The requirement of ATP metabolic processes for acidic resistance in *Escherichia coli*

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Abbreviations

AR: acidic resistance (acid resistance)

E.coli: Escherichia coli

H. pylori: Helicobacter pylori

ATP: Adenosine-5’- triphosphate

ADP: Adenosine diphosphate

AMP: Adenosine monophosphate

Crp: cyclic AMP receptor

PCR: polymerase chain reaction

RT-PCR: reverse transcription polymerase chain reaction

H-NS: histone-like nucleoid structuring protein

HU: heat-unstable nucleoid protein

SspA: the stringent starvation protein A
Summary

Since the normal human stomach averages pH 2 for approximately 2 h after it becomes empty, both commensal and pathogenic enteric bacteria have resistance systems to protect themselves against acidic stress. Four acidic resistance (AR) systems have been proposed in *Escherichia coli*. The other AR systems are amino acid-dependent system except AR1 system.

The maintenance of energy is required for many metabolic processes, including biosynthesis of cellular materials, membrane transport of ions and organic compounds, DNA repair, cell division and cell motility, and degradation of macromolecules. *E. coli* has two major energy sources, ATP and the proton-motive force. The latter is generated via the respiratory chain and used mainly for ATP synthesis and various membrane transports. Since *E. coli* can survive at low pH without the oxygen supply, ATP may be more important for survival under extremely acidic conditions.

Our research group suggested that multiple metabolic processes are required for survival under acidic conditions, and I have investigated the ATP metabolic processes under extremely acidic condition in the present research. The deletion of *purA* or *purB*, each of which encodes enzymes to produce AMP from inosinate (IMP), markedly decreased the AR. The content of ATP in these mutants decreased rapidly at pH 2.5 compared to that of the wild type. The AR was again decreased significantly by the mutation of *adk*, which encoded an enzyme to produce ADP from AMP. The DNA damage in the *purA* and *purB* mutants was higher than that in the wild type. These results demonstrated that metabolic processes that require ATP participate in survival under extremely acidic conditions, and that one such system is the ATP-dependent DNA repair system.

When I investigated the effect of nucleotides and nucleosides on AR in *E. coli*, and adenosine was found to increase the survival at pH 2.5 after cells had been adapted at pH 5.5 for 4 h. The deletion of adenosine deaminase which encoded by *add* decreased AR, and adenosine did not increase AR in the *add* mutant. The *add* expression was increased at pH 5.5 and the expression was further increased by the addition of adenosine at pH 5.5. These results indicated that adenosine induced AR in *E. coli* via the conversion into inosine and NH3 mediated by adenosine deaminase.
Introduction

Acidic resistance in enterobacteriaceae is an important aspect of bacterial survival of acid flux both within the animal host and in the natural environment. With pH values as low as 1.5–2.5 after it became empty, the stomach is one of the most inhospitable environments of the mammalian anatomy, in order to survive in the mammalian host, both commensal and pathogenic enteric bacteria have resistance systems to protect themselves against acidic stress. Many bacterial pathogens, such as *Escherichia coli*, *Salmonella Typhimurium*, and *H. pylori*, can circumvent the acid conditions of the stomach by developing adaptive mechanisms that allow these bacteria to survive in acid environments (10, 51).

Four acid resistance systems that are induced under different conditions have been proposed in *E.coli* (46). Acid resistance system 1 (AR1), which is induced in cells grown to the stationary phase, requires the sigma factor RpoS (4, 43) and the cyclic AMP receptor protein CRP (3). It had reported that the Rpos and CRP regulated the expression of these genes of AR2. However, the mechanism of AR1 is still unknown. The other three systems depend on the presence of particular amino acids. The second AR system (AR2) is glutamate-dependent system that requires two glutamate decarboxylase (GadA and GadB) and a putative glutamate:γ-aminobutyric acid(GABA) antiporper named GadC (4, 15, 49). The gadA/BC genes, activated in response to acid stress and in stationary phase cells, are subjected to complex circuits of regulation involving σ70, σS, cAMP receptor protein, H-NS, EvgAS, TorRS, GadE, GadX, GadW, and YdeO. The AR3 is arginine-dependent system, which is induced by low pH under anaerobic conditions, requires arginine decarboxylase (AdiA) and arginine/agmatine antiporter AdiC (yjdE) (11, 17). The AR 4 is lysine-dependent system, which requires lysine decarboxylase (CadA) and lysine/cadaverin antiporter (CadB) (35, 36), Acid-sensing protein CadC is a transcriptional regulator that activates the cadBA operon encoding lysine decarboxylase CadA and lysine-cadaverine antiporter CadB, under conditions of low external pH and exogenous lysine (27).

In addition to above enzymes, multiple global regulators (H-NS, CysB, SspA and HU) (13, 29, 52), small RNAs (DsrA and GadY) (26, 40), topoisomerase I (52), membrane
protein (50), and many other genes are reported to have some role in the induction of AR directly or indirectly. Furthermore, small molecules, such as indole, acetate and CO2 induce the AR (16, 53). There are multiple systems involved in survival under acidic conditions.

Adenosine-5'-triphosphate (ATP) is a multifunctional nucleotide used in cells as a coenzyme. ATP is the most important energy form to supply energy for many metabolic processes in biology, such as degradation of macromolecules, biosynthesis of cellular materials, DNA repair, cell division and cell motility (5, 6, 56). Furthermore, ATP is used as a substrate to phosphorylate proteins in signal transduction pathways and to produce the second messenger molecule cyclic AMP (20). Metabolic processes that use ATP as an energy source convert it back into its precursors. ATP is therefore continuously recycled in organisms.

ATP is produced from inorganic phosphate and ADP. ADP is biosynthesized from purine biosynthesis pathway (fig. 2-1). According to the current model of ATP synthesis in *E.coli*, ATP biosynthesis there was two main pathways: glycolysis and oxidative phosphorylation. Glycolysis generates a net two molecules of ATP through substrate phosphorylation catalyzed by two enzymes: PGK and pyruvate kinase (41). Oxidative phosphorylation, the proton-motive force across the inner membrane, generated by the respiring chain, drives the passage of protons through the membrane via the F_0 region of ATP synthase, creating ATP via the F_1 –part of ATP synthase. Under some conditions, the enzyme reaction can also be carried out in reverse, with ATP hydrolysis driving proton pumping across the membrane (9, 24).

