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Accumulation of Glutamate by Osmotically Stressed *Escherichia coli* Is Dependent on pH

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In the present study, we measured the accumulation of glutamate after hyperosmotic shock in *Escherichia coli* growing in synthetic medium. The accumulation was high in the medium containing sucrose at a pH above 8 and decreased with decreases in the medium pH. The same results were obtained when the hyperosmotic shock was carried out with sodium chloride. The internal level of potassium ions in cells growing at a high pH was higher than that in cells growing in a neutral medium. A mutant deficient in transport systems for potassium ions accumulated glutamate upon hyperosmotic stress at a high pH without a significant increase in the internal level of potassium ions. When the medium osmolarity was moderate at a pH below 8, *E. coli* accumulated γ-aminobutyrate and the accumulation of glutamate was low. These data suggest that *E. coli* uses different osmolytes for hyperosmotic adaptation at different environmental pHs.

Since bacterial habitats encompass a wide range of environmental conditions, bacteria are useful species for investigating the environmental adaptation of living things. Osmolarity of growth medium is one of the important conditions for bacterial growth, and there have been numerous reports concerning bacterial adaptation to changes in external osmolarity (1).

Many materials have been identified as being accumulated after hyperosmotic shock in order to increase the cytoplasmic osmolarity or to protect cellular metabolism under high osmotic pressure. For example, potassium ion, proline, betaine, trehalose, glutamate, glutamine, glutamyl peptide, and taurine, etc., have been reported to function as compatible solutes or osmoprotectants (1, 5–8). Some of them are probably not absolutely essential for osmotic adaptation, but they may increase the growth rate of hyperosmosis-stressed cells. Thus, in order to clarify the mechanism of bacterial osmotic adaptation, examination of the minimum requirement for the adaptation would be required.

Potassium glutamate is known to be a major osmolyte of *Escherichia coli* growing in hyperosmotic minimal medium (1, 7). In the present study, we investigated the accumulation of glutamate at various pH values of the minimal medium and found that the internal level of glutamate was increased at a pH above 8. γ-Aminobutyrate was accumulated when the glutamate accumulation was low upon moderate hyperosmotic shock at a pH below 8. Thus, *E. coli* seems to use different osmolytes at different pH levels.

MATERIALS AND METHODS

Bacterial strains and growth media. *E. coli* W3110 and TK2642 [F^- *thi lac rha trkA405 trkD1* del(*kdpABC*)*54 nagA malT*] were generously supplied by Y. Anraku (Tokyo University) and W. Epstein (Chicago University), respectively. *E. coli* was grown at 37°C in minimal medium containing 5 mM K_2PO_4, 20 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1% glucose, and thiamine (2 μg/ml). Tricine [tris(hydroxymethyl)methylglycine] (60 mM) or HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) was used as a buffer, and the medium pH was adjusted by the addition of KOH. Growth was monitored by measuring the A_{600} of the culture medium.

Analysis of amino acids, γ-aminobutyrate, and potassium ions. Amino acids and γ-aminobutyrate were extracted from cells by heat treatment with 5% trichloroacetic acid, and their contents in the extract were analyzed with an amino acid analyzer (Hitachi 835) as described previously (10). The standard deviation of this measurement was less than 10%. In some experiments, glutamate and γ-aminobutyrate were also analyzed by high-performance liquid chromatography (Toso Co., Tokyo, Japan) by the same method as the polyamine determination described previously (3), except that the elution buffer used contained 0.1 M citrate, 0.5 M NaCl, 0.01% *N*-caproic acid, and 0.1% Brij 35, pH 3.5. The amounts measured by this method were close to the values obtained by the first method; the difference was less than 10%. The peak of γ-aminobutyrate was identified by two analysis programs, using different buffers. Furthermore, γ-aminobutyrate was added to the cellular extract as an internal standard, and the resulting mixture was analyzed.

For the measurement of potassium ion content, 1.5 ml of growth medium was centrifuged, and the potassium ion content of the pellet was measured as described previously (10).

Other methods and chemicals. Protein was determined as described previously (4). Reagents used were of analytical grade.

