The role of OmpC and OmpF in acidic resistance in *Escherichia coli*

Amany Abdelrehim Bekhit Abdelrehim

2011
Contents

Abbreviation

Introduction

Chapter I
The role of OmpC and OmpF in acidic resistance in *Escherichia coli* in logarithmic phase

Introduction

Results

Chapter II
The role of OmpC and OmpF in acidic resistance in *Escherichia coli* in stationary phase

Introduction

Results

Discussion

Conclusions

Materials and methods

References

List of publications

Acknowledgements
Abbreviations

AR, Acidic resistance
C.F.U, Colony forming units
Crp, Cyclic AMP receptor protein
E. coli, Escherichia coli
GABA, γ-aminobutyrate
LPSs, Lipopolysaccharides
MES, 2-(N-Morpholino) ethanesulfonic acid
OMPs, Outer membrane proteins
PBS, Phosphate buffered saline
PCR, Polymerase chain reaction
RT, Reverse transcription
RpoS, RNA polymerase sigma factor
SD, Standard deviation
Introduction

An important property of microbial pathogens associated with oral-fecal routes of transmission is the ability to survive extremely acidic environments which threaten their viability. This observation further supports the role for acidic resistance (AR) as an important virulence feature of successful gastrointestinal pathogens (Lin et al., 1996). *E. coli* exhibits a high degree of AR via two mechanisms: an amino acid dependent mechanism and independent mechanism (Castanie-cornet et al., 1999 and Foster, 2004). The amino acid-independent pathway (AR1) sometimes referred to glucose repressed system (Fig.1). Activation of this system occurs when cells are grown in mild acidic complex media (LB pH5.5) in absence of glucose, key regulator of this system include alternative sigma factor, cAMP, $\sigma^8$ is critically important for survival during various environmental stresses including acid challenge. It is not known whether regulation of AR1 by RpoS or CRP is direct or indirect. The specific target genes controlled by these regulators are also unknown. Unlike amino acid dependent AR systems it provides the least level of protection (Castanie-Cornet et al., 1999).

The amino acid dependent system (AR2) requires two glutamate decarboxylase (GadA and GadB) enzymes and is induced at acidic pH under aerobic conditions, while arginine (AR3) and lysine (AR4) decarboxylase enzymes (AdiA, CadB) are induced by low pH under anaerobic conditions (Richard and Foster, 2004). As shown in (Fig.2) these reactions result in production of GABA, agmatine, and cadaverine, respectively, which are then extrude via membrane-bound specific
antiporters with concomitant uptake of exogenous amino acids (Casalino et al., 2010). These amino acid-dependent acidic resistance systems have been through to increase internal pH by production of amines, but the consumption of protons during decarboxylation was recently proposed to be a more important acidic resistance strategy (Foster, 2004). The cell envelope of Gram-negative bacteria is composed of two membranes, the inner membrane and the outer membrane, which are separated by the periplasm layer. While the inner membrane is a phospholipid bilayer, the outer membrane is highly asymmetrical, containing glycerophospholipids in the inner leaflet and lipo-polysaccharides (LPSs) exposed to the cell surface. The outer membrane functions as a permeability barrier protecting the bacteria against harmful compounds, such as antibiotics and bile salts, from the environments (Nikaido, 2003). Gram-negative bacteria are generally more tolerant to stress conditions than Gram-positive bacteria due to the outer membrane barriers (Zhang et al., 2010).

Most nutrients pass the outer membrane barrier via a family of integral outer membrane proteins (OMPs), collectively called porins (Tommassen, 2010). Among these OMPs, OmpC, this has a molecular mass of 36 kDa, and OmpF which has a molecular mass of 35 kDa, are often regarded as classical porins and are present in large quantities (Osborn and Wu, 1980, Lugtenberg and Van Alphen, 1983). Normally, the total amount of OmpC and OmpF proteins is fairly constant, but the relative proportion of the two varies, subject to factors such as osmolarity of the medium, temperature, the concentration of certain antibiotics, and growth phase (Liu and Ferenci, 1998). Medium with high osmolarity, high temperature, or toxic ingredients favors the expression of OmpC, and medium of low osmolarity and low
temperature increases OmpF and diminishes the level of OmpC (Pratt et al., 1996). Although it has been proposed that OmpC and OmpF are required under some harsh conditions in Gram-negative bacteria (Özkanca et al., 2002), *E. coli* cells are able to grow in the absence of these porin proteins (Kaeriyama et al., 2006). An *E. coli* mutant that was deficient in *ompR*, a gene that regulates the expression of *ompC* and *ompF*, demonstrated poor survival under strong acidic conditions (Bang et al., 2000). However, the roles of OmpC and OmpF are still unknown because *ompR* has been proposed to regulate many genes other than *ompC* and *ompF* (Gibson et al., 1987, Higashitani et al., 1993). The outer membrane is the first important barrier between Gram-negative bacteria and the surrounding environment. Özkanca and Flint (2002) reported that any little changes in relative amounts of OMPs conditions effect *E. coli* survival.