Cells cannot function if DNA damage corrupts the integrity and accessibility of essential information in the genome (but cells remain superficially functional when so-called “non-essential” genes are missing or damage). DNA damages are physical abnormalities in the DNA, such as single- and double-strand breaks, 8-hydroxydeoxyguanosine residues, and polycyclic aromatic hydrocarbon adducts. DNA damages can be recognized by enzymes, and, thus, they can be correctly repaired if redundant information, such as the undamaged sequence in the complementary DNA strand or in a homologous chromosome is available for copying. If a cell retains DNA damage, transcription of a gene can be prevented, and, thus, translation into a protein will
also be blocked. Replication may also be blocked and the cell may die. Double-strand DNA breaks in bacteria are repaired by the RecBCD pathway of homologous recombination. The RecBCD pathway is the main recombination pathway used in bacteria to repair double-strand breaks in DNA (14, 55, 59). RecBCD pathway include a series of reactions known as branch migration, in which single DNA strands are exchanged between two intercrossed molecules of duplex DNA, and resolution, in which those intercrossed molecules of DNA are cut apart and restored to their normal double-stranded state (8).

Although these roles of ATP have been found in cells growing at under near neutral pH, ATP may have an essential role in growth and survival under extremely acidic conditions. In this study, we investigated in the role of ATP under acidic condition, and found that genes required for the ATP biosynthesis were required for AR induction, indicating that the ATP-dependent systems play an important role for *E.coli* to survive under extremely acidic condition. And we found that one of the ATP requirement DNA repair system (14), RecBCD system which required for the DNA repair after acid challenge.

It had been reported that AR2 and AR3 protect the cell from acid stress by consuming intracellular protons during each decarboxylation reaction. It was indicated that *E. coli* cells take up some of amino acid from the surroundings to survive under acidic stress if the surroundings contain such amino acid like glutamate, arginine and lysine. Bacteria generally infect to human stomach with foods, and hence an enough amount of amino acid and other nutrition would be available in the stomach. Are there other nutrition dependent acidic resistance system? In this research we found that the adenosine mediated by the adenosine deaminase (*add*) that converts adenosine to inosine and NH$_3$ have a function for *E.coli* to survive against extremely acidic stress.
Fig. 1  Biosynthesis pathway of ATP
Chapter 1

ATP requirement for acidic resistance in *Escherichia coli*

ABSTRACT

Adenosine 5’-triphosphate (ATP) participates in many cellular metabolic processes as a major substrate to supply energy. Many systems for acidic resistance (AR) under extremely acidic conditions have been reported, but the role of ATP has not been examined. To clarify whether or not ATP is necessary for the AR in *E. coli*, the AR of mutants deficient in genes for ATP biosynthesis was investigated in this study. The deletion of *purA* or *purB*, that encodes enzymes to produce AMP from IMP, markedly decreased the AR. The content of ATP in these mutants decreased rapidly at pH 2.5 as compared with the wild type. The AR was again decreased significantly by the mutation of *adk* that encoded an enzyme to produce ADP from AMP. The DNA damage in the *purA* and *purB* mutants was higher than that in the wild type. These results demonstrated that metabolic processes which require ATP participate in survival under extremely acidic conditions and that one such system is the ATP-dependent DNA repair system.
**MATERIALS AND METHODS**

**Bacterial strains and culture media**

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown at 37°C in EG medium, i.e., minimal E medium (18) containing 0.4% glucose. The medium pH was adjusted by the addition of NaOH or HCl. LB medium was also used as a rich medium. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml.

**Measurement of the AR**

The AR of the logarithmic phase cells was measured as previously described (2). After the cells had been pre-cultured overnight in LB medium with antibiotics if necessary, the cells were diluted 1000 fold with EG medium at pH 7.5 and cultured at 37°C until the optical density at 600 nm (OD$_{600}$) reached 0.3-0.4. For the adaptation to acidic pH, cells collected by centrifugation at 5000xg for 5 min were suspended with a two-fold volume of EG medium at pH5.5 and then incubated for 4 h under anaerobic culture conditions. The adapted cells were washed with fresh EG medium at pH5.5 and then suspended with 40-fold of EG medium at pH2.5. After incubation at 37°C for 1 to 2 h, the cells were diluted with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, and 1.4 mM KH$_2$PO$_4$, pH 7.4) and spread on LB agar plates. Colonies appearing after overnight culture at 37°C were counted and viability was expressed as the percentage of viable cell number to the cell number before the acidic challenge.

**Measurement of the ATP content**

After *E. coli* cells had been cultured as indicated, the cells were chilled on ice and then centrifuged at 10,000xg for 5 min at 4°C. The pellets were treated with the solution containing 20 mM glycine, 50 mM MgSO$_4$, 4 mM EDTA, and 50% methanol at pH7.4 for 30 minutes at 70°C (31), and then centrifuged at 10,000xg for 5 min. The ATP content of the supernatant was measured using a luminometer (Turner Designs, Inc) as described previously (25). Luciferase and standard ATP were purchased from Sigma Chemical Co.
**Genomic DNA damage test**

Genomic DNA damage was measured as described previously (19) with modifications. The genomic DNA was extracted using the standard method (48). One µg of the isolated chromosomal DNA was digested with *Bal*31 endonuclease (0.2 units) at 30°C for 30 min. After the enzyme had been inactivated at 75°C for 10 min, the mixture was chilled on ice, and the resulting DNA fragments were analyzed using 0.8% (w/v) agarose gel electrophoresis and ethidium bromide staining.