RESULTS AND DISCUSSION

Ohyama et al. (10) reported that *E. coli* can adapt itself to a hyperosmotic medium with a negligible proton motive force in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and that glutamate was accumulated similarly in the presence and in the absence of this drug. We measured again the accumulation of glutamate of cells stressed osmotically by 0.6 M sucrose in the presence and in the absence of CCCP, and the results sometimes showed that the hyperosmosis-stressed cells accumulated a smaller amount of glutamate in the presence of CCCP than in its absence. The medium pH decreases drastically during the growth of *E. coli* in medium containing glucose and CCCP. We therefore assumed that this discrepancy was due to the difference in medium pH when the cells were harvested and carried out the following experiments to confirm this.

The accumulation of glutamate by cells growing in the presence of 0.4 M sucrose varied considerably with changes in the medium pH (Table 1). When *E. coli* was stressed osmotically by 0.6 M sucrose, the accumulation of glutamate was again dependent on pH (Table 1). Since the medium pH was adjusted by KOH, the medium osmolarity was high at an alkaline pH; the growth media with pH values 7.3 and 8.5 contained approximately 180 and 220 mosM solutes, respectively. This small change in medium osmolarity would have little effect on

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TABLE 1. Accumulation of glutamate and γ-aminobutyrate in cells growing in the presence of sucrose[a]

Growth condition					Cellular content (nmol/mg of protein)	
Sucrose (M)	KCl (mM)[b]	A_{600}[c]	pH[d]			
			Start	End	Glu	GABA
In the absence of CCCP						
—[e]	—	0.27	8.5	8.3	54.5	5.7
—	—	0.27	8.0	7.8	22.6	13.2
—	—	0.23	7.3	7.2	8.3	35.8
0.4	—	0.40	8.5	7.9	258	151
0.4	—	0.19	8.0	7.8	59.1	170
0.4	10	0.47	8.0	7.5	87.8	153
0.4	—	0.80[f]	8.0	7.3	130	125
0.4	—	0.26	7.3	7.1	70.7	116
0.4	20	0.26	7.3	7.1	96.1	74.8
0.4	—	0.85[f]	7.3	6.2	47.8	98.4
0.6	—	0.41	8.5	8.0	566	62.1
0.6	—	0.37	8.0	7.6	247	110
0.6	—	0.38	7.3	6.7	188	88.9
In the presence of 0.1 mM CCCP						
0.4	—	0.20	8.5	8.2	237	42.6
0.4	—	0.19	8.0	7.6	87.1	176
0.4	—	0.26	7.3	7.0	17.5	148

[a] *E. coli* W3110 was grown in the medium containing Tricine. After the A_{600} reached 0.2 to 0.3, the cells were shocked osmotically by the addition of high-osmolarity medium and allowed to grow for another 3 to 5 h. For the first three entries in this table, precultured cells were inoculated at the A_{600} of 0.02 to 0.05 and the cells were cultured for 3 to 5 h. After the cells were harvested, the cellular contents of glutamate (Glu) and γ-aminobutyrate (GABA) were measured as described in Materials and Methods, and the data obtained by a single assay are represented.

[b] KCl was added to the medium in order to adjust its osmolarity to the value at pH 8.5.

[c] A_{600} of the growth medium when the cells were harvested. The growth medium at the A_{600} of 0.2 contained approximately 10^8 cells per ml.

[d] The medium pH "start" and "end" values were measured soon after the hyperosmotic shock and at the time the cells were harvested, respectively. For the first three entries in this table, the pH start values were measured soon after the cells were inoculated.

[e] —, no addition.

[f] Cells were grown for 10 h after hyperosmotic shock and harvested at the late exponential growth phase.

the accumulation of internal osmolytes. In fact, when the accumulation of glutamate was measured using the same osmolar medium at different pH values, the data showed again that the accumulation was dependent on pH (Table 1). The stimulation of glutamate accumulation at a high pH was observed when *E. coli* was stressed osmotically by sodium chloride (Table 2). Our data suggested that the growth phase affected the accumulation of glutamate (Table 1), but its effect was little in comparison to the effect of pH. As expected, the dissipation of the proton motive force by CCCP had little effect on the accumulation of glutamate, and the accumulation was again high at a pH above 8 in the presence of this drug (Table 1). These results demonstrated that the accumulation of glutamate is dependent on pH.

