Bacterial responses to alkaline stress

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Studies of bacterial adaptation to alkaline pH have been less extensive to date compared with those of acidic pH. Recent development of novel methods for global analysis of gene expression under various conditions revealed that many genes were induced at high pH. These data led us to question why so many genes are required for adaptation to alkaline pH. The internal pH of bacteria growing at extremely high pH remains unclear because the methods for measuring interior acidic ΔpH developed to date are not so accurate, but it is generally accepted that cytoplasmic pH increases with medium alkalization, although the increase is lower than that of the change in medium pH. Therefore, activities of enzymes working in neutral cytoplasm may decrease with cytoplasmic alkalization under extreme alkaline conditions. Based on these findings, we propose in this article that genes whose products have an optimum activity at high pH are induced under alkaline stress to compensate for the decrease in activities of systems functioning at neutral pH.

Keywords: bacterial adaptation, alkaline stress

Introduction

There are a lot of places with extremely high pH values on the surface of the earth and bacteria inhabit such harsh places. There have been numerous reports concerning bacterial growth and survival under acidic conditions, but adaptation mechanisms to alkaline environments have remained largely unclear to date. Bacterial habitats with high pH are limited in the human body and conditions for industrial fermentation are generally acidic. These are the main reasons why research concerning bacterial adaptation to alkaline pH has been less actively studied to date. Studies on bacterial strategies to survive under alkaline environments are important for promoting our understanding of not only microorganisms, but also higher organisms. Novel

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methods for global analysis of genes induced at alkaline pH revealed that numerous genes are required for adaptation to alkaline environments.[1–2] In this article, recent advances in our knowledge of how bacteria adapt themselves to high pH stress are summarized.

Cytoplasmic concentration of protons

Bacteria are small organisms and hence their cytoplasmic volume is very small. On the assumption that *Escherichia coli* is rod shaped of 1.5 μm in length and 1 μm in diameter, the cytoplasmic volume of one cell is calculated to be 1.2×10^{-15} liter. Therefore, one cell contains 7.2 molecules of protons at pH 8 and less than one at pH above 9.

Proteins have a lot of proton binding sites and the binding protons are in the equilibrium with free protons. An enzyme's activity is dependent on the binding of protons to its specific sites and consequently enzyme activity is dependent on pH. Is protonation of proteins the same in bacterial cytoplasm at any pH value above 9 because there is no free proton in the cytoplasm? If this is true, it means that the activity of all enzymes is always the same at pH values above 9, although this is unlikely. At alkaline pH above 9, protons released from proteins are immediately bound to proteins or OH^- and the chance of enzymes binding protons released decreases with the increase in the OH^- concentration. Therefore, if protonation at a specific site activates the enzyme activity, active molecules of the enzyme decrease with increases in pH. In any case, the enzyme activity may be affected by the increase in cytoplasmic pH under alkaline environments although no free proton is present.

Measurement of cytoplasmic pH

Since bacterial cells are very small, direct measurement of cytoplasmic pH using a pH electrode is difficult and the following indirect methods have been developed. The first involves using florescence probes.[3] The second calculates internal pH based on the concentration gradient of weak bases or acids between the cytoplasm and external medium. The theoretical background and detailed method have been described in a previous report.[4] The last approach uses the chemical shift of a NMR signal which is dependent on the protonation of a probe.[5]

The problem with the first method is that the loading of fluorescence probes into the cytoplasm is difficult because bacterial membranes are usually high barriers for moving such probes across the

membranes. Furthermore, no fluorescence probe to measure pH values above 8 has been available until now. When cytoplasmic pH is lower than medium pH, a basic probe has been often used in the second method. Many measurements obtained using basic probes demonstrated that cytoplasmic pH was lower than external pH in bacteria growing at pH above 8.[6] Basic compounds charged positively are bound to cellular materials such as proteins and nucleic acids that are mostly charged negatively. Therefore, internal pH measured by this method would be lower than the true value. Internal pH was also measured using acidic probes in *E. coli* with the result suggesting that internal pH was only slightly lower than medium pH in cells growing above pH 8.[7] The binding of acidic compounds to cellular materials is negligible, but the cytoplasmic concentration of probes lower than that of the medium should be measured and hence the standard deviations are generally large.

