Involvement of phospholipids in resistance and adaptation of *Escherichia coli* to acid conditions and to long-term survival

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Abstract

In *Escherichia coli* membranes, three major phospholipids are formed: phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL). We report here the survival of mutants lacking either PE or both PG and CL at an acid pH and during long-term survival experiments. Stationary phase cultures of *E. coli* lacking PE are much more sensitive to acid shock (pH 3) than the wild-type strain. Moreover, in the strain lacking PE, long-term survival in stationary phase is impaired and after 5 days no viable cells are recovered. The survival of an exponential phase culture to acid shock is known to be increased if the culture is exposed to moderately acid conditions (pH 5) prior to a shift to pH 3. If either PE or both PG and CL are missing, the exposure to pH 5 does not increase the survival at pH 3.

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1. Introduction

In nature, enteric bacteria are frequently subjected to adverse conditions such as starvation, high temperature, osmotic stress or exposure to acidic environments such as those encountered in the mammalian stomach. *Escherichia coli* entry into the stationary phase is associated with the acquisition of cross-protection to a variety of stresses [1,2].

*E. coli*, grown in Luria Broth (LB) up to the stationary growth phase, can resist several hours of exposure to pH 3. On the other hand, exponential growing cells are very sensitive to an exposure at pH 3 [3]. If, prior to this exposure to pH 3, cells are adapted during one doubling time to pH 5 and then shifted to pH 3, they are able to significantly resist this change in hydrogen ions concentration. This acid tolerance response in *Salmonella typhimurium* and *E. coli* involves acid-induced proteins that protect the cells from a lower acid shock (pH 3) [4,5].

Recently, a strong correlation was reported between the resistance of *E. coli* to a rapid decrease of the pH and the level of cyclopropane fatty acids (CFA), a major component of the phospholipids [6]. The main phospholipids found in *E. coli* membranes are composed of 79% phosphatidylethanolamine (PE), 19% phosphatidylglycerol (PG) and 2% cardiolipin (CL) [7,8]. Strains carrying the null allele of the *pss* gene (encoding phosphatidylserine synthase) completely lack PE, which is replaced by PG and CL [9]. These mutants are viable if grown in the presence of 50 mM MgCl₂ or if they carry a plasmid-borne copy of the *pss* gene on plasmid pDD72 [10]. Strains carrying the null allele of the *pgsA* gene (encoding phosphatidylglycerophosphate synthase) completely lack both PG and CL. These mutants are viable if a null allele of the *lpp* gene, encoding a major lipoprotein, is introduced into the cell [11]. Indeed, during its maturation, the lipoprotein receives the diacylglycerol moiety of PG on the Cys-21 residue, rendering it susceptible to signal peptidase II. In the absence of PG, this modification does not occur and the lipoprotein accumulates in the inner membrane. This accumulation would lead to a covalent linking between the inner membrane and peptidoglycan through the COOH-terminal lysine of the lipoprotein. This covalent linking disrupts the cell envelope integrity. Conse-
sequently, a *pgsA* mutant is viable only if Lpp is removed from the cell to avoid the disruption of cell envelope integrity [11].

Better survival of stationary phase cells to different stresses [12] and the involvement of CFA in acid resistance have prompted us to characterize long-term stationary phase survival and acid resistance and adaptation of *E. coli* cells lacking either PE or both PG and CL.

2. Materials and methods

2.1. Bacterial strains and plasmids

All bacterial strains were derivatives of *E. coli* K-12 and are listed in Table 1. Genetic markers were transferred among strains by phage P1vir transduction as described by Miller [13]. The Δ*lpp*-254 allele was introduced into MA3960 by selection for a linked transposon (zdh::Tn10).

The presence of the *lpp* deletion was detected by testing outer membrane permeability. Cells were streaked on LB plates containing 1.5% Torula Yeast RNA (Sigma). The leakage of periplasmic RNase I was estimated after overnight incubation by addition of 10% trichloroacetic acid.

The *pss-93::kan* and *pgsA::kan* alleles were introduced into MA3960 by selection for kanamycin resistance. Strains carrying the *pss-93::kan* null allele require, for viability, either a functional plasmid-borne copy of the *pss* gene (plasmid pDD72, temperature-sensitive for replication) or a growth medium containing 50 mM MgCl2. To produce PE-deficient derivatives, plasmid pDD72 was cured at 42°C from strain GPH12175 as described by De-Chavigny et al. [10].

Standard procedures were used for plasmid purification, restriction analysis, ligation and transformation.

2.2. Growth of bacteria

Cells were grown in LB medium at 37°C [13]. Media were supplemented with chloramphenicol (20 µg ml⁻¹), tetracycline (15 µg ml⁻¹) or kanamycin (50 µg ml⁻¹) when necessary.