The present study was carried out in order to investigate the survival of *E. coli* and a number of OMP-deficient mutants in acidic conditions in logarithmic and stationary phases. This may help to understand the role played by OmpC and OmpF during acidic resistance in *E. coli*. 
Fig. 1. The amino acid independent system (AR1) in *E. coli*
Fig. 2. The three main amino acid decarboxylase systems in *E. coli*
Chapter I

The role of OmpC and OmpF in acidic resistance in 

*Escherichia coli* in logarithmic phase
Introduction

*E. coli* has numerous strategies for survival under acidic stress (Kobayashi *et al.*, 2000). One of the strategies may be the change in porin expression at different pH values (Heyde and Portalier, 1987, Thomas and Booth, 1992 Heyde *et al.*, 2000). *E. coli* is unable to grow at pH less than 4, but the ability to survive under such acidic conditions was enhanced by adaptation in mild acidic environments whose pH is 5 to 6 in many cases (Small *et al.*, 1994).

The four acidic resistance systems in *E. coli* include oxidative, glutamate-dependent, arginine-dependent, and lysine-dependent systems (Casalino *et al.*, 2010). These systems could contribute to provide a different level of protections against gastric acidity (Richard and Foster, 2003). Thus, an important question as to whether OmpC and OmpF have an essential role in the acidic resistance in *E. coli* remains unclear. I therefore focused this chapter on (i) assay of the activity of AR systems of *E. coli* wild type, single mutants, and double mutant in OmpC and OmpF in logarithmic phase using two different growth medium and (ii) investigation of the expression of amino acid decarboxylases and their activity in *ompC* and *ompF* deficient mutants. I found that OmpC and OmpF play essential roles in the acidic resistance of *E. coli* in the presence of arginine and lysine. This result suggests that the transport of some substrates is required for survival under acidic conditions.
Results

Effect of glucose on the expression of *ompC* and *ompF* in *E. coli*

It was known that porin levels are sensitive to a wide variety of environmental parameters including osmolarity, temperature, pH, growth phase, and cell density (Pratt *et al.*, 1996 and Buckler *et al.*, 2000). In this study, we examined whether glucose can control *ompC* and *ompF* expression. When 0.4% glucose was added to LB medium, no significant reduction in *ompC* expression was observed at pH 5.5 compared to LB medium without glucose (Fig. 3). Interestingly, the expression of *ompF* was decreased to about half by the addition of glucose under acidic pH. So, I suggest that glucose has a mild repressive effect on *ompF* expression under acidic environments.

Acidic resistance in mutants deficient in *ompC* and *ompF*

In order to investigate the roles of OmpC and OmpF in the survival of *E. coli*, we initially analyzed how OmpC and OmpF deficiency affects AR. AR was somewhat decreased by the deletion of either *ompF* or *ompC*, and the AR of the mutant that was deficient in both *ompC* and *ompF* was lower than that of the strains containing both OMPs (Fig. 4).

The attenuation of the AR of the single mutants was recovered by the addition of plasmid containing *ompC* or *ompF*, and the multicopy expression of *ompF* or *ompC* gene increased the viability more than the wild type MKW505 (Fig. 4). These results clearly indicated that OmpC and OmpF have a significant impact on *E. coli* survival in extremely acidic
environments. The recovery of the AR by the addition of plasmids containing \textit{ompC} or \textit{ompF} in the double mutant ABC2010 was lower than that in the single mutants, indicating that both OmpC and OmpF are required for maximum survival, although either OmpC or OmpF alone can support the survival at acidic pH.

\textbf{Effect of OmpC and OmpF on glutamate, arginine, and lysine dependent AR systems}

According to previous reported study, \textit{E. coli} strains have the ability to induce acidic resistance systems under different physiological conditions (Bhagwat \textit{et al.}, 2005). AR2 requires extracellular glutamate and is induced at acidic pH under aerobic conditions, while AR3 and AR4 are induced at low pH in the presence of arginine and lysine under anaerobic conditions (Richard and Foster, 2004). Therefore, I examined the effect of OmpC and OmpF on the glutamate dependent AR under aerobic conditions, while the effect on the arginine and lysine dependent AR was studied under anaerobic conditions. \textit{E. coli} strains MKW505, MH621, KAEC5, and ABC2010 were treated with 1mM glutamate (AR2) in EG medium with a pH of 5.5 under aerobic conditions and then challenged in EG medium with a pH of 2.5. The addition of glutamate enhanced the AR 12-fold in the MKW505 cells, 13-fold in the MH621 cells, 15-fold in the KAEC5 cells, and 8-fold in the ABC2010 cells (Fig. 5). The similar increases in the AR of the four strains suggested that the roles of OmpC and OmpF in AR are not mediated by the glutamate-dependent system (AR2).

In contrast, the survival rate of the double deficient mutant was not significantly increased after the addition of 1mM arginine (AR3) or lysine (AR4) to EG medium under anaerobic conditions (Fig. 6). On the other
hand, the presence of arginine or lysine protected the single mutants and the wild type strains against acidic stress (Fig. 6). Furthermore, the protection induced by arginine or lysine in the double deficient mutant was restored by complementation with plasmids containing \textit{ompC} or \textit{ompF} (Fig. 6). These results strongly suggest that OmpC and OmpF play essential roles in the arginine and lysine-dependent AR systems.

