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Acid adaptation of *Escherichia coli*: a genetic study

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E. coli log-phase cells are capable of inducing of log-phase acid tolerance response (ATR), but stationary-phase cells do not exhibit this adaptive ability. To study the genetic systems responsible for the induction of log-phase ATR, we investigated *E. coli* mutants deficient in the known acid resistance systems. Our results show that RpoS-dependent oxidative and arginine decarboxylase systems are not induced during acid adaptation of log-phase cells. Glutamate decarboxylases were found to be induced during adaptation and result in a more efficient acid-protection. However, our study shows that other highly efficient protection mechanisms are induced, operate and play the main role in log-phase ATR.

Key words: *E. coli* acid tolerance response (ATR), acid resistance systems (AR), RpoS-dependent oxidative system, glutamate and arginine decarboxylase systems

INTRODUCTION

A sublethal environmental acid stress (pH 5.5–4.5) induces an adaptive tolerance response in many bacteria and provides protection against subsequent exposure to a lethal stress (pH < 4.0) by a mechanism known as acid tolerance response (ATR). ATR has been identified and studied in a vide variety of gram-negative and gram-positive bacterial species [1]. ATR includes both log-phase and stationary-phase systems [2], however, stationary-phase ATR varies greatly among species [3] and strains of the same species [4]. The ATR of *Salmonella* requires a number of acid-shock proteins that are thought to prevent and repair cellular damage caused by acid stress [5]. The main defense strategies protecting the cell from acid are changes in membrane composition [6, 7], homeostasis systems for internal pH [2, 8] and pathways to repair or protect the essential cellular components [9, 10].

Although acid tolerance response was observed in *E. coli* first [1], it was not studied in detail. It has been shown that *E. coli* log-phase cells are capable of inducing log-phase ATR [3]. Paul and Hirshfield have found that pre-exposure of *E. coli* log-phase cells to pH 5.5 and pH 4.3 induces the synthesis of acid shock proteins, which most likely are responsible for the induction of ATR [11], but largely are not defined yet.

In contrast, the *E. coli* acid resistance (AR) phenomenon, which is based on three complex cellular systems, has been studied extensively [3]. These systems are RpoS-dependent oxidative system and the inducible amino acid decarboxylases – arginine decarboxylase and glutamate decarboxylase. The latter two are presumed to consume intracellular protons at low pH via decarboxylation reaction, after which a membrane antiporter exchanges the reaction product for more of the amino acid substrate [8, 12]. The protection mechanism of the oxidative system remains to be a mystery. All three systems were found to function effectively in stationary phase cells [8].

The aim of the present study was to investigate the induction of ATR in *E. coli* and to determine if acid resistance systems could be involved in cell protection during log-phase ATR.

MATERIALS AND METHODS

Bacterial strains, culture media and acid survival experiments. The *E. coli* K-12 strain used was MG1655. The derivatives of MG1655 generated and used in this study are listed in Table. Unless otherwise indicated, *E. coli* cells were grown in Luria–Bertani (LB) medium [13]. When necessary, antibiotics were added at the following concentrations: kanamycin 60 μg ml^{-1}, tetracycline 10 μg ml^{-1}.

For ATR analysis, cultures were grown in LB to an A_{600} of 0.35 for the log phase ATR assay and to A_{600} of 1.5 for the stationary phase ATR assay. To induce acid adaptation, the cells were resuspended in fresh LB adjusted by HCl to pH 4.45. Control (unadapted) and adapted cell cultures were inoculated into a medium acidified with HCl to pH 2.0 to approximately 1×10^8–3×10^8 CFU ml^{-1}. Viable cells

Table. ***E. coli*** **strains used in the study**

Strain	Genotype / Description	Reference / Source
MG1655	F- lambda- *ilvG- rfb*-50 *rph*-1	*E. coli* Genetic Stock Center, CGSC 6300
RH90	MC4100 *rpoS*359::Tn10	[15]
JE100	MG1655 *rpoS*359::Tn10	This study
JE101	MG1655*gadC*	This study
JE102	MG1655*adiA*	This study

were determined by plating aliquots of serially diluted cultures.