**Intracellular pH measurement.**

Internal pH was determined by the distribution of salicylic acid between outside and inside the cells as described previously (21). After the cells had been cultured in EG medium at pH5.5 for 4 h, the cells collected by the centrifugation at 10,000xg for 5 min were suspended in EG medium at pH5.5 or pH2.5 at approximately 1x10⁹ cells per ml, and [¹⁴C] salicylic acid (10 µM, 0.2 µCi/ml) was added as an indicator. After incubation at 37°C for the times indicated, 1 ml of the medium was centrifuged at 10,000xg for 5 min through the oil mixture (laurulbromide/liquid paraffin). The radioactivity of the supernatant and the pellet were measured to obtain the indicator concentrations outside and inside the cells, respectively. The protein amount of the pellet was measured, and the radioactivity of the pellet was divided by the water content of the pellet calculated from the protein content of the pellet. The intracellular pH was calculated by the following equation.

\[
\text{pH}_i = \log \left( \frac{[A]_{\text{in}}}{[A]_{\text{out}}} \left( 10^{\text{pKa}} + 10^{\text{pH}_{\text{out}}} \right) - 10^{\text{pKa}} \right),
\]

where \([A]_{\text{in}}\) and \([A]_{\text{out}}\) are concentrations inside and outside the cells, respectively, and the pKa of salicylic acid used was 2.89.

**Other methods.**

Transduction with P1kc (28), transformation with CaCl₂ (48), and plasmid isolation (48) were performed as described previously. Internal levels of K⁺ and Na⁺ were measured as described previously (39). Protein was measured as described previously (30), and bovine serum albumin was used as a standard.
RESULTS

The effect of the deletion of genes for purine nucleotide biosynthesis on the AR and the intracellular ATP level

_E. coli_ cells grown to the stationary phase have been mainly used for measurements of AR, and such cells may be more resistant to various stresses (44). To minimize the responses to stresses other than acidic stress in the stationary phase, cells growing exponentially were used in this study.

ATP was produced from inorganic phosphate and adenosine diphosphate (ADP) that was synthesized through metabolic pathways as described in Fig. 1-1. We first examined the effect of the deletion of genes for adenosine monophosphate (AMP) synthesis from inosinate (IMP), adenylosuccinate synthetase (purA) or adenylosuccinate lyase (purB), on the AR. Since _purA_ and _purB_ mutants were unable to grow without the addition of adenine or adenosine in EG medium at pH7.5, these mutants were cultured in EG medium at pH7.5 containing 0.1 mM adenine until OD_{600} reached 0.3. After the cells had been washed with EG medium at pH5.5, the cells were suspended in the same medium at pH 5.5 without the addition of adenine. The resulting cells grew in EG medium at pH5.5 for at least 6 h at a slower rate than the wild type (Fig. 1-2). We therefore adapted the cells at pH 5.5 for 4 h in the absence of adenine, and the survival at pH 2.5 was measured.

The survival rates after 1 h challenge at pH 2.5 were decreased 5 fold and 15 fold in _purA_ and _purB_ mutants, respectively (Fig. 1-3). The internal level of ATP was increased during the adaptation at pH 5.5 and decreased after the cells had been transferred to medium of pH 2.5 in the wild type strain W3110 (Fig. 1-4). The ATP content in the _purA_ or _purB_ mutant was lower at pH 5.5 and decreased more rapidly at pH 2.5 compared with those of W3110 (Fig. 1-4). The plasmids having _purA_ or _purB_ recovered the decrease in the AR (Fig. 1-3) and the ATP content of the deficient mutants (Fig. 1-4). When 0.1 mM adenine was added to the culture medium at pH 5.5 for the adaptation, the AR and the ATP contents of the _purA_ and _purB_ mutants were recovered (Fig. 1-3 and 1-4).

For further confirmation of the above suggestion that ATP biosynthesis is required for the AR in _E.coli_, the effect of the deletion of adnylate kinase encoded by _adk_, that synthesizes ADP from AMP, was investigated. Since adenylate kinase is indispensable
for growth, we used a temperature sensitive mutant. W3110 \textit{adk} (ts) was grown in EG medium at pH 7.5 and 30° C and then adapted in EG medium at pH 5.5 and 40° C to inactivate adenylate kinase. The acidic challenge at pH 2.5 was carried out at 37° C. After the acidic challenge, the viable cell count was carried out at 30° C. A control experiment was carried out at 30° C for all steps. The AR was markedly decreased by the inactivation of adenylyl kinase (Fig. 1-5). Since \textit{E. coli} has multiple enzymes to produce ATP from ADP, and ATP synthesis is essential for growth, the effect of the deletion of these genes could not be examined.

In contrast to genes for AMP biosynthesis, IMP dehydrogenase (\textit{guaB}) and guanosine monophosphate (GMP) synthetase (\textit{guaA}), enzymes for GMP synthesis from IMP, had no significant role in the AR (Fig. 1-5). Furthermore the deletion of enzymes for purine deoxynucleotide synthesis showed no significant effect on the AR (data not shown). The deletion of enzymes for IMP biosynthesis, such as \textit{purL}, \textit{purC}, or \textit{purK}, had no significant decrease in the AR (Fig. 1-5). The ATP level of these mutants was higher than that of the \textit{purA} or \textit{purB} mutant, but lower than that of the wild type (Fig. 1-6). These results suggested that the ATP level is important for survival at pH 2.5

**DNA damage in the \textit{purA}, \textit{purB}, \textit{adk} and \textit{recB} mutants at pH 2.5.**

The above data indicated that metabolic processes consuming ATP are required for survival at pH 2.5. Which metabolic process is required? The DNA repair system is a candidate system because cells would not grow if DNA damage corrupts the integrity and accessibility of essential information in the genome. A variety of repair strategies has evolved to avoid the loss of DNA information, and the DNA repair systems need ATP as a substrate to supply energy(56, 58, 60). Rapid DNA damage was previously observed at acidic pH in mutants deficient in genes for DNA repair systems with concomitant decrease in the AR(19), suggesting that the DNA repair process are active for survival under acidic conditions. We found that the mutant deficient in \textit{recB}, whose function requires ATP, decreased the AR of W3110 (Fig. 1-5). Furthermore, TH1559 having multiple mutations (\textit{recB}, \textit{recC} and \textit{sbcB}) brought the low survival rate at pH 2.5 (Fig. 1-5).

