytoplasmic pH regulation.

The cytoplasmic pH increases proportionally to an increase in the medium pH in alkaline conditions above pH 8 and the pH difference between the outside and inside spaces of the cells is approximately 0.4 (internally acidic) (Figure 6). This pH gradient may be established by a physical power, as a protonophore that accelerates proton movement across the membranes was shown not to affect the pH gradient (Mugikura et al. 1990). There are a huge number of negatively charged large molecules, such as proteins and nucleic acids, in the cytoplasmic space, and hence the cytoplasm is negatively charged—these negatively charged molecules are impermeable across the cytoplasmic membranes, while cations, such as K^+ and Na^+, are rather permeable. This is called the Donnan potential. Positively charged protons are accumulated through the Donnan potential, resulting in the generation of a pH gradient —inside acidic—at pH values above 8 in the medium.

3. Cytoplasmic pH regulation in Enterococci

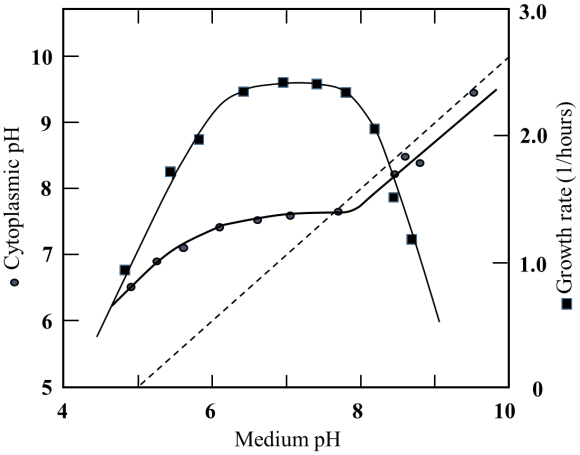

Figure 7. Cytoplasmic pH values and growth rates in *Enterococcus hirae*. Closed squares—growth rates; closed circles—cytoplasmic pH values; broken line—cytoplasmic pH equal to medium pH.

Enterococci have no respiratory chain and oxidative phosphorylation does not take place, hence ATP is produced only by glycolysis. This bacterium grows within a medium pH range of 4.5 to 9.5. The internal pH of this bacterium is almost constant in a medium pH range of 6 to 7.5 and decreases gradually at a medium pH below 6 (Figure 7).

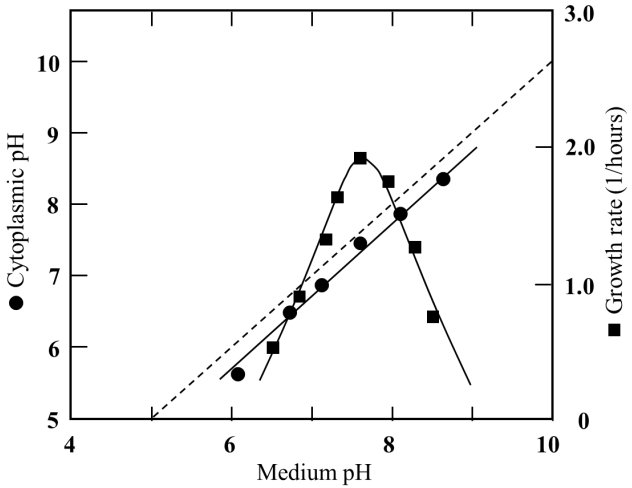

Figure 8. Cytoplasmic pH values and growth rates in *Enterococcus hirae* growing in the presence of gramicidin D. Closed squares—growth rates; closed circles—cytoplasmic pH values; broken line—cytoplasmic pH equal to medium pH.

A protonophore acts as a channel for protons, allowing them to move freely across the membranes. As can be seen in Figure 8, when gramicidin D, a protonophore, was added, the cytoplasmic pH became lower than the medium pH with a pH difference of approximately 0.4 pH units at all pH values. These data lead us to assume the presence of a regulatory system to keep the cytoplasmic space at a pH of around 7.5, which is essential for growth in acidic conditions.