AR assays were performed as described [8]. Briefly, for the analysis of RpoS-dependent oxidative system, cells were grown overnight in LB-MES (pH 5.5) and diluted in M9 (pH 2.0) [13] to 1×10^8–3×10^8 CFU ml^{-1}. For the analysis of the glutamate-dependent AR system, cells were grown overnight in LBG (LB 0.4% glucose, 0.1M MOPS, pH 7.4) and diluted in M9 (pH 2.0) supplemented with glutamate (1.5 mM). The arginine-dependent AR system was tested by growing cells in LBG overnight and diluting the culture into M9 (pH 2.0) supplemented with arginine (0.6 mM). Viable-cell counts were determined at 0 and 2 h after the acid challenge by plating serially diluted (in PBS [13]) cell suspensions.

Construction of *gadC, adiA* and *rpoS* mutants. The *gadC* and *adiA* gene knockouts were constructed according to the method of Datsenko [14]. The PCR products needed to create targeted deletions in *gadC* and *adiA* genes were made from oligo-GadC1 (5'-ttccgacaggaataccgttttaggggataatatggctacatcaggtgtaggctggagctgcttc), oligo-GadC2 (5'-tggttagtgtttcttgtcattcatcacaatatagtgtggtgaacgtcatatgaatatcctccttagt-3') and oligo-AdiA1 (5'-tagtggtt acgctttcacgcacataacgtggtaaataccgtcaatcatatgaatatcctccttagt-3'), oligo-AdiA2 (5'-ttgcccgcaacg aagattccttcataaccgggtaagcaatgatgagtgtaggctggagctgcttc), respectively. Verification of the mutations was made by PCR using locus-specific primers: oligo-GadC3 (5'-ggagcgatga attatcgctc-3') and oligo-GadC4 (5'-aacaaaaacaggtgcggttcc-3') for *gadC* and oligo-AdiA3 (5'-a cagcccgaaaaggccggaa-3') and oligo-AdiA4 (5'-tcagcggaatagtggttac-3') for *adiA*. The mutations and Kan^r marker were further transferred into *E. coli* K-12 strain MG1655 by phage P1*vir*. The mutation of *rpoS* was created by transferring *rpoS*359::Tn10 insertion [15] from strain RH90 into MG1655 by phage P1*vir*. *rpoS* mutations were verified by glycogen staining.

RESULTS AND DISCUSSION

ATR in *E. coli* cells. Our results have demonstrated that when the *E. coli* K-12 strain MG1655 is cultured in a rich medium, protection against lethal acid challenge of pH 2.0 is provided by pretreatment of the exponentialy growing cells at pH 4.5. During this experiment cells were grown to exponential phase in LB medium at pH 7.0 and were acid-adapted at pH 4.5 as described in Material and Methods. The adapted cells of the exponential growing culture showed a 10000-fold better survival as compared to the acid-unadapted cells (Fig. 1). This indicates that exponential phase cells of *E. coli* induce an ATR under given conditions. Exposure to pH 4.5 for 30 min was sufficient to increase the survival of exponentialy growing cells (Fig. 2). The survival of *E. coli* was increased up to 1000-fold as a result of ATR induction. The further extension of adaptation time only slightly improved the protection to acid challenge at pH 2.0 (Fig. 2). Paul and Hirshfield found that *de novo* protein synthesis was required for ATR in *E. coli*, as chloramphenicol-treated and subsequently adapted cells were unable to survive acid stress. They have shown that under protein two-dimensional gel electrophoresis at least 17 proteins are induced at pH 4.3 [11]. Some of them could be key acid shock proteins required for stress protection. Several studies showed the Dps mutant (DNA-binding protein from starved cells) to exhibit a decreased survival in log phase [10]; the Fur (ferric uptake regulator, a master regulator of iron metabolism) mutant was unable to trigger the acid-tolerance response [16], and the *asr* gene encoding an acid-inducible protein of unknown function, was shown to be required for the induction of acid tolerance in log-phase cells [17].

Conversely, unadapted *E. coli* stationary-phase cells showed a high survival. There was only a 50% loss of viability upon 80 min exposure to pH 2.0 (Fig. 1). Adaptation at pH 4.5 did not improve the acid-survival. Most likely the high survival was conferred by acid resistance systems such as the RpoS-dependent oxidative system, glutamate decarboxylase and arginine decarboxylase systems, which have been shown to function in stationary phase cells [8].