The third method may be more reliable. In *E. coli*, internal pH was measured in a medium pH range from 5.5 to 9 and the results showed that the internal pH value varied from 7.4 to 7.8 within this range of medium pH.[5] However, *E. coli* produces a lot of acidic compounds in media with pH above 8 and hence the medium pH drops rapidly during the measurements even if a powerful buffer is used because a high concentration of cells is used for this measurement,[5] suggesting that the internal pH might be above 7.8 in a medium of pH 9. There is no report on measuring internal pH in media of pH above 9 with this method.

Therefore, it is still difficult to measure the internal pH of bacterial cells in a medium of pH above 8, so the precise value of bacterial cytoplasmic pH remains unclear. However, it is now generally accepted that the internal pH is lower than the medium pH in alkaline environments. The important point is that internal pH is not maintained at a constant value and the cytoplasm is alkalinized with the increase in external pH, even if the change in cytoplasmic pH is smaller than that of the external pH due to the internal pH regulation mechanism.[8] Therefore, bacteria may have some strategies to adapt themselves to the alkalinity of cytoplasm.

Genes required for growth at high pH

E. coli has 4288 open reading frames[9] and the functions of approximately 2000 genes still remain to be identified. Some of these unknown genes may work for growth at pH above 8, as most studies with *E. coli* to date have been carried out at pH below 8.[8]

Enterococci grew in a wide range of medium pH from 4 to 11. This species has no respiratory chain and the F-type ATPase extrudes

protons using ATP produced metabolically instead of ATP synthesis.[10] The ATPase extrudes protons to maintain internal pH in acidic media, resulting in the generation of proton motive force. [10-12] Therefore, the proton motive force is available only at acidic pH. Bacteria have numerous transport systems driven by the proton motive force and enterococci are not exceptional,[13] leading us to argue that enterococci must have transport systems operating without the proton motive force in alkaline environments. In fact, an ATP-driven extrusion system for sodium ions functions instead of a sodium/proton antiporter at high pH.[14-15] Enterococci may have other ATP-driven transport systems for nutrients and ions. [13]

Base-induced proteins of *E. coli* were revealed by two-dimensional gel electrophoresis

In agreement with the above argument, two-dimensional electrophoresis (2-D gels) analysis in combination with the *lac* fusion gene analysis revealed alkaline-inducible proteins under aerobic growth conditions.[16] The periplasmic protein YceI, outer membrane porins OmpA and MalE, and membrane-bound redox modulator DsbA were induced in extreme bases. In addition, high pH induced amino acid metabolic enzymes TnaA(tryptophan deaminase) and CysK(*o*-acetylserine sulfhydrylase A), which generate NH_3 and acids. AstD(succinylglutamic semialdehyde dehydrogenase) and GabT(γ-amino butyric acid transaminase), participating in arginine and glutamate catabolic pathways, were also expressed at a high level under alkaline stress.

Proteins with elevated expression at high pH under anaerobic conditions included metabolic enzymes and periplasmic proteins providing substrates for catabolism, in addition to stress proteins.[17] Only two of these proteins, TnaA and DsbA, are induced at high pH anaerobically as well as aerobically. High-pH induction of the glycolytic DhaKLM enzymes, which are three major components of the dihydroxyacetone kinase system, and GapA, which directs phosphorylated dihydroxyacetone to glyceraldehyde 3-phosphate, was observed. Sugar transporters MalB and MglB for the uptake of hydrolyzed glycogen and lactose were expressed at high pH. Glycolysis and fermentation of available sugars would proceed more rapidly at high pH due to the induction of these enzymes. These findings may be related to data showing that the respiratory activity decreases dramatically at high pH.[18]

Glutamate decarboxylase genes *gadA* and *gadB* were induced at high pH anaerobically, but not with aeration. GadA and GadB generate GABA, which is directed into production of succinate by GabT at high

pH.[19] Interestingly, higher expression of these genes was reported at acidic pH, as compared with the expression at pH 7.[20] Amino acid decarboxylases have been thought to alkalinize external acidic surroundings.[21] Why are GadA and GadB induced at high pH? The production of GABA might be important for metabolism at high cytoplasmic pH. An alternative explanation is that glutamate decarboxylation provides carbon dioxide that is essential for bacterial growth, as proposed previously.[22] Carbon dioxide can be supplied from air under aerobic growth conditions, while the supply is limited under anaerobic conditions. The decarboxylation of amino acids might be a useful pathway to supply carbon dioxide under anaerobic conditions, and different amino acid decarboxylases may be used under different pH conditions based on their pH-dependent activity. It can be assumed that the function of GadA and GadB is less active at neutral pH if the CO_2 supply from other systems is adequate for growth.

