Membrane Homeostasis in Bacteria upon pH Challenge

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Abstract

Bacteria are frequently exposed to acid stress. In order to survive these unfavorable conditions, they have evolved a set of resistance mechanisms, which include the pumping of protons out of the cytoplasm, the production of ammonia, proton-consuming decarboxylation reactions, and modifications of the membrane lipid composition. In this chapter, I will discuss the changes in membrane composition that have been described in bacteria to be part of the adaptation to acid stress conditions. The cytoplasmic membrane is a major barrier to proton influx in acid-treated cells. However, there is no single one membrane adaptation used by all

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bacteria in response to acid stress. Rather, different bacteria seem to use different strategies to adjust their membrane lipid composition in response to an increase in proton concentration.

1 Introduction

Bacteria are frequently exposed to varying environmental conditions such as, for example, changes in temperature, pH, or osmotic conditions and the presence or absence of nutrients and toxins. In order to cope with these stresses and to survive, they have adopted different response mechanisms. Examples for acidic environments in which bacteria are found are acidic soils, the gastric compartments of animals, the plant rhizosphere, and lytic vacuoles inside macrophages. In unprotected cells, a decrease in intracellular pH causes lowered enzyme activities, protein unfolding and denaturation, DNA damage, and membrane damage (Lund et al. 2014). The presence of an acid stress response provides the bacteria with an advantage in these otherwise life-threatening conditions. The most common mechanisms to resist acidic conditions are the pumping of protons out of the cytoplasm, the production of ammonia, proton-consuming decarboxylation reactions, and modifications of the membrane lipid composition (Kanjee and Houry 2013; Lund et al. 2014). In this chapter I will discuss the changes in membrane composition that have been described in bacteria to be part of the adaptation to acid stress conditions.

There is a lot of diversity of membrane lipid structures in the bacterial world, and there is no such thing as a typical bacterial membrane lipid composition (Sohlenkamp and Geiger 2016). Membranes are formed by amphiphilic lipids which in most cases and conditions are studied glycerophospholipids, but other lipids such as sphingolipids, hopanoids, and aminoacyl lipids have also been described. Membrane properties are defined in large part by the fatty acid structures that are incorporated into membrane lipids, although they also depend on other factors, such as headgroup composition. The cytoplasmic membrane is a major barrier to proton influx in acid-treated cells, and the modification of phospholipids in the inner membrane is a way to decrease proton permeability. Therefore, the ability to adjust membrane properties by modifying the types of fatty acids that are present in membrane lipids by altering the structure of preexisting membrane lipids or synthesizing new lipids is an important factor for bacterial survival under changing environmental conditions (Parsons and Rock 2013). As there is no typical bacterial membrane lipid composition, there is no single one membrane adaptation used by all bacteria in response to acid stress. Rather, different bacteria seem to use different strategies to adjust their membrane lipid composition in response to acid stress. Existing strategies can be classified in two groups: the modification of existing membrane lipids allows a quick response to acid stress, whereas the de novo synthesis of membrane lipids is a slower response.

2 Modification of Fatty Acids Composition

Membrane properties are defined in large part by the fatty acid structures that are incorporated into membrane lipids. The fatty acid composition of the membrane lipids depends on the growth conditions and the growth phase of the culture. The probably best-known example of how growth conditions affect the fatty acid composition is the maintenance of a similar membrane fluidity at different growth temperature (homeoviscous adaptation): membranes of *Escherichia coli* cells grown at 16 °C have a higher proportion of unsaturated fatty acids to saturated fatty acids than the membranes of cells grown at 37 °C. These adjustments allow that the fluidity of the membrane stays approximately constant at both growth temperatures although the fatty acid composition is changed. The acyl chain composition of the membrane lipids determines the viscosity of the membrane and adjustments in acyl chain composition are made to maintain the required biophysical properties. The proton permeability of the membrane of mesophilic bacteria is also controlled by the lipid composition.

Under conditions of acid stress and in the stationary growth phase, a conversion of unsaturated fatty acids to cyclopropane fatty acids (CFAs) (Fig. 1) has been observed in *E. coli* and several other bacteria. CFAs are formed from unsaturated fatty acids by adding a methyl group from *S*-adenosylmethionine (SAM) to the double bond, a reaction catalyzed by the enzyme cyclopropane fatty acid synthase (Cfa) (Chang and Cronan 1999; Kim et al. 2005). Transcription of the *cfa* gene is induced in *E. coli* under acidic conditions (Chang and Cronan 1999). *E. coli* membranes lacking CFAs have been shown to be more permeable to protons and showed a decreased ability to extrude protons (Shabala and Ross 2008), and mutants deficient in Cfa are very sensitive to a shift to low pH, but this sensitivity can be overcome by feeding CFAs exogenously. CFAs have biophysical properties similar to unsaturated fatty acids, but apparently they are more stable to environmental insults, such as acid stress. The acid tolerance of individual *E. coli* strains appears to be correlated with membrane CFA content (Brown et al. 1997).