The survival without the addition of amino acids was higher under anaerobic conditions (Fig. 5 and 6). Previous studies on survival of \textit{E. coli} reported that the survival was longer in anaerobic than aerobic conditions. Several factors may explain the apparent poor survival of \textit{E. coli} in aerobic conditions. Endogenous energy reserves may be burned faster under aerobic conditions. The damage of cellular materials due to the accumulation of reactive oxygen species will contribute to the decreasing survival under aerobic conditions (Roslev \textit{et al.}, 2004).

\textbf{Expression of genes for AR systems in the \textit{ompC} and \textit{ompF} deficient mutant}

The results described above suggested that the expression of the genes encoding for decarboxylases was affected by the deletion of \textit{ompC} and \textit{ompF}. I measured the mRNA levels in the double deficient mutant. After the addition of 1 mM glutamate, the expression levels of \textit{gadA} and \textit{gadB} were not significantly affected by the deletion of both \textit{ompC} and \textit{ompF} under aerobic conditions, while the expression levels of \textit{adiA} and \textit{cadB} in the presence of 1mM arginine and lysine, respectively, were significantly repressed in the double deficient mutant (Fig. 7).
These results indicate that OmpC and OmpF are essential for the full induction of arginine and lysine decarboxylases, while the expression of glutamate decarboxylases is not significantly affected by the deletion of ompC and ompF.

**The activities of arginine, lysine, and glutamate decarboxylations in ompF and ompC deficient mutant**

As described above, the addition of lysine or arginine did not significantly increase survival in the ompC and ompF double mutant. Next, the conversion of amino acids to amines was assayed (Fig.8). After membrane had been disrupted with Triton X-100, the enzyme activity of the double deficient mutant was slightly lower than that of the wild type. In contrast, the conversion of arginine and lysine was repressed in the double deficient mutant in the absence of Triton X-100. The conversion of glutamate was not repressed in the double mutant with and without TritonX-100. These results suggested that OmpC and OmpF support the survival via the transport of arginine and lysine through these porins. Glutamate may be able to move across the outer membrane via other routes.

**Effect of growth medium on the AR in ompC and ompF deficient mutant**

It was previously reported that E.coli is resistant to more extreme acidity in complex medium (Gorden and Small, 1993). I have asked whether or not
acidic resistance of \textit{ompC} and \textit{ompF} deficient mutant in complex medium is parallel to that observed in EG medium. Therefore the AR of both MKW505 and ABC2010 were measured in LB medium. Contrary to the formal finding, only AR in LB medium without glucose and amino acid supplementation had a significant reduction by \textit{ompC} and \textit{ompF} deletion, while the addition of glucose and amino acid increased AR (Fig.9). These results suggest that AR of double deficient mutant depends on the growth medium.
Fig. 3. Effect of glucose on the expression of *ompF* and *ompC* in the logarithmic phase. *E. coli* was grown at pH 5.5 in LB medium with or without 0.4% glucose until their absorbance at 600 nm reached 0.3. The β-galactosidase activity was assayed in duplicated in at least three independent experiments, and is expressed in Miller units. Each bar represents mean ± S.D.
Fig. 4. The acidic resistance of mutants deficient in OMPs. MKW505 (ompC\(^+\) ompF\(^+\)), MH621 (ompC\(^+\) ompF\(^-\)), MH621 containing pompF, KAEC5 (ompC\(^-\) ompF\(^+\)), KAEC5 containing pompC, ABC2010 (ompC\(^-\) ompF\(^-\)), ABC2010 containing pompF, and ABC2010 containing pompC were grown aerobically in EG medium at pH 5.5 and challenged in EG medium at pH 2.5 for 1 hr, and then their viability was measured, as described in Materials and Methods.
Fig. 5. Effect of glutamate on the AR of various mutants. MKW505 (ompC⁺ ompF⁺), MH621 (ompC⁺ ompF⁻), KAEC5 (ompC⁻, ompF⁺), and ABC2010 (ompC⁻ ompF⁻) were grown aerobically at pH 5.5, and their viability was measured after acidic challenge at pH 2.5 for 1 h, as described in Materials and Methods. One mM glutamate was added to the culture medium at pH 5.5 (black bars). The white bars represent the AR measured without glutamate.
Fig.6. Effect of arginine and lysine on the AR of various mutants. MKW505 ($ompC^+ ompF^+$), MH621 ($ompC^+ ompF^-$), KAEC5 ($ompC^-, ompF^+$), ABC2010 ($ompC^- ompF^-$), ABC2010 containing $pompF$, and ABC2010 containing $pompC$ were grown anaerobically at pH 5.5, and their viability was measured after acidic challenge at pH 2.5 for 1 h, as described in Materials and Methods. One mM arginine or lysine was added to the medium at pH 5.5, as indicated below. Symbols: white bars, the viability measured without amino acids; grey bars, the viability measured in the presence of arginine; black bars, the viability measured in the presence of lysine.
Fig. 7. Expression of genes for AR systems. The levels of mRNA were measured with RT-PCR. MKW505 (*ompC*⁺ *ompF*⁺, W) and ABC2010 (*ompC*⁻ *ompF*⁻, M) were cultured aerobically in EG-medium pH 5.5 containing 1 mM glutamate (A) *gadA/B*: genes for AR2, or anaerobically in EG-medium pH 5.5 containing 1 mM arginine (B) *adiA*: gene for AR3 or lysine (C) *cadB*: gene for AR4. (D) The level of 16S rRNA was used as a control.
Fig. 8. Activity of arginine, lysine, and glutamate decarboxylases. MKW505 (ompC\(^+\) ompF\(^+\)) and ABC2010 (ompC\(^-\) ompF\(^-\)) were grown anaerobically in the presence of 1 mM arginine (A and D) lysine (B and E), or aerobically in the presence of 1 mM glutamate (C and F) until their absorbance at 600 nm reached 0.3. After the cells had been harvested, the pH change was measured in the presence of arginine (A and D), lysine (B and E), or glutamate (C and F), as described Materials and Methods. TritonX-100 was added in A, B, and C. Symbols: ●, MKW505 with amino acids; ■, ABC2010 with amino acids; ○, MKW505 without amino acids; □, ABC2010 without amino acids. The mean values and standard deviations obtained from three independent experiments are represented.
Fig. 9. Effect of supplements on the AR in *ompC* and *ompF* deficient mutants during the logarithmic phase. MKW505 (*ompC*+ *ompF*+) (white bars) and ABC2010 (*ompC*− *ompF*−) (black bars) were grown in LB medium at pH 5.5 until their absorbance at 600 nm reached 0.3 and their viability was measured after the acidic challenge at pH 2.5 for 1 hr, as described in Material and Methods. Glucose (0.4%) was added to all medium except the left experiment, and amino acids (1mM) were added to the medium of pH 5.5 as indicated. The mean ± S.D. of duplicate determination from at least three independent cultures are represented.
Chapter II