Alkaline shock induces the *Bacillus subtilis* δ^W regulon

DNA macroarray analysis revealed that alkaline shock caused dramatic changes in the gene expression profile, and that more than 80 genes were clearly induced at least fourfold in *B. subtilis*.[1] Many of these alkaline-inducible genes were under control of the alternative sigma factor δ^W, which belongs to the so-called extracytoplasmic function subfamily. The analysis with a *sigW* knockout strain supported the idea that a pH-upshift is a specific stressor for the δ^W regulon.[1]

Run-off transcription/macroarray analysis, a novel method to identify additional target promoters, indicated that δ^W controls genes that protect the cell against agents impairing cell wall biosynthesis, but failed to reveal any connection to operons likely to function in alkaline adaptation. As the *sigW* knockout strain showed no enhanced alkali sensitivity compared with the wild type,[2] further work should elucidate whether induction of δ^W-dependent genes is crucial for survival at alkaline pH.

Why are many genes induced at high pH?

Enhanced production of fermentation acids and consumption of bases might neutralize alkalinity and enhance survival in extremely alkaline environments, as proposed.[17,19] The change in external pH was observed only at a high density of cells *in vitro*, and there is no evidence to show that genes induced under alkaline environments

minimize the shift in external pH in natural habitats. As described above, internal alkalization decreases the activity of many enzymes through the deprotonation of proteins, so additional enzymes being active at high internal pH may be required for cellular metabolism.[8,23] Therefore, functions of individual genes induced at alkaline pH should be analyzed to understand bacterial strategies for adaptation to high pH.

Extrusion systems for potassium ions are required for growth at high pH

Bacteria have various kinds of transport systems for potassium ion accumulation. However, no specific transporter functioning at alkaline pH has been identified until now. An extrusion system for potassium ions functioning at high pH was reported in *E. coli*.[24] Enterococci had a system for potassium ion extrusion and the system was essential for growth at high pH.[25] The negativity of cellular materials increases at alkaline pH, resulting in the accumulation of potassium ions without the energy supply. In fact, the internal level of potassium ions increased with the increase in medium pH when enterococcal cells were grown in the presence of ionophores.[8] The internal level was increased by the addition of an ionophore in cells growing in a medium of pH 9.4 containing 170 mM potassium ions,[8] suggesting that the extrusion system for potassium ions was working under such conditions. Furthermore, no growth reduction of enterococci by the addition of valinomycin was observed in an alkaline medium containing 50 mM potassium ions.[8] These results suggest that the extrusion for potassium ions, rather than the accumulation of these ions, is required at high pH.

In *B. subtilis*, a putative $K^+(Na^+)/H^+$ antiporter operon (*yhaU, yufU, yufV*) was induced at alkaline shock in a more or less δ^W-independent manner.[1] This putative antiporter might be involved in pH homeostasis. As the operon was strongly induced by alkaline pH-plus salt-induced stress,[26] the physiological role of this system is thought to be extrusion of K^+ and/or Na^+ only when the medium level of these ions has increased at high pH.

Sodium/proton antiporters may participate in adaptation to alkaline environments

Sodium/proton antiporters have been proposed to have an essential role in the growth of not only alkalophilic bacteria, but also neutro-

philes.[27] *E. coli* has three systems for sodium ion extrusion and a mutant deficient in the three genes showed no detectable ability to extrude sodium ions.[28] Interestingly, such a mutant grew at the same rate as the wild type in an alkaline medium of pH above 8 containing a low level of sodium ions, although the mutant was very sensitive to sodium ions.[28] Thus, in disagreement with the previous proposal,[27] it is less likely that a sodium/proton antiporter has an essential role in pH homeostasis at alkaline pH.

The three sodium ion extrusion systems were shown to work under different environmental conditions in *E. coli*.[23] ChaA, one of the systems, functioned at alkaline pH[29] and its gene expression was induced under high osmotic stress at alkaline pH.[30]

Alkalophilic bacteria have also sodium/proton antipoters.[31] A mutant deficient in a sodium/proton antiporter derived from alkalophilic bacilli was reported to be sensitive to high pH.[32] Since numerous proteins of the mutant were different from those of its parent strain,[33–34] it may be essential to analyze the genomic sequence to conclude that the sodium/proton antiporter has an indispensable role in the growth of alkalophilic bacteria.