Mutants deficient in synthesis of CFA also have been described and characterized in other bacteria, such as *Salmonella typhimurium*, *Sinorhizobium meliloti*, and *Brucella abortus* (Kim et al. 2005; Basconcillo et al. 2009; Palacios-Chaves et al. 2012). Gene expression under acid stress conditions is induced in *S. meliloti*, or mutants deficient in synthesis of CFA are affected in survival under acidic growth conditions in *Brucella* and *Salmonella*. CFAs in *S. meliloti* increase by 10–15% under acid stress, and the transcription of the *cfa2* gene is induced under these conditions (Basconcillo et al. 2009). A *Salmonella* mutant deficient in CFA production was sensitive to low pH. Kim et al. (2005) concluded that unsaturated fatty acids as well as CFAs are necessary for full acid resistance in *Salmonella*, although CFAs contribute more to acid resistance. In *Chlorobium tepidum*, the inhibitor sinefungin, an analog of *S*-adenosyl-L-methionine, was used to show the importance of CFAs in glycolipids for the acid resistance of chlorosomes (Mizoguchi et al. 2013). Among *Helicobacter* isolates those identified as gastric colonizers apparently tend to

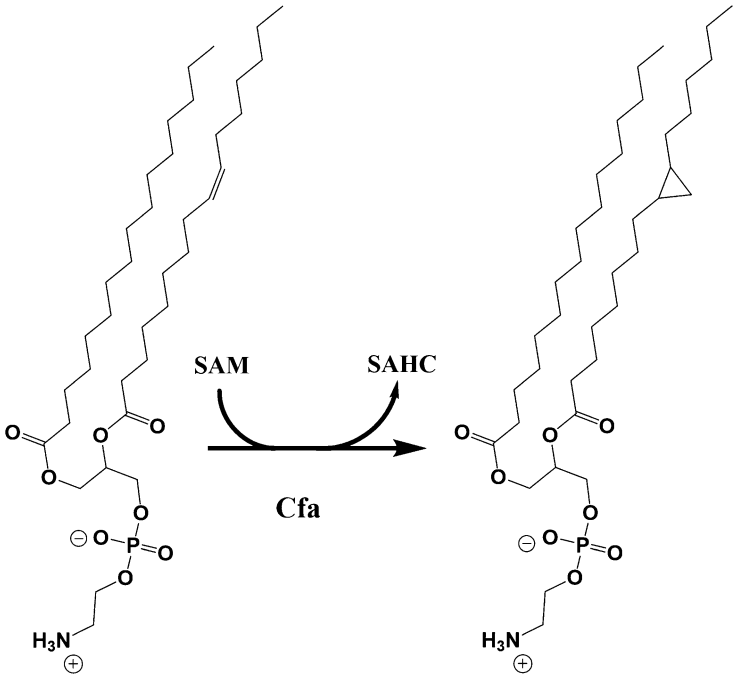

Fig. 1 Cyclopropanation of membrane lipid-bound unsaturated fatty acids. Depending on the growth phase or in response to acid stress, many bacteria can convert monounsaturated fatty acid into cyclopropane fatty acids (*CFAs*). These CFAs have biophysical properties similar to unsaturated fatty acids but are more stable to environmental stress conditions, such as low pH. Cyclopropanation occurs on membrane lipid-bound fatty acids, not on free fatty acids. In this example modification of the common phospholipid phosphatidylethanolamine is shown. The enzyme cyclopropane fatty acid synthase uses the methyl donor *S*-adenosylmethionine (*SAM*). There is no known mechanism to reverse the cyclopropanation of fatty acids; their content is diluted when the cells reenter logarithmic growth (Zhang and Rock 2008)

produce large amounts of CFAs. The isolates identified as intestinal colonizers do not produce large amounts of CFAs (Haque et al. 1996). But the presence/absence of CFA does not always influence acid resistance: *Pseudomonas putida* DOT-T1E mutants deficient in CFA formation were not affected under acidic growth conditions (Pini et al. 2009).