The role of OmpC and OmpF in acidic resistance in *Escherichia coli* in stationary phase
Introduction

The acidic resistance of *E. coli*, as measured by its ability to survive in low pH, was highly influenced by prior growth conditions (Jarvis and Russell, 2001). As *E. coli* enters to stationary phase, morphological and genetic changes occur to prolong survival and increase resistance to a variety of stress conditions, and these changes are mediated by a global regulatory system in which a putative stationary phase sigma factor encoded by *rpoS* plays a key role (Bohannon *et al.*, 1991). When RpoS level begins to raise, it triggers expression of many genes participating in AR1 in which the specific target genes are still unknown (Richard and Foster 2004). Moreover, it can induce *gadX* which is positive regulator of *gadA/B* expression in AR2 system (Castanie-Cornet *et al.*, 1999).

My results in chapter I show that the deletion of *ompC* and *ompF* are significantly decreased both of AR3 and AR4 and a little change was observed in AR2 at pH 5.5 in EG medium. However, the amino acid dependent systems have no significant alteration in LB medium at pH5.5 and AR1 can not be induced in double mutant strain in the logarithmic phase.

In this chapter, I first examine the effect of glucose in the stationary phase. Then, I study the ability of *ompC* and *ompF* mutants to survive under extremely acidic conditions in the stationary phase.
Results

Effect of glucose on the expression of ompC and ompF in *E. coli*

To determine whether glucose positively or negatively regulates the expression of *ompC* and *ompF* gene under acidic conditions in the stationary phase, I added 0.4% glucose to culture medium at pH5.5, and the the expression of *ompC* and *ompF* was measured. In contrast to logarithmic phase, glucose greatly repressed both of *ompC* and *ompF* expressions in the stationary phase, as shown in (Fig.10). Thus, it was suggested that glucose had a significant repressive effect on *ompC* and *ompF* expressions but its effect was higher in the stationary phase. Furthermore, the induction of AR by glucose is independent on *ompC* and *ompF* expressions.

Effect of growth medium on the AR systems in ompC and ompF deficient mutant

It was reported that stationary phase cultures showed 100% acidic resistance when grown at pH 5 to 8 (Small *et al.*, 1994). It was illustrated that *ompC* and *ompF* mutant can significantly reduce AR1, but both OmpC and OmpF were not required for AR3, AR4. Moreover, glucose can support the AR in *ompC* and *ompF* mutant grown in LB medium without amino acid supplementation during the stationary phase (Fig.11).

I analyzed the expression of *rpos* and *crp* genes. Unexpectedly, neither *rpos* nor *crp* levels appeared to change in *ompC* and *ompF* deficient mutant
(data not shown). These results indicated that RpoS-Crp mechanism was not responsible for AR1 reduction in \textit{ompC} and \textit{ompF} deficient mutant.