Our results confirm that *E. coli* log-phase cells are capable of inducing log-phase ATR, but cells in stationary phase do not exhibit this response. To elucidate the genetic systems responsible for the induction of log-phase ATR, we decided to investigate ATR in *E. coli* mutants deficient in acid resistance systems, listed above. It has been shown earlier that log-phase cells do not show glutamate decarboxylase activity [8], but it is possible that AR systems are induced during the adaptation process, since the ex-

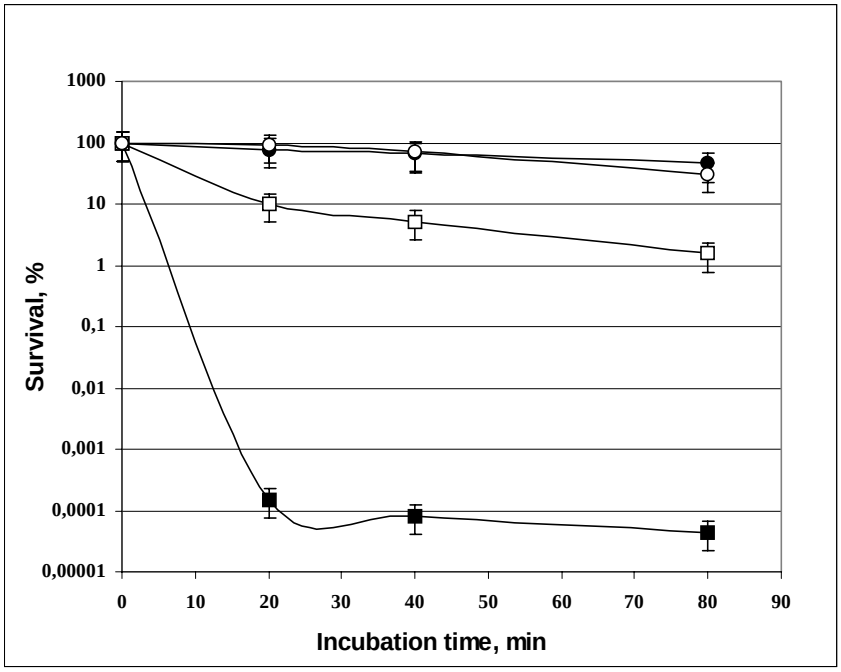

Fig. 1. Acid tolerance response of *Escherichia coli* strain MG1655. Culture was grown in LB medium pH 7.0. Exponential phase (A_{600} = 0.35) and stationary phase (A_{600} = 1.5) cells of *E. coli* were either unadapted (log phase – ■, stationary phase – ●) or adapted at pH 4.5 for 2 h (log phase – □, stationary phase – ○) prior exposure to acidified broth at pH 2.0. The percentage of survival at indicated time points was calculated using CFU. 100% viability equals to the viable counts obtained immediately after exposure to pH 2.0. The values are the means of at least three independent experiments. Bars indicate SD

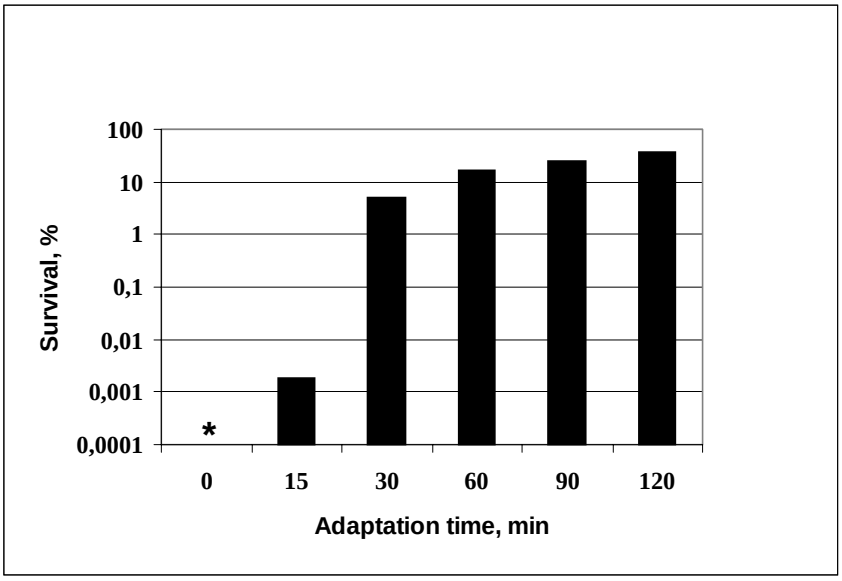

Fig. 2. Effect of adaptation time on acid tolerance response induction. Culture was grown in LB medium pH 7.0. Exponential phase (A_{600} = 0.35) cells of *E. coli* were adapted at pH 4.5 for indicated time period prior exposure to acidified broth at pH 2.0. The percentage of survival after 2 h of incubation at pH 2.0 was calculated using CFU. 100% viability equals the viable counts obtained immediately after exposure to pH 2.0. The values are from one representative experiment. Asterisks indicate survival below detection limits