Regulation of tryptophan biosynthetic genes in *Bacillus halodurans*

B. halodurans is an alkaliphilic *Bacillus* species that grows optimally at above pH9.5. Recently, regulation of the genes involved in tryptophan metabolism in *B. halodurans* has been investigated and compared to the extensively studied regulatory system in *B. subtilis*.[35] *B. halodurans* and *B. subtilis* use very similar RNA binding proteins called TRAP (*trp* RNA-binding attenuation protein) to regulate transcription of the *trp* operon, but the range of regulation is far lower in *B. halodurans*; *B. halodurans* does not regulate the translation of these genes, unlike *B. subtilis*. The attenuation mechanism that controls transcription in *B. halodurans* is similar to that in *B. subtilis*, but there are some differences in the predicted RNA secondary structures in the *B. halodurans trp* leader region. These differences likely reflect the different habitats of these two bacilli.

Change in outer membrane porin proteins is required for growth under alkaline stress

Sato et al.[36] reported that the outer membrane porin proteins OmpC and OmpF were induced under hypo-osmotic stress at acidic pH, and that

these porin proteins are essential for growth under such conditions. Since the OmpC level elevated with the increase in medium osmolarity at alkaline pH, OmpC may be required for growth at high pH. In fact, a mutant deficient in *ompC* grew more slowly than the wild type in a high osmolar medium of pH above 8 (Kaeriyama *et al*., unpublished data).

Mechanisms for alkaline tolerance in *E. coli*

Foster's group investigated the ATR (acid tolerance response) mechanism by which bacteria retain the ability to survive under extreme acidic conditions.[37-38] How do bacteria become tolerant to alkaline pH? Rowbury's group found that some kinds of extracellular small substances induced alkaline tolerance.[39] The characterization of such materials has been carefully carried out by his group.[39] Two compounds have been identified so far, a protein and a non-protein component.

The pretreatment of *E. coli* cells at acidic pH increased alkaline sensitivity, but the induction was inhibited by the addition of NaCl to the pretreatment medium.[40,41] *E. coli* expressed *ompC* at a low level in an alkaline medium and the expression increased at acidic pH, as the *ompC* expression was maximum at low osmolarity in the acidic medium.[36] Why does OmpC affect alkaline tolerance? Since some small materials enhanced the alkaline tolerance as described above, transport of such materials through outer porin proteins might be involved in the tolerance.

Defense against alkaline stress through the Tor phosphorelay system in *E. coli*

TnaA tryptophanase was shown to be induced at high pH.[19] In a genome-wide transcriptional analysis to identify new targets of the TorS/TorR phosphorelay system, the *tnaLAB* operon was found to be regulated positively by this system.[42] The TorS/TorR phosphorelay system detected trimethylamine *N*-oxide (TMAO) and induced many genes under anaerobic conditions.[43-44] The survival of wild type *E. coli* under high pH stress was enhanced in the presence of TMAO, and a *tnaA* mutant showed a decrease in survival even if TMAO was present.[42] Based on these results, the authors argued that the TorS/TorR phosphorelay triggers alkaline-stress defense to limit alkalization. However, alkaline pH induction was not reported in other genes controlled by the TorS/TorR system. In any case, the TorS/TorR phosphorelay system may participate in survival under alkaline stress through the induction of *tnaA*.

Conclusion

In contrast to studies concerning acidic stress, analysis of alkaline adaptation mechanisms is still limited. Novel methods for global analysis of gene expression developed recently have revealed induction of numerous genes at alkaline pH. As argued in many papers, it is possible that high pH induction of these genes participates in pH homeostasis under alkaline stress. Bacteria have various systems for pH homeostasis,[6,45] but cytoplasmic pH was not constant in the pH range supporting bacterial growth; internal pH varies under extreme pH conditions.[8,23] Our hypothesis is that different systems with different optimum pH values are working in the cytoplasm with different pH values. Studies concerning bacterial adaptation to alkaline pH may not proceed without the characterization of individual genes induced in extreme bases. Progress is now being made with many target genes, mostly unknown genes, that will lead us to that goal. Investigation of their physiological roles will elucidate the mechanisms of bacterial adaptation to high pH.

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