It is known that the cyclopropane bond is more stable than a double bond and that its presence affects proton permeability of the membrane, but the mechanism of how CFAs increase the resistance to acid stress is not completely clear yet. A molecular dynamics simulation has suggested that CFAs may fulfill a dual function: they stabilize membranes against adverse conditions while at the same time promoting their fluidity (Poger and Mark 2015).

Other adjustments in the fatty acyl composition of bacteria occurring under acid stress conditions also have been described. A few bacteria, especially gram-positives can also form branched chain fatty acids in addition to linear fatty acids. These

usually occur as iso- or anteiso-fatty acids. In general, the anteiso-fatty acids promote a more fluid membrane structure than the iso-fatty acids, because the methyl branch is further from the end of the fatty acid (Zhang and Rock 2008). *Listeria monocytogenes* has been shown to modify their iso- to anteiso-fatty acid ratio in response to temperature and pH stress. Under acid stress conditions, the formation of anteiso-fatty acids is increased in *L. monocytogenes* (Giotis et al. 2007).

In response to acidification, *Streptococcus mutans* increases the proportion of long chained monounsaturated fatty acids in its membrane with a concomitant decrease in short chained saturated fatty acids (Fozo et al. 2004; Fozo and Quivey 2004a). It has been shown that monounsaturated fatty acids are essential for survival at low pH (Fozo and Quivey 2004b). A *fabM* mutant deficient in the formation of unsaturated fatty acids is extremely sensitive to low pH in comparison to the wild type. This phenotype can be relieved by adding long-chain monounsaturated fatty acids to the medium (Fozo and Quivey 2004b). Similarly, in *Lactobacillus casei* ATCC 4646, an increase in long-chain monounsaturated fatty acids was observed when grown at acid pH, but in this strain also, the cyclopropane fatty acid lactobacillic acid was accumulated. Apparently, the acidic conditions caused a shift toward longer fatty acids, leading the authors to suggest that increased fatty acid length is an important membrane alteration to increase survival in acidic environments (Fozo and Quivey 2004b). A different response was reported for *L. casei* ATCC 334 (now called *Lactobacillus paracasei*): an increase in saturated and cyclopropane fatty acids was observed, whereas monounsaturated fatty acids showed a decrease (Broadbent et al. 2010).

Cyclopropane fatty acids apparently also play an important role in acidophilic bacteria such as *Acidithiobacillus ferrooxidans* and *Thiobacillus* sp. Levin (1971) and Kerger et al. (1986) have reported that the fatty acid profile of *Thiobacillus* species is characterized by high levels (>60%) of CFAs. Similar levels of CFAs are also reported in *A. ferrooxidans*, although lowering the pH from the optimal pH 2.0 further does not increase the amount of CFAs formed (Mykytczuk et al. 2010).

3 Changes in Membrane Lipid Types

In addition to modifications of fatty acids, other membrane modifications have been described in acid-stressed bacteria. There is a lot of diversity of membrane lipid structures in the bacterial world. Not all bacteria can form the same lipids and have an identical lipid composition. This might be the reason why different bacteria seem to use different strategies to adjust membrane lipid composition to changing environmental conditions. Most membrane lipids are glycerophospholipids, but other lipids such as sphingolipids and aminoacyl lipids have also been described. A function for a specific membrane lipid or membrane lipid modification under acid stress conditions has been usually concluded from the observation of increased formation of the specific lipid under acidic conditions or from the phenotype of mutants deficient in the formation of a specific membrane lipid. The presence of the following membrane lipids or an increase in their concentration under acidic stress

conditions has been related to a possible function in acid stress response: (A) formation of aminoacylated derivatives of phosphatidylglycerol, (B) presence of hopanoids, (C) increase of (hydroxylated) ornithine lipids, (D) presence and increase of sphingolipids, (E) modifications of lipopolysaccharide structure, and (F) modifications of cardiolipin concentration.