pression of genes encoding glutamate decarboxylases was significant in the cells grown at pH 4.5 [18].

Generation of mutants defective in AR. It has been reported previously that individual acid resistance systems are completely abolished in the following bacterial mutants: RpoS-dependent oxidative system in *rpoS* (encoding alternative RNA polymerase sigma factor) mutant, glutamate decarboxylase system in *gadC* (encoding glutamate:GABA antiporter) mutant and arginine decarboxylase system in *adiA* (encoding arginine decarboxylase) mutant [8]. The *gadC*, *adiA*, *rpoS* mutants in MG1655 strain were constructed as described in Materials and Methods. First, we have tested the effects of the mutations on the functionality of the three AR systems. The mutants JE100, JE101, JE102 and parental strains were grown overnight in an appropriate medium as described in Materials and Methods according to the Castanie–Cornet protocol [8]. To test the efficiency of the oxidative system (Oxi), cell survival was monitored in M9 medium, pH 2.0. The arginine decarboxylase (Adi) and glutamate decarboxylase (Gad) systems were tested in M9 medium, pH 2.0, supplemented either with arginine or glutamate. As shown in Fig. 3, the *rpoS* mutant lacked oxidative system; the activity of Adi system was reduced 1000-fold in the *adiA* mutant as compared to the parental strain, and the activity of the Gad system was reduced 10000-fold in the *gadC* mutant. These results confirm that the created mutants are defective in respective acid resistance systems. Interestingly, *rpoS* mutant showed a reduced glutamate and arginine-dependent AR. This can be explained partly by the fact that the expression of glutamate decarboxylases is dependent on RpoS [8]. However, there are no data to show that RpoS is invol-

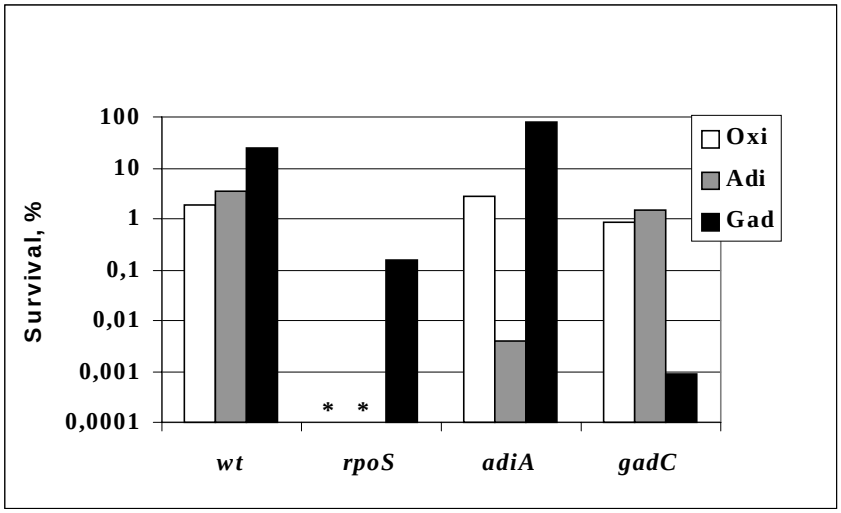

Fig. 3. Acid resistance analysis of *rpoS*, *adiA*, *gadC* mutants. The test for the oxidative system (open bars) involved overnight growth in LB-MES (pH 5.5) followed by dilution in M9 (pH 2.0). The arginine (gray bars) and glutamate (black bars) systems required overnight LBG (LB 0.4% glucose, 0.1M MOPS, pH 7.4) cultures, which were diluted in M9 (pH 2.0) containing 0.6 mM arginine and 1.5 mM glutamate, respectively. The percentage of survival after 2 h of incubation at pH 2.0 was calculated using CFU. 100% viability equals the viable counts obtained immediately after exposure to pH 2.0. Asterisks indicate survival below detection limits. The values are from one representative experiment. Experiments were repeated two or three times. Variations were within 50% of the stated value