To clarify whether or not a low ATP level in the \textit{purA} or \textit{purB} mutant affects the DNA
repair systems, we investigated the DNA damage using Bal31 as described previously (19). Bal31 cleaves DNA at nicks, gaps, single-stranded regions, or other lesions of duplex DNA. As shown in Fig. 1-7, the DNA damage was significantly increased in purA, purB, and recB mutants as compared with the parental strain. These results indicated that maintenance of the ATP level was essential for protection against the DNA damage caused under acidic stress.

Since pH$\text{i}$ regulation was proposed to be important for survival under acidic conditions, we measured the pH$\text{i}$ of the purA and purB mutants showing low survival at acidic pH. pH$\text{i}$ was not affected significantly by the deletion of purA or purB (Table 2). The low ATP levels in these mutants may still be high enough for pH$\text{i}$ regulation under acidic conditions. Alternatively, ATP may not be essential for the pH$\text{i}$ regulation under such conditions.

**Glutamate, arginine and lysine enhanced the ATP concentration and the AR.**

We next examined whether or not ATP was required for amino acid dependent AR (AR2, AR3, and AR4). When the cells were adapted at pH5.5 and challenged at pH 2.5 in the presence of 0.5mM glutamate, arginine or lysine, the AR was increased as reported previously(10) (Fig. 1-8), and the ATP level was higher than that of the cells adapted in the absence of these amino acids (Fig. 1-9). The deletion of purA or purB decreased the AR in the presence of 0.5mM glutamate, arginine or lysine (Fig. 1-8). These results indicated that the AR2, AR3, and AR4 systems enhance the ATP-requiring systems through elevation of the ATP level.
DISCUSSION

Many systems are proposed to be involved in the AR of both logarithmic and stationary phase cells (10). The well-studied systems are amino acid-dependent systems. It has been reported that the amino acid-dependent systems enhance survival under acidic conditions via the consumption of cytoplasmic protons by amino acid decarboxylation (45). Why is such pH regulation required for the survival of non-growing cells under acidic stress? Some metabolic processes may work under such non-growing conditions. In addition to amino acid-dependent induction, many studies have demonstrated that various genes participate in AR (10). Carbon dioxide, a substrate for nucleic acid and amino acid biosynthesis, induces AR (53), leading us to assume that nucleotide biosynthesis induce the AR, but there has been no report to show the role of nucleotides in the AR. We therefore examined the participation of purine nucleotide biosynthesis in survival under acidic conditions in the present study.

The present study with cells growing exponentially revealed that the survival of the cells under acidic stress required ATP in both the presence and absence of amino acids such as glutamate, arginine, and lysine. The ATP level increased during adaptation of E. coli cells at pH 5.5 and decreased during acidic challenge at pH 2.5 (Fig. 1-3). The ATP level was low at pH 5.5 and ATP was lost rapidly at pH 2.5 in the purA and purB mutants, and these mutants showed a low survival rate at pH 2.5. The defect of the adenylate kinese activity decreased the ATP level and the AR. In contrast, the deletion of genes for IMP biosynthesis did not decrease the AR. Furthermore, the ATP levels in these mutants are higher than the purA and purB mutants, but lower than the wild type. The requirement of genes for GMP or purine deoxyribonucleotide biosynthesis was suggested to be less significant for the AR. These data implied that the ATP level is more important for survival under acidic conditions than the levels of other purine nucleotides and deoxynucleotides.

All pur mutants tested were unable to grow without the addition of adenine at pH 7.5, and hence 0.1 mM adenine was added to EG medium at pH 7.5. The experimental conditions for the acidic adaptation and challenge were the same in all pur mutants, but
the ATP levels of the purA and purB mutants were lower than the levels of the purC, purK, and purL mutants (Fig. 1-5). It may be possible that the level of IMP produced from adenine at pH 7.5 is enough for maintain the ATP level required for survival at pH 2.5 in purC, purK, and purL mutants. The other possibility might be that E. coli has alternative enzymes or pathways functioning at acidic pH instead of PurC, PurK, and PurL. The functions of over 2000 genes in E. coli are still unknown.

It is not clear why cells grown at pH 5.5 have a higher ATP level than that at pH 7.5. The greater pH gradient at pH 5.5 may account for the high level of ATP. However, the magnitude of proton motive force (the sum of the membrane potential and the pH gradient) that drives ATP synthesis may be affected little by the pH change, because the magnitude is dependent on the redox potential of respiration. Alternatively, some of the metabolic processes consuming ATP decline at low pH because of the decrease in enzyme activities. In fact, growth rate was low at pH 5.5.

ATP is a substrate to supply energy for various metabolic processes. Which ATP-dependent metabolic process supports the survival at pH 2.5? Jeong et al. (19) showed that mutants deficient in the genes required for the DNA-repair had low survivals at low pH and that mutation caused more DNA damage. We found in the present study that the single deletions of recA, recD, sbcB, urvA and urvB had no significant effect on the AR (data not shown), but the AR of the recB mutant was lower than that of the parent strain. Furthermore, multiple mutations (recB, recC and sbcB) brought about low survival at pH 2.5 (Fig. 1-5). These data confirmed the suggestion that the DNA repair system is indispensable for survival in acidic conditions. The DNA damage analysis showed that deletion of purA or purB caused more DNA damage in acidic conditions, suggesting that ATP keeps the DNA repair systems active.

E. coli has many other ATP-requiring systems such as ion transport systems and macromolecule biosynthesis. We found that the intracellular levels of Na\(^+\) and K\(^+\) decreased at acidic pH, but the levels of these ions in the purA and purB mutants were almost the same as those of their parent strain (data not shown), suggesting that the low ATP level shown in the mutants is sufficient for maintenance of the cytoplasmic levels of these cations in acidic conditions. Biosynthesis of macromolecules may not occure under non-growing conditions at pH 2.5.
It was reported that pH of the wild type was 3.6 to 3.7 in medium of pH 2.3 to 2.4 without the addition of amino acid (45). In contrast, pH was 3.7 to 4.0 under our experimental conditions (Table 2). In the previous study, the cells grown to the stationary phase were transferred to pH 2.5 medium and pH was measured. We adapted the cells in the logarithmic phase at pH 5.5 and pH was measured after the cells had been transferred to pH 2.5 medium. Therefore, cells growing logarithmically at pH 5.5 may have an increased ability to regulate pH in the absence of amino acids.