3.1 Formation of Aminoacylated Derivatives of Phosphatidylglycerol

The anionic phospholipid phosphatidylglycerol (PG) can be aminoacylated in some bacteria. Amino acids such as arginine, lysine, or alanine are transferred from a charged tRNA to the glycerol headgroup of phosphatidylglycerol thereby modifying an anionic lipid depending on the amino acid transferred into a zwitterionic lipid or a cationic lipid (Slavetinsky et al. 2016) (Fig. 2). This amino acid transfer is catalyzed

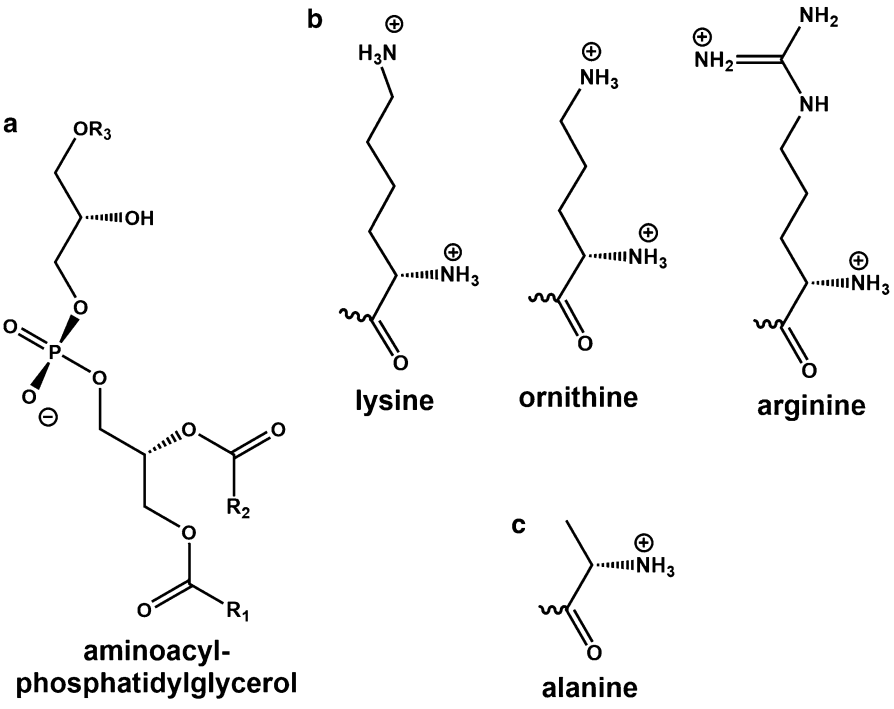

Fig. 2 Structure and headgroup variation of aminoacylated phosphatidylglycerol. Depending on which amino acid is ester bound to phosphatidylglycerol, the resulting lipid can be cationic (lysine, ornithine, arginine) or zwitterionic (alanine). (**a**) phosphatidylglycerol backbone and (**b** and **c**) amino acids that have been found in aminoacyl phosphatidylglycerol in different species. R_1 and R_2 alkyl chains of *sn1*- and *sn2*-bound fatty acids and R_3 amino acid ester bound to the glycerol headgroup of PG (shown in **b** and **c**)

by enzymes called multiple peptide resistance factor (MprF) or low pH inducible (LpiA). MprF has also been shown to have a flippase activity, flipping the cationic lipid lysyl-PG (LPG) from the cytoplasmic layer of the membrane to the periplasmic layer of the membrane (Ernst et al. 2009; Slavetinsky et al. 2012). In *Staphylococcus aureus*, *Enterococcus faecalis*, and *Rhizobium tropici*, the formation of the cationic phospholipid LPG is induced under acidic growth conditions (Houtsmuller and van Deenen 1965; Vinuesa et al. 2003; Sohlenkamp et al. 2007), and *P. aeruginosa* synthesizes significant amounts of alanyl-PG (APG) when exposed to acidic growth conditions but not under neutral growth conditions (Klein et al. 2009). Consistently, in a transcriptional study of the *Sinorhizobium meliloti* acid stress response, the *lpiA* gene was among the strongest induced genes (Hellweg et al. 2009). The transcriptional induction of the *mprF/lpiA* gene and the increase in the formation of the different aminoacylated PGs in different species indicate a possible function under acid stress, but growth of *P. aeruginosa* mutants deficient in APG was not affected under acidic growth conditions (Klein et al. 2009). In gram-negative bacteria, the gene encoding for LpiA is in most cases forming an operon with a gene coding for a putative serine lipase that has been shown to be a periplasmic protein in *Agrobacterium tumefaciens* and *P. aeruginosa* (Kang et al. 1994; Arendt et al. 2013). Often genes encoded in operons encode enzymes acting in the same metabolic pathway. Consistently, in *P. aeruginosa*, the enzyme PA0919 encoded by the second gene of this operon appears to have some minor APG hydrolase activity that should lead to the liberation of alanine in the periplasm (Arendt et al. 2013). It is not clear how this activity might be conferring acid resistance to the bacteria.