Similar to the results obtained from the experiments with the logarithmic phase cells, the glutamate dependent AR was not affected by \textit{ompC} and \textit{ompF} mutant in the stationary phase cells grown in minimal EG (pH5.5). However, the \textit{ompC} and \textit{ompF} mutant strain could not induce arginine dependent AR under the same conditions (Fig.12). The results suggested that \textit{E. coli} with \textit{ompC} and \textit{ompF} deletion exhibits the same mechanisms in both logarithmic and stationary growth phases. Furthermore, the results represented clearly indicated that the medium components can affect AR systems in \textit{ompC} and \textit{ompF} deficient mutant.
Fig.10. Effect of glucose on the expression of \textit{ompC} and \textit{ompF} in the stationary phase. \textit{E. coli} was grown at pH5.5 in LB medium with or without 0.4% glucose for 22 hr. The $\beta$-galactosidase activity was assayed in at least three independent experiments, and is expressed in Miller units. Each bar represents mean ± S.D.
Fig.11. Effect of supplements on AR in *ompC* and *ompF* deficient mutants during the stationary phase in complex medium. MKW505 (*ompC*+ *ompF*+) (white bar), and ABC2010 (*ompC* *ompF*-) (black bar) were grown in LB medium at pH 5.5 for 22 hr, and their viability was measured after the acidic challenge at pH2.5 for 1 hr, as described in Material and Methods. Glucose (0.4%) was added except of the left experiment, and amino acids (1mM) indicated were added. The mean ±S.D. is represented.
Fig. 12. Effect of supplements on the AR in *ompC* and *ompF* deficient mutants during the stationary phase in minimal medium. MKW505 (*ompC*<sup>+</sup> *ompF*<sup>+</sup>) (white bar), and ABC2010 (*ompC*<sup>−</sup> *ompF*<sup>−</sup>) (black bar) were grown in E medium at pH 5.5 for 22 hr and their viability was measured after the acidic challenge at pH 2.5 for 1 hr, as described in Material and Methods. Glucose (0.4%) was added in all experiments, and amino acids (1 mM) indicated were added. The mean ± S.D. is represented.
Discussion

E. coli is generally considered to be a neutralophilic organism, but both commercial and pathogenic E. coli are capable of surviving in extremely acidic environments (pH 2.5) for several hours (Foster 2004, and Large et al., 2005). E. coli grown to the stationary phase became more resistant to acidic stress (Datta and Benjamin, 1999)

Several amino acid decarboxylases have been identified in E. coli (Gale, 1946), and glutamate, arginine, and lysine decarboxylases have been linked to the AR mechanisms dependent on these amino acids (Guilfoyle and Hirshfield, 1996). It was shown in a previous study that the glutamate-dependent AR system is essential for maximal protection of E. coli O157:H7 against both oxidative and acidic stress (Bearson et al., 2009). On the contrary, acidic adapted S. Typhimurium survived better at pH 2.5 in the presence of arginine or lysine, but glutamate did not exert any protective effect (Álvarez-Ordóñez et al., 2009). The prevailing hypothesis has been that protons entered into the cells are consumed by decarboxylase reaction of its α-carboxyl group (Molenner et al., 1993). However, the underlying mechanism has not been established.

OmpC and OmpF play important roles in the membrane transport of solutes such as amino acids in E. coli and other Gram-negative bacteria (Nikaido and Vaara, 1985). It was previously reported that a lack of porin proteins had a significant impact on E. coli in filtered natural sea water (Darcan et al., 2003). However, an E. coli mutant that was deficient in both ompC and ompF was shown to grow under conventional growth conditions (Kaeriyyama et al., 2006).
The roles of these porins may differ under different pH conditions because
the expression levels of ompC and ompF were obviously altered by
changes in pH (Heyde and Portalier, 1987). Wu et al (2009) have reported
the pH-related characterization of OstA, TolC, OmpT, OmpF, LamB,
OmpC, and OmpW, which have been reported to function as a porin.
Previous study had clarified that phosphorylation of OmpR (protein
regulates ompC and ompF expression) is dependent on pH and carbon
source (Heyde et al., 2000).
These previous observations led me to argue that bacterial OMPs play
multiple roles during bacterial response to pH changes. Concerning the
importance of OmpC and OmpF in acidic resistance, I constructed an
ompC/ompF double mutant to investigate their roles in the AR and
examined how the deletion of ompC and ompF affects the major acidic
resistance systems in this study. Our results clearly indicated that OmpC
and OmpF play integral roles in the induction of both AR3 (arginine-
dependent AR) and AR4 (lysine-dependent AR) in the logarithmic phase
under anaerobic conditions. In addition, OmpC and OmpF have a similar
role on AR3 in the stationary phase cells.
Prior work has shown that both arginine and lysine decarboxylases play a
crucial roles in acidic resistance in E. coli accompanied by Gad system
(Hirakawa et al., 2010). Our findings revealed that the expression of
gadA/gadB was not significantly affected by the deletion of ompC and
ompF, while adiA and cadBA were repressed in the ompC and ompF
double mutant. These results suggested that repression of AR3 and AR4 in
ompC and ompF deficient mutant is due to decreased expression of adiA
and cadB.
A previous observation suggested that porin proteins control the transportation of small hydrophilic molecules through the outer membrane (Pratt et al., 1996), but no previous report has shown that the transport activity of amino acids is influenced by the absence of OmpC and OmpF under near neutral conditions. Our results demonstrated that the conversion of arginine and lysine was repressed by the deletion of \textit{ompC} and \textit{ompF} more strongly than that in cells treated with Triton-X100 (Fig. 8), suggesting that the membrane permeability of arginine, lysine, and their decarboxylated amines through OmpC and OmpF was important for conversion of external amino acids to amines.