2.3. Acid shock

The protocol was adapted from Chang and Cronan [6]. Acid shock was carried out on overnight cultures grown in LB medium. Cultures were 25-fold diluted and split into separate aliquots. 1 N HCl was added until a pH of 3–3.2 was reached. The pH values were monitored by pH measurements on an identical, but separate, culture. The same volume of LB was added to the untreated control. After acid addition, cultures were shaken at 37°C for 40 min, then diluted and plated for colony formation.

2.4. Acid adaptation

The protocol of acid adaptation was adapted from Chang and Cronan [6]. Acid adaptation was carried out on exponential growing cells in LB medium. Overnight cultures were 100-fold diluted and shaken for several hours at 37°C. As soon as the cultures reached an OD₆₀₀nm of 0.5, they were 100-fold diluted and shaken at 37°C until the OD₆₀₀nm reached 0.15. The cultures were adjusted to pH 5 by addition of 1 N HCl; unadapted cultures received LB. Growth was allowed to continue for 40 min, under shaking, at 37°C. Adapted and unadapted cultures were subjected to an acid shock (pH 3) as described above. At each step of adaptation and subsequent acid shock, cultures were diluted and plated for colony formation.

2.5. Colony formation

Viability was determined by plating 100 µl of serial dilution of cultures on three LB plates and counting the colonies after 24 h at 37°C. 50 mM MgCl2 was added to GPH12188 growth media.

### Table 1

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Source or derivation</th>
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<tr>
<td>Strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD903</td>
<td><em>pss-93::kan lacY lacZ recA srl::Tn10</em>pDD72 (pss⁺ Cam₈)</td>
<td>[10]</td>
</tr>
<tr>
<td>GPH12174</td>
<td>MA3960 Δ<em>lpp</em>-254 zdh::Tn10</td>
<td>This study</td>
</tr>
<tr>
<td>GPH12175</td>
<td>MA3960 *pss-93::kan pDD72 (pss⁺ Cam₈)</td>
<td>This study</td>
</tr>
<tr>
<td>GPH12177</td>
<td>MA3960 Δ<em>lpp</em>-254 zdh::Tn10 *pgsA::kan</td>
<td>This study</td>
</tr>
<tr>
<td>GPH12188</td>
<td>MA3960 *pss-93::kan</td>
<td>This study</td>
</tr>
<tr>
<td>HDL1001</td>
<td>*pgsA::kan [Δ(lacOP-pgsA¹) lacZ⁺ lacY::Tn9 recA srl::Tn10]</td>
<td>[9]</td>
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<tr>
<td>JC9780</td>
<td>ΔlacU169 metB hsdS supE gal Δ<em>lpp</em>-254 zdh::Tn10</td>
<td>This laboratory</td>
</tr>
<tr>
<td>MA3960</td>
<td>Hfr P4X thi metBI relA spoT1 phoS Δ(lacU169Δ(phoA20)</td>
<td>[14]</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pDD72</td>
<td>Carrying the wild-type <em>pss</em> gene, temperature-sensitive for replication, Cam (20 µg ml⁻¹)</td>
<td>[10]</td>
</tr>
</tbody>
</table>
3. Results and discussion

3.1. Role of phospholipids in long-term stationary phase survival

We investigated the viability of E. coli strains lacking either PE or both PG and CL during the stationary phase in LB medium (Fig. 1). GPH12174 (\(\Delta\)lpp-254) and GPH12175 (\(\Delta\)lpp-254 \(pss\)-93::\(kan\)/\(pss\)+) were the parental strains for GPH12177 (\(\Delta\)lpp-254, \(pss\)-93::\(kan\))/\(pss\)+) and GPH12188 (\(pss\)-93::\(kan\))/\(pss\)+) strains, respectively. A strain lacking PG and CL (GPH12177) exhibited a similar decrease in viability as the parental strain (GPH12174) during 7 days of stationary phase. After 7 days, the culture lost more than 95% viability. A strain lacking PE (GPH12188) reached a cellular density 2.5 times lower than that reached by the parental strain (GPH12175). During the same time, no loss in viability was observed for the parental strain (GPH12175). In the absence of PE, viability decreased regularly during the first days in stationary phase with, at each time point, a viability at least 100 times lower for the strain lacking PE than for the parental strain. After 6 days in stationary phase, no viable cells could be recovered from GPH12188 strain. The survival of parental strain was 10^8 colony-forming units (CFU) ml\(^{-1}\) after 6 days and still more than 10^5 CFU ml\(^{-1}\) after 7 days (Fig. 1).

3.2. In stationary phase, strains lacking PE are more sensitive to an acid shock

We tested the acid sensitivity of stationary phase cultures of E. coli strains lacking either PE or both PG and CL (Fig. 2). Overnight cultures grown in LB medium (pH 7) were adjusted to pH 3, held at this pH for 40 min and then samples were spread on LB plates. Our results showed that cells lacking Lpp (strain GPH12174) were as resistant to pH 3 as Lpp+ cells and that the presence of 50 mM MgCl\(_2\) in the growth medium had no effect on the survival rate of the parental strain (data not shown). The \(pss\)+ derivative of GPH12174 (strain GPH12177) had an acid survival rate two-fold lower than the parental strain whereas the \(pss\)- derivative of GPH12175 (strain GPH12188) became highly acid-sensitive (less than 0.1% survival). These data indicate that during the stationary growth phase, the phospholipid composition of the membrane plays a major role in the protection of E. coli from acid shock. PE provides the greatest protection and its absence cannot be compensated for by PG and CL.