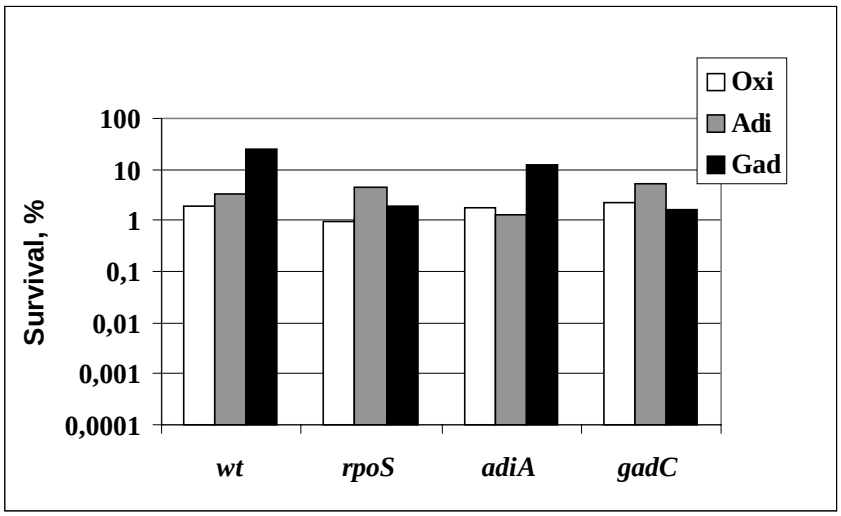

Fig. 4. Log-phase acid tolerance response of *Escherichia coli rpoS*, *adiA*, *gadC* mutants. Cultures were grown in LBG medium pH 7.4. Exponential phase (A_{600} = 0.35) cells of *E. coli* were adapted in LBG at pH 4.5 for 2 h. To test for the oxidative system (open bars), the cells were diluted in M9 (pH 2.0), for arginine (gray bars) and glutamate (black bars) systems cells were diluted in M9 (pH 2.0) containing 0.6 mM arginine and 1.5 mM glutamate respectively. The percentage of survival after 2 h of incubation at pH 2.0 was calculated using CFU. 100% viability equals the viable counts obtained immediately after the challenge to pH 2.0. Values are from one representative experiment. Experiments were repeated two or three times. Variations were within 50% of the stated value

ved in the regulation of expression of the Adi system components.

Analysis of log-phase ATR in the *rpoS*, *adiA* and *gadC* mutants. *rpoS*, *adiA* and *gadC* mutants were grown to exponential phase in LBG medium and were acid-adapted at pH 4.5 or unadapted as described in Materials and Methods. To further test the efficiency of the oxidative system (Oxi), cell survival was monitored upon exposure to M9 medium, pH 2.0. To test the arginine decarboxylase system (Adi), pH 4.5 adaptated and unadapted cells were shifted to an M9 medium, pH 2.0, supplemented with arginine, and to test glutamate decarboxylase system (Gad) the cells were exposed to M9 medium, pH 2.0, supplemented with glutamate. As shown in Fig. 4, the *rpoS* mutant, deficient in oxidative system, survived acid stress similarly as did the parental strain. These results indicate that RpoS is not involved in triggering log-phase ATR and suggest that other protective mechanisms are induced during adaptation in log-phase cells. However, the survival of acid-adapted *gadC* mutant in M9 medium supplemented with glutamate was reduced 10-fold as compared to the parental strain, indicating that the *gadC* mutant was more susceptible to acid stress and that the GAD system was induced during adaptation. The activity of the glutamate decarboxylase system was reduced in the *rpoS* mutant as a result of a reduced expression of the Gad system components [8] as reported before. The acid-adapted *adiA* mutant did not show a reduced acid survival as compared to the *wt*, indicating that the arginine decarboxylase system is not involved in log-phase ATR.