Why are OmpC and OmpF the main route for the amino acid transportation across the outer membranes under acidic conditions? Porins, which support the transportation at neutral pH, may not work under acidic conditions except of OmpC and OmpF. The expression of such porins might be repressed at acidic pH. Their pore size may decrease at low pH as reported by Todt and McGroarty (1992).

In contrast to arginine and lysine decarboxylations, the glutamate decarboxylation was not affected by such deletion. These results suggested that \textit{E. coli} has another route for glutamate transportation. In the absence of amino acids supplementation, the AR was decreased by the deletion of both \textit{ompC} and \textit{ompF}, leading me to conclude that the membrane transport of solutes other than amino acids is also essential for survival under acidic conditions. The direct measurement of the transport activity across the outer membrane may improve the conclusion. However, the rate limiting step of the solute import across the cell envelope may be the transport across the inner membranes in \textit{E. coli}. Furthermore, we have no way to prepare outer membrane vesicles for the transport activity. Thus, no method
for the direct measurement of the amino acid transport across the outer membranes is now available.

I found that glucose significantly repressed the expression of $ompC$ and $ompF$ at pH 5.5 in the stationary phase, while its repressive effect was particularly marked only on $ompF$ expression in the logarithmic phase. My results clearly indicated that the AR in the stationary phase is independent of $ompC$ and $ompF$ expression in presence of glucose. The extreme acidic resistance of *E. coli* in stationary phase is regulated by RpoS (Diez-Gonzalez and Russell 1999, Cheville *et al.*, 1996). I found that the expression level of $rpoS$ and $crp$ were not significantly altered in $ompC$ and $ompF$ deficient mutant (data not shown). It appears that OmpC and OmpF can control AR1 via unknown mechanism.

Recently Kieboom and Abee (2006) suggested that cells grown in rich medium at pH 5.5 to the stationary phase in the presence of glucose survive in minimal medium at pH 2.5 only in the presence of glutamate or arginine. Our results predict that ompC and ompF deletion has not any inhibitory effect on AR2 and AR3 in LB medium with and without glucose. My finding is in contrast with previous results that neither of $ompC$ nor $ompF$ gene, either singly or in combination, had any effect on the stationary phase AR of *S. Typhimurium* (Bang *et al.*, 2000). It is interesting that the stationary phase AR in the $ompC$ and $ompF$ deficient mutant is close to the logarithmic phase AR in minimal as well as complex media. It may be better to think that logarithmic phase and stationary phase are not well separated categories, the similar mechanisms for the AR are working in both phases.
Materials and Methods

Bacterial strains and growth conditions

*E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* cells were grown at 37°C in minimal EG medium consisting of E medium and 0.4% glucose. E medium contained 73 mM K$_2$HPO$_4$, 17mM NaNH$_4$HPO$_4$, 10 mM citric acid, and 0.8 mM MgSO$_4$ (Jensen, 1993). The medium pH was adjusted with HCl or KOH. When required, ampicillin (100 μg/ml), kanamycin (25 μg/ml), and chloramphenicol (20 μg/ml) were added.

Bacterial growth was monitored by measuring the absorbance of the culture media at 600 nm. The cell density at an absorbance of 0.3 was approximately 2×10$^8$ colony-forming units CFU/ml under our experimental conditions.

β-galactosidase assay

β-galactosidase assay was performed according to Miller (1972). Cells were cultured in LB medium to both of logarithmic and stationary phases. Then, 1ml of a culture was centrifuged 12000 rpm for 5min at 0°C. The pellet was suspended in 1ml Z buffer pH7.0 containing 100mM sodium phosphate, 10mM KCl, 1mM MgSO$_4$, and 50mM β-mercaptoethanol. Cells were treated by the addition 20ml of chloroform and 20ml of 0.1% sodium dodecylsulfate and were assayed for β-galactosidase activity by addition of 200ml of ONPG (4mg/ml) and stopped with 500ml Na$_2$CO$_3$ (1M) at predefined intervals. The activity was expressed in Miller Units.

Acid resistance assay

EG medium: Cells were precultured in LB medium at pH 7 were diluted
1,000-fold with EG medium at pH 5.5 and then cultured at 37°C until their absorbance at 600 nm reached 0.3 for the logarithmic phase or for 22 hours for the stationary phase. The cells were grown either aerobically with shaking or anaerobically in tightly capped 6-ml glass tubes filled with growth medium without shaking in a static incubator.