To investigate this idea, various mutants deficient in the ability to grow at an acidic pH were isolated. The first mutant was isolated from *Streptococcus faecalis* (now classified as *Enterococcus hirae*) in 1980 (Kobayashi and Unemoto 1980). This mutant lacked F-ATPase activity.

This enzyme was first reported by Abrams et al. (1960) and its structure is the same as F-ATPase in *E. coli* and mitochondria (Shibata et al. 1992). As described on page 8, this enzyme catalyzes in both directions. ATP synthesis is coupled with proton movement driven by the electrochemical potential of protons, which are transported against the electrochemical potential using energy released from ATP hydrolysis in an opposite reaction. The data obtained with the mutants imply that F-ATPase functions as a regulator to keep the bacterium's cytoplasmic pH at a constant value when the medium's pH drops and its function is essential for growth under acidic conditions. This may be the reason why F-ATPase is present in bacteria without oxidative phosphorylation.

The underlying mechanism for pH regulation mediated by enterococcal F-ATPase was studied in the 1980s and has been revealed in minute detail. The first discovery was that the membrane level of this enzyme increases under acidic conditions and protons are extruded. After the cytoplasmic pH returns to around pH 7.5, the enzyme level decreases and, simultaneously, enzyme activity declines, resulting in a reduction in proton extrusion (Kobayashi et al. 1984). If the synthesis of F-ATPase was suppressed by a protein synthesis inhibitor, the cytoplasmic pH was lower than that of the untreated cells, indicating that an increase in the enzyme is essential for pH regulation (Kobayashi, Suzuki, and Unemoto 1986).

The next question is how the enzyme level increases at an acidic pH. One possibility is that gene expression is stimulated by a decrease in pH. However, the relevant gene expression was found to be almost constant at both acidic and near neutral pH values. F-ATPase consists of 8 subunits, α,

β, γ, δ, ε, a, b, and c, as described on page 7. Western blot analysis has suggested that the cellular contents of these subunits are not significantly increased by a decline in cytoplasmic pH, leading us to conclude that the assembly of the enzyme from its subunits is elevated at acidic pH values (Arikado et al. 1999, Figure 9).

Hydrolyzation of ATP is optimal at pH 6.8 and declines at around pH 7.5. Based on these observations, it can be concluded that cytoplasmic pH is regulated by the pH-dependent assembly and pH-dependent activity. The question remains as to which factor is more dominant in this regulation. To discover the answer, the best way will probably be to isolate a mutant in which enzyme activity is pH-independent or the activity to assemble the whole enzyme from its subunits does not vary within the pH range we observe; however, it is difficult to obtain such a mutant and computer simulation analysis may be the best, most practical option.

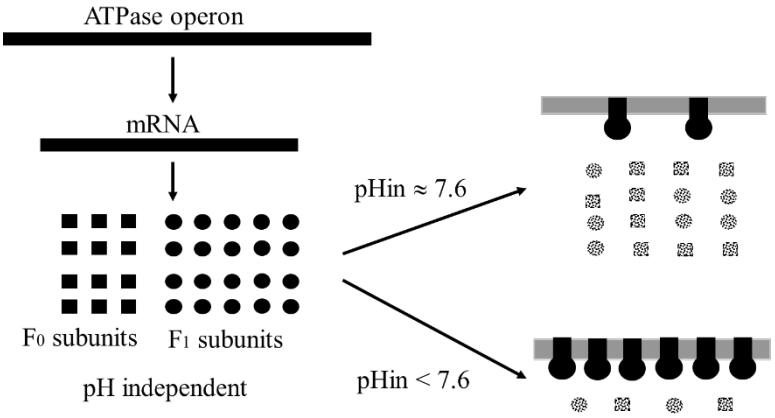

Figure 9. The mechanism for the regulation of the membrane level of F-ATPase. Symbols with dots represent subunits that are not assembled.