Accumulations of other amino acids were comparatively insignificant after hyperosmotic shock (Fig. 1). However, γ-aminobutyrate was accumulated in cells stressed osmotically (peak 3 of Fig. 1). It should be noted that peak 1 in Fig. 1 is different from any peak of an amino acid. Cells stressed by 0.4 M sucrose accumulated a greater amount of this compound than cells growing in the presence of 0.6 M sucrose (Table 1). The same result was also obtained when sodium chloride was used as the osmolyte; a large accumulation of γ-aminobutyrate was observed in the presence of 0.2 M sodium chloride, especially at a pH below 8, and the accumulation was lower in the presence of 0.3 M sodium chloride (Table 2). When *E. coli* was grown in the presence of 0.5 M sodium chloride, the internal level of γ-aminobutyrate was close to the value for cells grown without the addition of sodium chloride (Tables 1 and 2). These results suggested that γ-aminobutyrate was accumulated instead of glutamate when the osmotic pressure was moderate, at less than 0.3 M sodium chloride and at a pH below 8.

In 1975, it was first reported that γ-aminobutyrate was accumulated after hyperosmotic stress in *E. coli* grown in complex medium (8). There has been no report to suggest the accumulation of γ-aminobutyrate in a synthetic medium until our present work. As described above, a large accumulation of γ-aminobutyrate was observed in the presence of 0.2 M sodium chloride, and its accumulation was very low when the concentration of sodium chloride was increased to 0.5 M (Table 2). In previous studies, the accumulation of osmolytes has often been observed in cells shocked by high osmolarity above 0.5 M sodium chloride at or near a neutral pH. This may account for the discrepancy between our present data and the previous observations.

An important question is whether the accumulation of glutamate is regulated by internal pH or by medium pH. Since we used a medium without the addition of glutamate, glutamate was obviously synthesized by the intracellular metabolism of cells growing in the hyperosmotic medium. It has been reported that internal pH stays at a level between 7.4 and 7.8 within a wide range of medium pH from 5.5 to 9.0 (11). In contrast, Mugikura et al. (9) have reported that the internal pH of growing *E. coli* cells is 7.2 to 7.4 within the range of medium

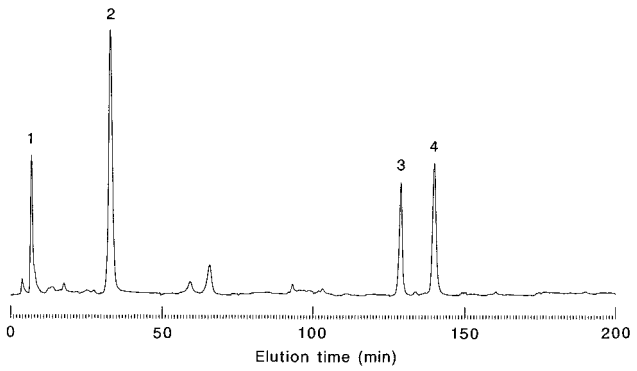

FIG. 1. Elution profile of amino acid analysis. *E. coli* W3110 was grown in the medium containing 0.6 M sucrose at pH 7.9 to 7.7, and the sample for amino acid analysis was prepared as described in Materials and Methods. Elution was carried out as follows: 0 to 65 min, 0.155 M lithium-citrate buffer (pH 3.0) at 33 to 47°C; 65 to 93 min, 0.255 M lithium-citrate buffer (pH 3.7) at 47°C; 93 to 133 min, 0.805 M lithium-citrate buffer (pH 3.3) at 47 to 65°C; 133 to 159 min, 1.0 M lithium-citrate buffer (pH 4.1) at 65°C; 159 to 200 min, 1.2 M lithium-citrate buffer (pH 7.0) at 65 to 68°C. All buffers contained 0.1% Brij 35 and 0.01% *N*-caproic acid. Amino acids were detected by ninhydrin. Peaks: 1, unknown; 2, glutamate; 3, γ-aminobutyrate; 4, NH_3.

pH from 6.5 to 7.5 and that the cytoplasmic pH increases with an increase in the medium pH from 7.5. Thus, it is quite possible that the accumulation of glutamate is stimulated by cytoplasmic alkalinization.