The deletion of purA did not affect pH, the deletion of purB have some effect on pH (Table 2). The mechanism for maintenance of the pH gradient remains unclear. It has been clarified that the pH gradient is generated by the F-type H+-ATPase in enterococci (22). The same mechanism might work in E. coli as proposed by Richard and Foster (45). However, the ATP hydrolysis activity of the H+-ATPase was negligible at pH less than 5 and Km for ATP was 0.6 mM in E. coli (23). 0.6 mM ATP corresponds to approximately 1.8 nmole ATP per mg protein. Therefore, the ATPase may be difficult to extrude protons at acidic pH. It may be possible that E. coli has an identified system, whose Km for ATP is less than 0.1 mM, to maintain the pH homeostasis. In any cases, the pH regulation is essential for the survival under acidic stress. In addition, our present results suggested that the ATP-dependent repair system has an essential role in the AR.
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W3110</td>
<td>$\lambda$ F- derived from <em>E. coli</em> K-12</td>
<td>(18)</td>
</tr>
<tr>
<td>BW25113</td>
<td>$lac^f, rrnB_{T14}, acZ_{W316}, hsdR514, araBAD_{AH35}, haBAD_{LD78}$</td>
<td>(7)</td>
</tr>
<tr>
<td>JW4135</td>
<td>BW25113 purA::Km$^r$(1)</td>
<td>Keio collection(2)</td>
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<td>BW25113 purB::Km$^r$</td>
<td>(42)</td>
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<td>JW2461</td>
<td>BW25113 purC::Km$^r$</td>
<td>Keio collection</td>
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<td>JW0511</td>
<td>BW25113 purK::Km$^r$</td>
<td>Keio collection</td>
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<td>Keio collection</td>
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<td>BW25113 guaB::Km$^r$</td>
<td>Keio collection</td>
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<tr>
<td>JW2788</td>
<td>BW25113 recB::Km$^r$</td>
<td>Keio collection</td>
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<td>TH1559</td>
<td>K12 recB recC sbcB</td>
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<td>IH125</td>
<td>W3110 adk(ts)</td>
<td>NIG(2)</td>
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<td>W3110 purA::Km$^r$</td>
<td>This study, W3110xP1(JW4135)</td>
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<td>SE1929</td>
<td>W3110 recB::Km$^r$</td>
<td>This study, W3110xP1(JW2788)</td>
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<td><strong>Plasmid</strong></td>
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<td>ppurA</td>
<td>pNTR-SD-ppurA</td>
<td>mobile plasmid collection(2)</td>
</tr>
<tr>
<td>ppurB</td>
<td>pNTR-SD-ppurB</td>
<td>mobile plasmid collection</td>
</tr>
</tbody>
</table>

(1)$\text{Km}^r$, resistant to kanamycin.

(2)Obtained from the National BioResource Project (NIG, Japan): *E. coli*
Table 2. The cytoplasmic pH (pHi)

<table>
<thead>
<tr>
<th>Strains</th>
<th>pHo = 5.5</th>
<th>pHo = 2.5</th>
</tr>
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<tr>
<td></td>
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<td>15 min</td>
</tr>
<tr>
<td>W3110</td>
<td>7.12 ± 0.09</td>
<td>4.25 ± 0.03</td>
</tr>
<tr>
<td>W3110 purA</td>
<td>7.05 ± 0.06</td>
<td>4.18 ± 0.05</td>
</tr>
<tr>
<td>W3110 purB</td>
<td>7.04 ± 0.09</td>
<td>4.11 ± 0.06</td>
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</tbody>
</table>

After the cells had been grown in EG medium at pH 7.5 until OD₆₀₀ reached 0.3, the cells were suspended with a two-fold volume of EG medium at pH5.5 and then cultured for 4 h. The cells were incubated with the indicator in EG medium at pH 5.5 or 2.5 for the times indicated, and then pHi was measured as described in Materials and Methods. pHo is the pH value of the medium.
Fig. 1-1. Metabolic pathway for purine nucleotide synthesis.
Fig. 1-2. Growth of mutants at pH 5.5 under anaerobic conditions. After W3110 and mutants indicated had been grown in EG medium at pH 7.5 until OD_{600} reached 0.3, the cells were harvested and suspended in EG medium at pH 5.5. The optical density was measured at time intervals indicated. For growth of pur mutants, 0.1 mM adenine was added only to medium at pH 7.5. Symbols: ⬤, W3110; △, W3110 purA; ▽, W3110 purB; □, w3110 purC; ⬤, W3110 purK.
Fig. 1-3. Survival of *purA* and *purB* mutants at pH 2.5. After the cells had been grown in EG medium at pH 7.5 until OD$_{600}$ reached 0.3, the cells were harvested and suspended in EG medium at pH 5.5. After the cells had been grown for 4 h at pH 5.5 under anaerobic conditions, the cells were challenged at pH 2.5 for 1 and 2 h under anaerobic conditions, and the viable cells were counted. Adenine (0.1 mM) was added to EG medium of pH 7.5 for the growth of *purA* and *purB* mutants. Adenine was also added at pH 5.5 but not at pH 2.5 in the experiments indicated by asterisks. Isopropyl -β-D-thiogalactopyranoside (0.25mM) was added when the cells containing plasmids were cultured. Symbols: open bars, W3110; grey bars, W3110 *purA*; black bars, W3110 *purB*; dotted bars, W3110 *purA* containing *ppurA*; striped bars, W3110 *purB* containing *ppurB*. The average values and standard deviations obtained from three experiments using separate culture are represented.
Fig. 1-4. The ATP content of *purA* and *purB* mutants at pH 5.5 and 2.5. The same strains as those used in Fig. 1-3 were cultured as described in the legend of Fig. 1-3, and harvested at the indicated times. The ATP contents were measured as described in Materials and Methods. Symbols are the same as for Fig. 1-3. The average values and standard deviations obtained from three experiments using separate culture are represented.
Fig. 1-5. Survival of various mutants at pH 2.5. W3110, W3110 guaA, W3110 guaB, W3110 purL, W3110 purC, W3110 purK, W3110 recB, TH1559 (recB, recC, sbcB), and W3110 adk(ts) were used. After the cells had been grown in EG medium at pH 7.5 until OD$_{600}$ reached 0.3, the cells were harvested and suspended in EG medium at pH 5.5. After the cells had been grown for 4 h at pH 5.5, the cells were challenged at pH 2.5 for 1 h, and the viable cells were counted. Adenine (0.1 mM) was added only to medium of pH 7.5 for the growth of the pur mutants. In the experiments indicated by “40°C”, the cells were cultured at 30°C in medium at pH 7.5, adapted at 40°C in medium at pH 5.5, and challenged at 37°C in medium at pH 2.5. After the acidic challenge at pH 2.5, the cells were cultured on LB agar plates at 30°C. In the experiments indicated by “30°C”, the cells were cultured at 30°C for all steps. The average values and standard deviations obtained from three experiments using separate culture are represented.
Fig. 1-6. The ATP content of various mutants at pH 5.5 and 2.5. W3110, W3110 purA, W3110 purB, W3110 purL, W3110 purC, and W3110 purK were used. The cells were cultured as described in the legend for Fig. 1-5, and harvested at the indicated times. The ATP contents were measured as described in Materials and Methods. The average values and standard deviations obtained from three experiments using separate culture are represented.
Fig. 1-7. DNA damage of *purA*, *purB*, *recB* mutants. W3110, W3110 *purA*, W3110 *purB*, and W3110 *recB* were cultured as described in the legend for Fig. 1-3. After the cells had been challenged at pH 2.5 for 1 and 2 h, the cells were harvested. The cells before the acidic challenge were also harvested, and the DNA fragmentation of these cells was analyzed as described in Materials and Methods.
Fig. 1-8. Effect of amino acids on the AR of the wild type and mutants. After W3110, W3110 purA, and W3110 purB had been grown as described in the legend for Fig. 1-3, the cells were challenged at pH 2.5 for 1 h, and the viable cells were counted. Amino acids (0.5 mM) were added to EG media of pH 5.5 and 2.5. The average values and standard deviations obtained from three experiments using separate culture are represented.
Fig. 1-9. Effect of amino acids on the ATP level of the wild type. W3110 cells were cultured as described in the legend for Fig. 1-8, and the ATP contents were measured at the times indicated. The average values and standard deviations obtained from three experiments using separate culture are representing.
Chapter 2