**LB medium:** Cells were precultured in LB medium at pH 7 were diluted 1,000-fold with LB medium buffered with 100mM MES at pH 5.5 and then cultured at 37°C until their absorbance at 600 nm reached 0.3 for the logarithmic phase or for 22 hours for the stationary phase. For glucose repressed oxidative system, cells were grown in unsupplemented LB-MES (pH5.5). The glutamate and arginine dependent AR systems were tested by growing cells in LB medium containing 0.4% glucose, which repress the RpoS- dependent oxidative systems, supplemented with 1mM glutamate or arginine (Castanie-Cornet *et al.*, 1999).

Next, the cultured cells from both media were then diluted 40-fold with EG medium at pH 2.4. The pH of the resulting medium was 2.5 ± 0.02. Acid challenge was carried out for 1 hour (logarithmic phase) and 2 hours (stationary phase) at pH 2.5 at 37°C, and then the culture medium was diluted with PBS consisting of 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄ at pH 7.4. The number of viable cells was determined after the cells had been plated onto LB agar plates, followed by overnight incubation at 37°C. AR was represented as the percentage of the number of viable cells after the acidic challenge at pH 2.5 to that before the challenge. Viability was measured three times in all experiments, and mean values and standard deviations were obtained.
RNA isolation and reverse transcription

After the *E. coli* cells had been grown in EG medium at pH 5.5 until their absorbance reached 0.3 under aerobic or anaerobic conditions, $5 \times 10^8$ cells were harvested. The total RNA was subsequently extracted using TRI reagent (Sigma) and contaminating DNA was digested using DNase I (Takara-Bio, Otsu, Japan) according to the manufacturer’s protocol. The concentration and purity of RNA were determined using a GeneQuant RNA/DNA Calculator (Pharmacia Biotech, Cambridge, England). Isolated RNA was stored at $-80^\circ$C until use. cDNA synthesis was performed in 20 μl of the reverse transcription (RT) buffer containing 0.2 μg of RNA, 60 ng of random hexamers (Invitrogen Corp, Carlsbad, CA, USA), 20 units of RNase inhibitor (TOYOBO, Osaka, Japan), 1 mM of each deoxynucleoside triphosphate, and 100 units of reverse transcriptase (ReverTra Ace; TOYOBO), according to the manufacturer’s protocol.

Reverse transcription-polymerase chain reaction RT-PCR.

The PCR mixture consisted of 0.5μl of Taq DNA polymerase (Bio-Academia), 2μl of 2 mM deoxynucleoside triphosphate mixture, 2μl of 10x ThermoPol buffer (NEB), 1μl of 10μM each primer, 1μl of cDNA, and 12.9μl of sterilized water. The Polymerase chain reaction (PCR) was performed using a PCR thermal cycler (Takara-Bio) under the following conditions: 94°C for 1 min, 58°C for 1 min, and 72 °C for 1.5 min. The similar results were obtained with the cycle numbers of 25 and 30, and the band densities were almost saturated with the cycle number of 35. Therefore, the cycle number of 30 was used in Fig. 4. The PCR products were stored at 4°C. The primer sets used are listed in Table 2. The PCR
products were separated on 6% polyacrylamide gel in 90mM Tris-borate buffer containing 2mM EDTA, at pH8.2, before being stained with ethidium bromide for visualization on a UV transilluminator.

**Measurement of decarboxylase activity**

Cells grown in the logarithmic growth phase in EG medium at pH 5.5 were harvested, washed with 0.9% NaCl, and resuspended at $5 \times 10^{10}$ cells/ml in the same solution. Decarboxylase activity was measured as described previously (Jung and Kim, 2003) with some modifications. An aliquot of cells ($2 \times 10^8$ cells) was suspended in 1 ml of the assay solution containing 0.9% of NaCl and 0.1% of glutamate, arginine, or lysine, with or without Triton X-100 (0.3%). The suspension was then incubated in a 37°C water bath, and the pH of the suspension was measured at 10 min intervals for 1 hour.