It has been suggested that the accumulation of glutamate is accompanied by the accumulation of potassium ions (1, 2, 7), and our data showed that the level of potassium ions was higher at a high pH (Table 3). Therefore, is possible that potassium ions stimulate the synthesis of glutamate. In order to examine this possibility, *E. coli* mutant TK2642 cells deficient in transport systems for potassium ions were stressed osmotically. Since the medium pH was adjusted by KOH, the media we used contained 20 to 60 mM potassium ions, and this level was enough for growth of this mutant; its growth rates were close to the rates of the wild-type strain at any of the pH values tested under our growth conditions. When TK2642 cells were stressed osmotically at an alkaline pH, the internal level of potassium ions did not increase but rather decreased (Table 3). Since *E. coli* cells are suggested to be shrunken by hyperosmotic shock (2, 7), the decrease in the internal amount of potassium ions would likely be due to the shrinkage. Under the same conditions, glutamate was accumulated at an alkaline pH (Table 4). These data suggested that glutamate synthesis is stimulated by osmotic stress in an alkaline medium without an increase in the internal level of potassium ions, ruling out the possibility that potassium ion is a primary regulator of glutamate synthesis.

Dinnbier et al. (2) have reported that the accumulation of potassium glutamate is transient, and McLaggan et al. (7) proposed that this subsequent decrease in the glutamate level occurs in the stationary phase. Our present data suggest another explanation, namely, that the decrease in the glutamate level is due to the rapid drop of medium pH in the late logarithmic growth phase together with the increase in cell density.

The accumulation of potassium glutamate plus that of γ-aminobutyrate is not enough to increase the internal osmolarity to the level of the external osmolarity, especially at a neutral pH. Thus, it is quite possible that some unknown compound is accumulated upon hyperosmotic stress, but it is still an open question as to what kind of osmolyte increases upon hyperosmotic stress under our experimental conditions. No significant increase in the levels of other amino acids was observed. Since our growth medium contains neither betaine nor choline, betaine would not be a candidate. *E. coli* mutant cells deficient in the *otsA* gene could adapt to hyperosmolarity

TABLE 2. Accumulation of glutamate and γ-aminobutyrate in cells growing in the presence of NaCl[a]

Medium				Cellular content (nmol/mg of protein)	
Buffer	NaCl (M)	pH[b]			
		Start	End	Glu	GABA
Tricine	0.2	8.5	8.4	163	92.5
Tricine	0.2	8.0	7.7	11.6	182
HEPES	0.2	7.3	7.0	19.9	163
Tricine	0.3	8.5	8.3	349	69.0
Tricine	0.3	8.0	7.8	104	97.1
HEPES	0.3	6.8	6.7	137	27.3
HEPES	0.3	6.4	6.3	51.8	33.1
Tricine	0.5	8.5	8.3	868	9.5
Tricine	0.5	7.1	6.9	309	40.4

[a] *E. coli* W3110 was cultured, and pH values and cellular contents of glutamate (Glu) and γ-aminobutyrate (GABA) were measured as described in footnote *a* of Table 1. The data obtained by a single assay are represented. The cells were harvested when the A_{600} was 0.2 to 0.3.
[b] See footnote *d* of Table 1.

TABLE 3. Accumulation of potassium ions after hyperosmotic shock[a]

Strain	pH		Potassium ion (μmol/mg of protein)[b]		
	Start	End	No addition	Sucrose (0.4 M)	NaCl (0.3 M)
W3110	8.5	8.1	1.20 ± 0.03	2.05 ± 0.13	3.72 ± 0.16
	8.0	7.6	1.14 ± 0.01	1.51 ± 0.05	ND[c]
	7.3	6.9	1.05 ± 0.01	1.12 ± 0.01	1.28 ± 0.21
TK2642	8.5	8.1	1.35 ± 0.01	1.01 ± 0.03	ND
	8.0	7.7	0.89 ± 0.01	0.82 ± 0.04	ND
	7.3	6.9	0.97 ± 0.01	0.63 ± 0.09	ND

[a] *E. coli* W3110 and TK2642 were cultured in the medium containing Tricine buffer as described in footnote *a* of Table 1. The cells were harvested when the A_{600} was 0.2 to 0.3. pH values of the media were measured as described in footnote *d* of Table 1. Potassium ion content was measured as described in Materials and Methods.
[b] Average of two determinations ± standard deviation.
[c] ND, not determined.