We can conclude that *E. coli* possesses different low-pH inducible acid survival systems depending upon whether the cells are in the exponential or in stationa-

ry-phase. Studies of acid survival in *S. typhimurium* demonstrated that different types of ATR systems exist in log-phase and stationary-phase cells. RpoS, PhoP and Fur proteins have all been shown to be needed for the log-phase ATR [5, 19, 20] and OmpR has been reported to be required for an efficient induction of the stationary-phase ATR of *S. typhimurium* [21]. Our results show that RpoS-dependent oxidative and arginine decarboxylase systems are not induced during acid adaptation of log-phase *E. coli* cells. Glutamate decarboxylases seem to be induced during adaptation and result in a more efficient protection. However, it is clear that other highly efficient protection mechanisms are induced, operate and play the main role during log-phase ATR. They need further investigation.

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References

1. Both IR, Cash P, Byrne CO. Ant von Leeuwenhoeck 2002; 81: 33–42.
2. Park YK, Bearson B, Bang SH et al. Mol Microbiol 1996; 20: 605–11.
3. Lin J, Lee IS, Frey J et al. J Bacteriol 1995; 177: 4097–104.
4. Buchanan RL, Edelson SG. Appl Environ Microbiol 1996; 62(11): 4009–13.
5. Lee IS, Slonczewski JL, Foster JW. J Bacteriol. 1994; 176: 1422–6.
6. Chang YY, Cronan Jr JE. Mol Microbiol 1999; 33: 249–59.
7. Jordan KN, Oxford L, O'Byrne CP. Appl Environ Microbiol 1999; 65(7): 3048–55.
8. Castanie-Cornet MP, Foster JW. Microbiology 2001; 147: 709–15.
9. Raja N, Goodson M, Smith DG, Rowbury RJ. J Appl Bacteriol 1991; 70(6): 507–11.
10. Choi SH, Baumler DJ, Kaspar CW. Appl Environ Microbiol 2000; 66: 3911–6.
11. Paul B, Hirshfield I. Res Microbiol 2003; 154(2): 115–21.
12. Gong S, Richard H, Foster JW. J Bacteriol 2003; 185(15): 4402–9.
13. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1989.
14. Datsenko KA, Wanner BL. Proc Natl Acad Sci USA 2000; 97: 6640–5.
15. Lange R, Hengge-Aronis R. Mol Microbiol 1991; 5: 49–59.
16. Zhu C, Ngeleka M, Potter AA, Allan BJ. Can J Microbiol 2002; 48(5): 458–62.
17. Ðeputienë V, Motiejûnas D, Suþiedëlis K et al. J Bacteriol 2003; 185: 2475–84.
18. Tucker DL, Tucker N, Conway T. J Bacteriol 2002; 184: 6551–8.
19. Hall HK, Foster JW. J Bacteriol 1996; 178: 5683–91.
20. Bearson BL, Wilson L, Foster JW. J Bacteriol 1998; 180: 2409–17.
21. Bang IS, Kim BH, Foster JW, Park YK. J Bacteriol 2000; 182: 2245–52.

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Escherichia coli RÛGÐTIES TOLERANCIJOS ATSAKO GENETINIS TYRIMAS

Santrauka

E. coli logaritminës fazës làstelës, adaptuotos vidutinio rûgðtinio streso (pH 4,5) sàlygomis, stiprø rûgðtiná stresà (pH 2,0) iðgyvena geriau nei làstelës, augusios neutralioje terpëje. Pasireiðkia rûgðties tolerancijos atsakas, ágytas adaptacijos metu. Nustatyta, kad jis nëra bûdingas *E. coli* stacionarios fazës làstelëms. Siekiant nustatyti genetinius mechanizmus, kurie yra indukuojami logaritminës fazës làstelëse rûgðties tolerancijos atsako indukcijos metu, buvo iðtirta, kaip ði adaptacija pasireiðkia *E. coli rpoS*, *adiA* ir *gadC* genø bakterijø mutantuose. Rasta, kad nuo *rpoS* priklausoma oksidacinë sistema ir arginino dekarboksilazës sistema nëra indukuojamos logaritminës fazës làstelëse tolerancijos atsako metu. Tuo tarpu glutamato dekarboksilazës sistema yra indukuojama ir apsaugo làsteles nuo stipraus rûgðtinio streso. Rezultatai taip pat liudija, kad logaritminës fazës làstelëse rûgðties tolerancijos atsako metu yra indukuojami ir pagrindiná vaidmená vaidina kiti efektyvûs apsaugos mechanizmai.