**Other Procedures**

P1 transduction was carried out using the P1kc phage as described previously (Lennox, 1955).
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKW505</td>
<td>MH621 <em>ompF</em>+</td>
<td>Kaeriyama et al., 2006</td>
</tr>
<tr>
<td>MH621</td>
<td>MH20 <em>Φ (ompF-lacZ) hyb.16-21</em> <em>ompF</em> <em>ompC</em>+</td>
<td>Sato et al., 2000</td>
</tr>
<tr>
<td>KAEC5</td>
<td>MH621 <em>ompF</em>+ <em>ompC</em>:: Km*</td>
<td>Kaeriyama et al., 2006</td>
</tr>
<tr>
<td>W3110</td>
<td>λ- F- derived from wild type</td>
<td>Lab stock</td>
</tr>
<tr>
<td></td>
<td><em>E. coli K-12</em></td>
<td></td>
</tr>
<tr>
<td>BW25113</td>
<td><em>lacIq rmnB</em>&lt;sub&gt;Y&lt;/sub&gt;ΔlacZ&lt;sub&gt;W&lt;/sub&gt;Δ hsrd514* ΔaraBAD&lt;sub&gt;AH33&lt;/sub&gt; ΔrhaBAD&lt;sub&gt;LD78&lt;/sub&gt;</td>
<td>Datsenko and Wanner, 2000</td>
</tr>
<tr>
<td>FBW25</td>
<td>BW25113 <em>ompF::Cm</em></td>
<td>Lab stock</td>
</tr>
<tr>
<td>WOF25</td>
<td>W3110 <em>ompF::Cm</em></td>
<td>Lab stock, W3110×P1 (FBW25)</td>
</tr>
<tr>
<td>ABC2010</td>
<td><em>MH621 ompF::Cm</em> <em>ompC::Km</em></td>
<td>This study, KAEC5×P1 (WOF25)</td>
</tr>
<tr>
<td>MF5.4</td>
<td>PS2209 *Φ(ompC-lacZ)16-13 <em>ompF</em>+</td>
<td>Lab stock</td>
</tr>
<tr>
<td>MF8.1</td>
<td>PS2209 *Φ(ompF-lacZ)16-13 <em>ompF</em>+</td>
<td>Lab stock</td>
</tr>
</tbody>
</table>

Plasmids

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pompF</td>
<td>pNT3-<em>ompF</em></td>
<td>mobile plasmid collection**</td>
</tr>
<tr>
<td>pompC</td>
<td>pNTR-SD-<em>ompC</em></td>
<td>mobile plasmid collection**</td>
</tr>
</tbody>
</table>

*Constructed by one-step gene disruption, as described previously (Datsenko and Wanner, 2000).

**Obtained from the National BioResource Project (National Institute of Genetics, Mishima, Japan): *E. coli*
Table 2. Primers used in this study

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer (5’ to 3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gadA</td>
<td>f: AGTTCGAAATGGACCAGAAGCTGT</td>
<td>266</td>
</tr>
<tr>
<td></td>
<td>r: GTCGATCCAGTTTTTATTGATCGAC</td>
<td></td>
</tr>
<tr>
<td>gadB</td>
<td>f: GGAGTTTTAAAATGGATAAGAAGCAAG</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td>r: TTGTCGATCCAGTTTTTGTTAATG</td>
<td></td>
</tr>
<tr>
<td>adiA</td>
<td>f: TAACTTCTCCCGCTTCAACC</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>r: AATCAACCCTTCGTCAATC</td>
<td></td>
</tr>
<tr>
<td>cadB</td>
<td>f: TCTTCTGTAATGGCGGCTTCC</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>r: CCTGGCCTACCAACATCATC</td>
<td></td>
</tr>
<tr>
<td>16S rRNA</td>
<td>f: GATCATGGCTCAGATTGAACG</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>r: CTACCGTTTCCAGTAGTTATCC</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 13. Schematic illustration of the mechanism of OmpC and OmpF role in AR
Conclusions

• OmpC and OmpF are essential for survival of *E. coli* under acidic stress.

• OmpC and OmpF play integral roles in the induction of both AR3 and AR4 under anaerobic conditions.

• OmpC and OmpF are the main route of entry for arginine and lysine under acidic conditions, but *E. coli* has another route for glutamate transportation.

• Glucose can increase AR via unknown mechanism.

• OmpC and OmpF have a role in the induction of AR1 during the stationary phase.

• Glucose has a greatly repressive effect on the expression of *ompC* and *ompF* in the stationary phase.

• AR of *ompF* and *ompC* deficient mutant is depended on the medium component more than the growth phase.
References


Higashitani A., Nishimura Y., Hara H., Aiba H., Mizuno T., Horiuchi K. (1993) Osmoregulatation of the fatty acid receptor gene *fadL* in


Lennox E.S. (1955) Transduction of linked genetic characters of the host by bacteriophage P1. *Virology.*, **1**, 190-206


- 47 -


List of publications

Acknowledgements

First, I would like to express my gratitude to my supervisor Professor Hiroshi Kobayashi, Department of Biochemistry, Graduate school of pharmaceutical science, Chiba University for his kindness, guidance, and continuous encouragement.

I express my thanks to Lecturer Hiromi Saito and Assistance Researcher Toshinhiko Fukamachi for valuable suggestion and helpful during laboratory work.

I am sincerely grateful to scholarship of the Ministry of High Education, Egypt.

I want to thanks all the students in the Biochemistry Department specially my close friend Yan for their kindness and assistance during my research work.

Sincere thanks and great gratitude for my husband Osama for his sustained support, understanding and helping me take care of my children throughout the accomplishment of this thesis.

Finally, I thank my children Reem, Kareem, and Nadeem for their patience, and bearing my absence from home for long periods during the study.
This doctoral thesis was reviewed by the following dissertation committee belongs to the Faculty of Pharmaceutical Sciences, Chiba University.

Members of the Doctoral Dissertation Committee which consists of a chief judge and two assistance

Chief examiner: Professor Naoto Yamaguchi

Assistant examiner: Professor Tomoko Yamamoto

Assistant examiner: Professor Toshihiko Murayama