TABLE 4. Accumulation of glutamate and γ-aminobutyrate in TK2642 growing in the presence of 0.4 M sucrose[a]

pH		A_{600}[b]	Cellular content (nmol/mg of protein)	
Start	End		Glu	GABA
8.5	8.3	0.22	226	46.1
8.0	7.8	0.22	116	45.6
7.7	7.5	0.21	26.7	98.3
7.3	7.2	0.21	46.2	82.4

[a] *E. coli* TK2642 was cultured in the medium containing Tricine buffer as described in Materials and Methods. Values of medium pH and cellular contents of glutamate (Glu) and γ-aminobutyrate (GABA) were measured as described in footnotes *d* and *a*, respectively, of Table 1. The data obtained by a single assay are represented.
[b] See footnote *c* of Table 1.

under the same conditions (data not shown), suggesting that trehalose is not essential for osmotic adaptation under our conditions.

Glutamate was accumulated without the accumulation of potassium ions in TK2642. Since glutamate is an anionic compound, either the accumulation of some cation or the release of some anion from cells should take place with the accumulation of glutamate in order to maintain the cytoplasmic electroneutrality. It is also an open question as to how the neutrality is maintained after hyperosmotic shock under our conditions. As shown in Fig. 1, an unknown compound (peak 1) was accumulated. Since this compound elutes very fast, it appears to be a strong anionic compound. The elution position of this compound was close to the position of phosphoserine, but its elution was too fast for identification. Finally, the important point remaining to be clarified is how glutamate synthesis is regulated by pH. Further studies will be required to clarify these points.

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REFERENCES

1. **Csonka, L. N.** 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. **53:**121–147.
2. **Dinnbier, U., E. Limpinsel, R. Schmid, and E. P. Bakker.** 1988. Transient accumulation of potassium glutamate and its replacement by trehalose during adaptation of growing cells of *Escherichia coli* K-12 to elevated sodium chloride concentrations. Arch. Microbiol. **150:**348–357.
3. **Igarashi, K., K. Kashiwagi, H. Hamasaki, A. Miura, T. Kakegawa, S. Hirose, and S. Matsuzaki.** 1986. Formation of a compensatory polyamine by *Escherichia coli* polyamine-requiring mutants during growth in the absence of polyamines. J. Bacteriol. **166:**128–134.
4. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
5. **McLaggan, D., and W. Epstein.** 1991. *Escherichia coli* accumulates the eukaryotic osmolyte taurine at high osmolarity. FEMS Microbiol. Lett. **81:**209–214.
6. **McLaggan, D., T. M. Logan, D. G. Lynn, and W. Epstein.** 1990. Involvement of γ-glutamyl peptides in osmoadaptation of *Escherichia coli*. J. Bacteriol. **172:**3631–3636.
7. **McLaggan, D., J. Naprstek, E. T. Buurman, and W. Epstein.** 1994. Interdependence of K^+ and glutamate accumulation during osmotic adaptation of *Escherichia coli*. J. Biol. Chem. **269:**1911–1917.
8. **Measures, J. C.** 1975. Role of amino acids in osmoregulation of non-halophilic bacteria. Nature (London) **257:**398–400.
9. **Mugikura, S., M. Nishikawa, K. Igarashi, and H. Kobayashi.** 1990. Maintenance of a neutral cytoplasmic pH is not obligatory for growth of *Escherichia coli* and *Streptococcus faecalis* at an alkaline pH. J. Biochem. **108:**86–91.
10. **Ohyama, T., S. Mugikura, M. Nishikawa, K. Igarashi, and H. Kobayashi.** 1992. Osmotic adaptation of *Escherichia coli* with a negligible proton motive force in the presence of carbonyl cyanide *m*-chlorophenylhydrazone. J. Bacteriol. **174:**2922–2928.
11. **Slonczewski, J. L., B. P. Rosen, J. R. Alger, and R. M. Macnab.** 1981. pH homeostasis in *Escherichia coli*: measurement by ^{31}P nuclear magnetic resonance of methylphosphonate and phosphate. Proc. Natl. Acad. Sci. USA **78:**6271–6275.