3.2 Presence of Hopanoids

Bacteriohopanepolyols, generally referred to as hopanoids, are triterpenoic, pentacyclic compounds. They are widespread in bacteria and can be found in the cytoplasmic and in the outer membrane. They are structurally similar to sterols such as cholesterol and are often considered their functional analogs in bacteria. The enzyme squalene-hopene cyclase is responsible for the formation of the simplest hopanoids, diplotene or diplopterol, from squalene. This basic structure can be modified leading to structural variations present in hopanoids: some are methylated, some are unsaturated, and the functionalization of the side chain can vary greatly (Sohlenkamp and Geiger 2016). It has been proposed that the presence of hopanoids enhances the membrane stability, decreases the membrane permeability, and increases stress tolerance in a few bacteria. A few studies have suggested that hopanoids are necessary for maintaining membrane integrity as well as coping with external stresses: ethanol tolerance in *Zymomonas mobilis*, oxygen diffusion in *Frankia*, and prevention of water diffusion in *Streptomyces* spores (Berry et al. 1993; Horbach et al. 1991; Poralla et al. 1984, 2000). In recent years many of the genes involved in hopanoid synthesis and modification have been identified, and mutants deficient in these genes have been constructed. Whereas *Burkholderia cenocepacia* and *Rhodopseudomonas palustris* mutants deficient in hopanoid formation showed

increased sensitivity to low pH and other environmental stresses; *Streptomyces scabies* mutants were not affected (Welander et al. 2009; Schmerk et al. 2011; Seipke and Loria 2009). In *Bradyrhizobium diazoefficiens* 2-methylated hopanoids contribute to growth under plant cell-like microaerobic conditions and acidic conditions in the free-living state. Hopanoids with a side chain are also required under various stress conditions including high temperature, low pH, and others (Kulkarni et al. 2015).

In vitro experiments have shown that hopanoids are efficient in condensing artificial membranes and enhancing the viscosity of liposomes (Poralla et al. 1984). It was demonstrated that lipid A undergoes a pH-dependent change in order to become less fluid at lower pH and to approach a gel state possibly causing the membrane to become leaky. Hopanoids are capable of interacting with lipid A and inhibiting this phase transition while at the same time helping to form a highly ordered bilayer (Sáenz et al. 2012, 2015). Hopanoid-producing gram-negative bacteria with disrupted hopanoid synthesis exhibit retarded growth at low pH (Welander et al. 2009; Schmerk et al. 2011).

Recently hopanoids have been found covalently bound to lipid A in a photosynthetic *Bradyrhizobium* strain (Silipo et al. 2014). These hopanoid lipid A hybrids reinforced the stability and rigidity of the outer membrane. A hopanoid-deficient strain displayed increased sensitivity to stressful conditions and reduced the ability to survive intracellularly in the host plant (Silipo et al. 2014).

Interestingly, the gram-negative bacteria lacking hopanoids were more sensitive to abiotic stresses than the respective wild type, whereas this was not the case for the gram-positive *S. scabies*. As there is no lipid A in *S. scabies*, hopanoid probably have a different function in the latter by interacting with other cellular structures, and this might be also the reason for the differences in phenotypes observed.

3.3 Increase of (Hydroxylated) Ornithine Lipids

Ornithine lipids (OLs) are amino acyl lipids, composed of an ornithine headgroup to the α-amino group of which a 3-hydroxy fatty acyl group is amide-bound. A secondary fatty acid is ester linked to the 3-hydroxy group of the first fatty acid. It has been predicted that OLs can be formed by about half of the sequenced bacteria, but they are absent from eukaryotes and archaea (Vences-Guzmán et al. 2015). OLs can be hydroxylated in various positions, but especially the 2-hydroxylation of the secondary fatty acid has been related to an increased acid and temperature resistance (Rojas-Jimenez et al. 2005; Gonzalez-Silva et al. 2011; Vences-Guzman et al. 2011). When *R. tropici* is grown at pH 4.5, more hydroxylated OLs are accumulated compared to growth at neutral pH. Mutants deficient in OlsC, the OL hydroxylase responsible for this modification, present a drastic growth phenotype at low pH (Vences-Guzmán et al. 2011). Another OL hydroxylase called OlsD was described in *B. cenocepacia* that is responsible for the 2-hydroxylation of the amide-linked fatty acid (Gonzalez-Silva et al. 2011). This species of OLs cannot be detected when cells are grown at neutral pH, but small amounts accumulate when the cells are grown at