Adenosine increases acidic resistance in *Escherichia coli* requirements adenosine deaminase

ABSTRACT

Acid resistance (AR) in *Escherichia coli* contributes to the persistence in its host and is thought to promote passage through the gastric barrier of humans. Adenosine taken up by *E. coli* is converted to either adenine or inosine and then utilized. In this study, adenosine was found to play a role in survival of *E. coli* under extreme acid stress. Deletion of add, a gene encoding adenosine deaminase that converts adenosine to inosine and NH$_3$, attenuated the AR induced by the addition of adenosine. The addition of adenosine increased intracellular pH of *E. coli* cells in the pH 2.5 medium. The addition of inosine or adenine did not increase the AR. These results implied that adenosine was used to survive under extremely acidic conditions and requirement the adenosine deaminase.
MATERIALS AND METHODS

Strains and growth media

The bacterial strains used in this study are listed in Table 1. *E. coli* was grown at 37°C in minimal E medium (57) containing 0.4% glucose that was designated EG medium. LB medium was used as rich medium. The pH of EG medium was adjusted at 7.5, 5.5 and 2.5 by the addition of NaOH or HCl. Kanamycin (25 µg/ml) was added when resistant strains to kanamycin were cultured.

Acid resistance (AR) assay.

The AR was measured as described previously (2, 54). After cells had been pre-cultured overnight in LB medium, the cells were diluted 1,000-fold with EG medium at pH 7.5 and cultured at 37°C until OD_{600} reached 0.3 to 0.4. The resulting cells were used for the measurement of the AR of unadapted cells. For the adaptation to acidic pH, the cells were collected by the centrifugation at 5,000xg for 5 min and then suspended with 2-fold volume of EG medium at pH 5.5. After incubation for 4 h under anaerobic culture conditions, the cells were used for AR measurement of the adapted cells. For AR measurement, the cells were suspended with 40-fold of EG medium at pH 2.5. After incubation at 37°C for 1 h, the cells were diluted with phosphate-buffered saline (pH 7.4) and spread on LB plates. After overnight culture at 37°C, colonies appeared were counted and viability was expressed as the percentage of viable cell number to the number before the acid challenge at pH 2.5.

Measurement of the ATP content

The cells cultured as indicated above were chilled on ice and collected by the centrifugation at 10,000xg for 5 min at 4°C. The cells were treated with hot methanol solution containing 0.02M glycine, 0.05M MgSO₄, 0.004M EDTA, and 50% methanol at pH 7.4 for 30 min at 70°C (31, 54), and centrifuged at 10,000xg for 5 min. The ATP content of the supernatant was measured using a luminometer as described previously (25). Luciferase and ATP were purchased from Sigma Chemical Co.
**Measurement of the mRNA level**

The cells were cultured at pH 7.5 in EG medium until OD$_{600}$ reached 0.3 to 0.4, collected by the centrifugation at 10,000xg for 5 min, and transferred to EG medium at pH 5.5. After 1 h culture, total RNA was isolated using TRI reagent (Sigma) according to the manufacturer’s protocol, and the reverse transcriptase-polymerase chain reaction was carried out as described previously (1, 34). The PCR primers used were listed in Table 1.

**Intracellular pH measurement**

Internal pH was determined by the distribution of salicylic acid between outside and inside of cells as described previously (21, 32, 54). After the cells had been cultured as indicated, the cells were collected by the centrifugation at 10,000xg for 5 min, suspended in EG medium at pH 5.5 or pH 2.5 at approximately 1x10$^9$ cells per ml, and cultured at 37°C. $[^{14}C]$ salicylic acid (10µM, 0.2µCi/ml) was added at indicated time. After 15 min, 1 ml of the medium was centrifuged at 10,000xg for 5 min through the oil mixture (laurulbromide/liquid paraffin). The radioactivities of the supernatant and the pellet were measured to obtain the indicator concentrations of outside and inside of cells, respectively. The water space of pellet was measured and the radioactivity of the pellet was divided by the water content of cells. The intracellular pH was calculated by the equation: $pHi = \log\{(\frac{[A]_{in}}{[A]_{out}})(10^{pK_a} + 10^{pH_{out}}) - 10^{pK_a}\}$, where $[A]_{in}$ and $[A]_{out}$ are concentrations of inside and outside of cells, respectively. pKa of salicylic acid used was 2.89.