3.3. Role of phospholipids in acid adaptation of exponentially growing cells

The data obtained with the parental strains confirm that early exponential phase cultures of E. coli were highly sensitive to an acid shock (Fig. 3). Indeed, exponential cells of parental strains GPH12174 (\(\Delta\)lpp-254) and GPH12175 (\(pss\)-93::\(kan\))/\(pss\)+) directly exposed to pH 3 exhibited survival rates of 0.05% and 0.04%, respectively (Fig. 3), compared to the almost 100% survival observed.
respectively (Fig. 3). As already described [3], the adapted to pHo 3, the survival rate became 6% and 34%, subjected to mild acid (pH 5) for one generation before incubation at pH 5. When parental strains were sub-synthesization step allowed for a $10^2$ to $10^3$ increase in the survival rate of the parental strain (data not shown).

Fig. 3. Acid survival of strains in the absence (–) of either PE or both PG and CL after acid adaptation. GPH12174 strain (Δlpp-254), GPH12177 strain (Δlpp-254 pgsA::kan), GPH12175 strain (pss-93::kan lpp-254) and GPH12188 strain (pss-93::kan) were grown at 37°C to early exponential phase (OD$_{600}$ nm 0.15) in LB medium, adapted or not to pH 5 for 40 min and then subjected to an acid shock (pH 3) for 40 min. The survival of adapted cultures (black column) relative to the acid shock was determined after 40 min at pH 3. The percentage of survival corresponds to the ratio between the number of viable cells present after the acid shock (pH 3) and after the adaptation step at pH 5 (or 7 for the control). Average values from three independent experiments were plotted. Error bars display the S.D.

during the stationary growth phase (Fig. 2). The tolerance of exponentially growing cells was previously described as being greatly increased if the culture was first adjusted to a moderately low pH (pH 5) and then allowed to grow during one doubling time before a shift to pH 3 [5]. This phenomenon, called acid adaptation, required the synthesis and accumulation of acid shock proteins (ASP) during the incubation at pH 5. When parental strains were subjected to mild acid (pH 5) for one generation before exposure to pH 3, the survival rate became 6% and 34%, respectively (Fig. 3). As already described [3], the adaptation step allowed for a $10^2$ to $10^3$ increase in the survival rate. When PG and CL (GPH12177 strain) or PE (GPH12188 strain) were lacking, the survival rates of early exponential phase cultures of E. coli were less than 0.1%, with or without adaptation at pH 5. The presence of 50 mM MgCl$_2$ in the growth medium had no effect on the survival rate of the parental strain (data not shown).

4. Conclusions

In this work, we demonstrate that acid adaptation was lost in exponential growing cells of strains lacking any of the phospholipids. The role of phospholipids may be to provide a membrane environment suitable for acid adaptation or may play a regulatory role which results from the induction of other gene products.

In stationary phase, the role of the major phospholipids (PE) may be more specific. Indeed, the substitution of the major phospholipid (PE) of the E. coli cell envelope, by the anionic phospholipids (PG and CL), results in reduced long-term survival and in a higher sensitivity to acid shock (pH 3) of stationary phase growing cells.

We demonstrate that the survival of an E. coli strain lacking the major phospholipids PE during long-term stationary phase is reduced during the first 5 days and from the day 6 no viable cells could be recovered. The absence of PG and CL does not reduce long-term stationary phase survival.

Chang and Cronan [6] have shown that the membrane CFA is a major factor in acid resistance of E. coli. CFA formation is a post-synthetic modification of bacterial membrane lipid bilayers produced by the onset of the stationary phase of a bacterial culture. CFA is formed by the CFA synthase encoded by the cfa gene. cfa derivatives are more sensitive to acid shock than wild-type cells and this higher sensitivity is associated with the lack of CFA [6]. It is not known if cyclopropane is incorporated into all phospholipids. The extreme sensitivity to an acid shock (pH 3) we observed in the absence of PE is very similar to the sensitivity observed with cfa strains. As CFA-containing phospholipids, PE may specifically contribute in the passive proton permeability of the membrane or in interactions with membrane proteins that are involved in proton efflux.

In stationary phase, compared to the parental strain, E. coli cells lacking the major phospholipids (PE) reach a lower cellular density, exhibit a higher sensitivity to acid shock and a decrease in long-term survival and are unable to recover after 5 days. An understanding of these mechanisms at the molecular level may allow for the development of new strategies to block the survival and virulence of pathogens.

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References