pH 4.0, indicating that expression of the *olsD* gene is induced under low pH conditions. Both OlsC and OlsD are homologs of the lipid A 2-hydroxylase LpxO responsible for the alpha-hydroxylation of the 3′-myristic acid residue occurring in *S. typhimurium* lipid A under conditions mimicking the inside of vacuoles of macrophages (Gibbons et al. 2000, 2008). Nikaido has speculated that the presence of additional hydroxyl group in membrane lipids (like the ones introduced by OlsC, OlsD, and LpxO) might allow for the formation of additional hydrogen bonds which would cause a decrease in membrane fluidity and membrane permeability (Nikaido 2003).

3.4 Presence and Increase of Sphingolipids

Sphingolipids were thought for a long time to be absent from bacteria. Apparently, only a few bacteria mainly belonging to the *Cytophaga-Flavobacterium-Bacteroidetes* (CFB) group of bacteria and to the alphaproteobacteria are able to form sphingolipids. Evidence that the presence of sphingolipids in bacteria is related to an increased stress resistance comes from *Acetobacter malorum*. Ogawa et al. (2010) have shown that the sphingolipid content of its membrane increases under acidic growth conditions and at increased temperature (Ogawa et al. 2010). Only minor amounts of sphingolipid can be detected when the cells are grown at neutral pH, but ceramide formation is strongly induced at pH 4 or lower. No mutants deficient in sphingolipid synthesis have been constructed to confirm these suggestions.

A *Saccharomyces cerevisiae* mutant that formed sphingolipids only in the presence of the long-chain base phytosphingosine was sensitive to various abiotic stresses in the absence of sphingolipids: it failed to grow at 37 °C, in the presence of high salt concentrations and at low pH (Patton et al. 1992). A possible explanation for these phenotypes is that sphingolipids have a function in the response to abiotic stress.

3.5 Modifications of Lipopolysaccharide Structure

In general the outer membrane is not considered to play an important role in protecting bacteria against acidic conditions. Therefore there are only few reports relating to the importance of this structure under low pH stress. In *Shigella flexneri*, a pathogen causing diarrheal disease and dysentery among young children, it was shown that a mutant lacking O antigen was significantly more sensitive to extreme acid conditions than the wild type. In this bacterium, moderate acidic conditions induced the addition of phosphoethanolamine to the 1-phosphate group of lipid A. This modification contributed to an increased resistance to extreme acid conditions (Martinić et al. 2011).

The ability of *Salmonella typhimurium* to adapt to the acidic pH and the low divalent cation concentrations found inside macrophage vacuoles is critical for the

infection process. Among other changes occurring in the cells, lipid A is modified with phosphoethanolamine and 4-amino-arabinose, the acyl chains of lipid A are remodeled, and LpxO introduces a 2-hyroxylation on the 3′-secondary myristoyl chain (Gibbons et al. 2008; Henderson et al. 2016).

3.6 Modifications of Cardiolipin Concentration

In *Streptococcus mutans* the transcription of the gene encoding cardiolipin (CL) synthase is induced under acidic conditions, and *S. mutans* mutants deficient in CL formation are acid sensitive. In *S. mutans* wild-type monounsaturated fatty acids are important for acid resistance (Fozo and Quivey 2004a, b), and CL is the membrane lipid with major concentrations of these monounsaturated fatty acids. In *S. mutans* mutants deficient in cardiolipin synthase (Cls) that only reduced amounts of these fatty acids can be detected (MacGilvray et al. 2012). It is possible that the acid sensitive phenotype of the Cls mutants is caused by the decrease in monounsaturated fatty acids in the membrane. Furthermore it was observed that the absence of CL reduced acid resistance but does not prevent an acid-adaptive response (MacGilvray et al. 2012).

4 Conclusions and Research Needs

When bacteria are exposed to acid stress, they have to adapt to these adverse conditions, one important response is to make their membrane less permeable to protons. This apparently can be reached by making the membrane less fluid, denser, or thicker. Clearly, the most common membrane modification occurring in bacteria in response to acid stress is the cyclopropanation of monounsaturated fatty acids. Other common responses involve hopanoid formation and the introduction of hydroxyl groups into existing membrane lipids such as ornithine lipids, lipid A, or sphingolipids. Work is needed to completely understand the molecular mechanisms especially how the interactions between membrane molecules affect membrane properties such as proton permeability.

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