**Other methods**

P1 transduction with P1kc (28) and the measurement of protein (30) were carried out as described previously. Bovine serum albumin was used as a standard.
RESULTS

Increase in the AR of *E. coli* by adenosine

When we studied the ATP biosynthesis pathway under acidic condition, we investigated the effect of purine nucleotides and nucleosides on the AR of *E. coli*, and adenosine was found to increase the survival of cells about 2 folds or 3 folds at pH 2.5 for 1 h or 2 h after cells had been adapted at pH 5.5 for 4 h (Fig. 2-1 and Fig. 2-3). When the cells grown at pH 7.5 were transferred directly to the pH 2.5 medium and challenged for 1 h, the addition of adenosine to the pH 2.5 medium increased the AR (Fig. 2-2). Furthermore, the AR was further increased by the addition of adenosine to both pH 7.5 and 2.5 media (Fig. 2-2). When we increase the concentration of adenosine from 0.1 mM to 0.5 mM, the viability of cells have no big different between the different concentration(Fig. 2-3).

As shown in Fig. 2-4, adenosine is converted into adenine by membrane-associated purine-nucleoside phosphorylase encoded by *deoD* or into inosine by adenosine deaminase encoded by *add* in *E. coli* (38, 42, 61). Neither adenine nor inosine increased the AR (Fig. 2-1), indicating that the adenosine increased the AR may not because to produce the substrate for purine biosynthesis pathway.

Decrease in the AR by the deletion of *add*

Based on the exogenous adenosine metabolic pathway (Fig. 2-4), we investigated the AR of gene deletions in the pathway. We found that the deletion of *add* decreased the AR, and adenosine did not increase the AR in the *add* mutant (Fig. 2-4). The deletion of *deoD* increased somewhat the AR both with and without the addition of adenosine (Fig. 2-4). The deletion of *gsk* encoding inosine/guanosine kinase did not decrease the survival of *E.coli*, and the addition of adenosine increased the AR little in the *gsk* mutant (Fig. 2-4), probably due to the accumulation of inosine that attenuated the conversion of adenosine into inosine or inhibit the activity of some enzymes in purine biosynthesis pathway.

These data imply that the pathway mediated by adenosine deaminase encoded by *add* increases the survival under acidic stress and the adenosine increase the AR requirement adenosine deaminase. The deletion of *deoD* increased a little survival of cells compared
the wild type strain W3110, further confirmed that adenosine metabolic mediated by the adenosine deaminase may usefully for cell to against the acidic stress. The deletion of add decrease AR seemed that endogenous adenosine also has important role in AR.

Effects of adenosine on intracellular pH (pHi) and ATP content.

It has been reported that one of the reasons to account the increase in the AR by amino acids is the increase in pHi via amino acid decarboxylation. We next examined whether the conversion of adenosine into inosine and NH3 increase pHi or not. The addition of adenosine increased pHi at 0.02 and 0.3 pH units in pH 5.5 and 2.5 media, respectively (Table 2). Both adenosine and adenine decreased the ATP level at pH 5.5 and 2.5 (Fig. 2-5). The ATP level may still high enough for *E.coli* for acidic resistance compared to the purA and purB mutants at acidic condition (54). The reason for the decrease remains unclear, but it was suggested that the increase in the AR by adenosine was not caused by the elevation of the ATP level.

The expression of add at acidic pH

It found that the add expression was increased at pH 5.5 and the expression was further increased by the addition of adenosine at pH 5.5 (Fig. 2-6). The results indicate that the expression of add was increased during the adaptation at pH 5.5 and that elevated adenosine deaminase participates in the survival under acidic environments containing adenosine. That may was one of the reasons why the pH 5.5 adaptation could increase the survival of *E.coli* under acidic stress.
DISCUSSION

In the present study, we found that the adenosine increased the AR and requirement adenosine deaminase. How does adenosine increase the survival under acidic conditions? There are several possible explanations. One is that the NH₃ production increases pHᵢ via the conversion of NH₃ into NH₄⁺. It had reported neutralization of gastric acid with NH₃ produced by the enzyme of urease might allow the bacterium to survive in the acidic milieu in the *H. pylori* (33). The pKa of adenosine is about 3.5 (47), and the pKa of inosine is about 8.7 (12), so the pHᵢ could be changed by the adenosine deamination.

In fact, pHᵢ was increased by the addition of adenosine. However, the increase in pHᵢ was only 0.3 pH units at pH 2.5 (Table 2), and it remains unclear why such small alkalization of cytoplasm increases the AR. NH₃ may be used for the production of a metabolite which increases the AR. For example, glutamate is synthesized from α-ketoglutarate and NH₃. Arginine is synthesized from carbamoylphosphate via urea cycle, and carbamoylphosphate is synthesized from NH₃, CO₂, and ATP. These amino acids increase the AR. NH₃ is an essential substrate for nucleotide synthesis such as ATP, but ATP level was not increased by the addition of adenosine, indicating that this possibility is less likely. In any cases, our present data clearly demonstrated that the nucleosides increase the AR of *E.coli*.

Many AR systems are proposed to function in both logarithmic and stationary phase cells (10, 50). The well-studied systems are amino acid-dependent systems that convert amino acids into amines and CO₂. It has been assumed that the conversion increases not only the internal pH but also alkalinize the surroundings via exchange of external amino acids with amines, but the internal pH was not the only factor to induce the AR (45). Our group found previously that CO₂ induced the AR (53), suggesting that besides amino acid decarboxylation, some other metabolisms using CO₂ as a substrate are involved in the survival under acidic conditions. In addition, our results demonstrated the involvement of the adenosine deamination in the survival under acidic stress. Contrary to our initial expectation, huge metabolisms may be working for survival under extremely acidic conditions.

It was suggested in the present study that *E. coli* cells take up adenosine from the
surroundings to survive under acidic stress if the surroundings contain adenosine. Is adenosine or nucleosides available in the natural habitat for *E. coli*? Bacteria generally infect to the human stomach with foods, and hence an enough amount of adenosine would be available in the stomach. The *add* expression was shown to increase during the adaptation at pH 5.5. Therefore, adenosine-dependent AR may be a useful strategy to survive under acidic stress in the human stomach.
Table 1. Bacterial strains and plasmids used in this study

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<td>BW25113</td>
<td>(lacIq) (rrnB_{T14}) (\Delta{\lambda{W316}}) (hsdR_{514}) (\Delta{\lambda{BAD}<em>{H33}}) (\Delta{\lambda{rhaBAD}</em>{D78}})</td>
<td>(7)</td>
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<tr>
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**Primers**

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<tr>
<td>Add-r</td>
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<tr>
<td>16sRNA-f</td>
<td>GATCATGGGCTCAGATTGAACG</td>
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<tr>
<td>16sRNA-r</td>
<td>CTACCCTTCCAGTTGTGTCC</td>
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¹\(Km^r\) is the gene conferring resistance to kanamycin

²Keio collections were obtained from the National BioResource Project (National Institute of Genetics, Mishima, Japan): *E. coli*. 


<table>
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<th>Strains</th>
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</table>

pHo, medium pH.
Fig. 2-1. Effects of adenosine, adenine, and inosine on the AR. After W310 cells had been grown in EG medium at pH 7.5 until the OD_{600} reached 0.3 to 0.4, the cells were harvested and suspended in EG medium at pH 5.5. After cells had been grown for 4 h at pH 5.5, cells were challenged at pH 2.5 for 1 h, and the viable cells were counted. Adenine (0.1 mM), adenosine (0.1 mM), or inosine (0.1 mM) was added to EG medium of pH 5.5 and 2.5.
Fig. 2-2. Survive of the unadapted cells with and without adenosine. After W3110 cells had been grown in EG medium at pH 7.5 until the OD$_{600}$ reached 0.3 to 0.4, the cells were harvested and suspended in EG medium at pH 2.5. The cells were challenged at pH 2.5 for 1 h, and the viable cell number was counted. Adenosine (0.1 mM) and adenine (0.1 mM) were added to EG medium of pH 7.5 and 2.5 as indicated.
Fig. 2-3. Acid Survival of W3110 with different concentration adenosine. After W3110 cells had been grown in EG medium at pH 7.5 until the OD_{600} reached 0.3 to 0.4, the cells were harvested and suspended in EG medium at pH 2.5. The cells were challenged at pH 2.5 for 1 h and 2 h, and the viable cell number was counted. Adenosine 0.1 mM and 0.5 mM were added to EG medium of pH 7.5 and 2.5 as indicated.
Fig. 2-4. Metabolic pathways of adenosine degradation. Genes: *add*, adenosine deaminase; *deoD*, purine-nucleoside phosphorylase; *gsk*, inosine/guanosine kinase; *apt*, adenine phosphoribosyltransferase.
Fig. 2-5. The AR of various mutants with and without adenosine. After mutants cells had been grown in EG medium at pH 7.5 until the OD$_{600}$ reached 0.3 to 0.4, the cells were harvested and suspended in EG medium at pH 5.5. After grown for 4 h at pH 5.5, the cells were challenged at pH 2.5 for 1 h, and the viable cell number was counted. Symbols: white bars, no addition; black bars, adenosine (0.1 mM) was added to EG medium of pH 5.5 and 2.5.
Fig. 2-6. Effect of adenosine and adenine on the ATP level. W3110 cells were cultured as described in the legend of Fig. 2-1, and the ATP content was measured as described in Materials and Methods. Symbols: white bars, no addition; gray bars, adenine (0.1 mM) was added; black bars, adenosine (0.1mM) was added. Both adenine and adenosine were added to both EG medium of pH 5.5 and 2.5.
Fig. 2-7. The mRNA level of *add*. W3110 cells cultured in EG medium of pH 7.5 and 5.5 with and without 0.1 mM adenosine, and the mRNA level of *add* was measured as described in Materials and Methods.
Conclusion

We investigate the purine biosynthesis pathway under extremely acidic condition in the research. It had found that part genes of the purine biosynthesis pathway for ATP biosynthesis have some effect on the AR. The deletion of purA or purB, that encodes enzymes to produce AMP from IMP, markedly decreased the AR. The content of ATP in these mutants decreased rapidly at pH 2.5 as compared with the wild type. The AR was again decreased significantly by the mutation of adk that encoded an enzyme to produce ADP from AMP. And the content of ATP also decreased by the deletion of adk. The DNA damage in the purA and purB mutants was higher than that in the wild type. These results demonstrated that metabolic processes which require ATP participate in survival under extremely acidic conditions and that one such system is the ATP-dependent DNA repair system.

It was found in this study that adenosine had a function to survive against extremely acidic stress. The deletion of add encoding adenosine deaminase that converts adenosine to inosine and NH₃ attenuated the AR induced by the addition of adenosine. The addition of adenosine increased intracellular pH of E. coli cells in the pH 2.5 medium. The addition of inosine or adenine did not increase the AR. These results implied that adenosine was used to survive under extremely acidic conditions via the production of NH₃.


disulfide bond formation system in aerobically growing *Escherichia coli* cells. Proc Natl Acad Sci U S A **94**:11857-11862.


58. **Wagner, K., G. F. Moolenaar, and N. Goosen.** 2010. Role of the two ATPase domains of *Escherichia coli* UvrA in binding non-bulky DNA lesions and interaction with UvrB. DNA Repair (Amst) 9:1176-1186.


List of publications

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Judgment of the possibility for the application to Doctoral Thesis was performed by following members of Doctoral Dissertation Committee in Graduate School of Pharmaceutical Sciences at Chiba University.

Chief examiner: Professor Naoto Yamaguchi

Assistant examiner: Professor Tomoko Yamamoto

Assistant examiner: Professor Toshihiko Murayama