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The Absence of the “GATC - Binding Protein SeqA” Affects DNA Replication in *Salmonella enterica* Serovar Typhimurium

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*Faculty of Sciences of Bizerta, Carthage University, Tunisia*

1. Introduction

The aim of this chapter is to show the consequences of seqA gene disruption in *Salmonella typhimurium*. Of special interest is the observation that this disruption causes asynchrony of DNA replication. In fact, lack of SeqA protein causes major changes in the lipid profile of the membrane cell which is implicated in the initiation of DNA replication. Moreover, during passage through the small intestine in the host, *Salmonella typhimurium* faces periodic release of bile and due to the absence of SeqA protein the sensitivity to this bile increases. This unknown sensitivity could be due to an altered membrane composition of phospholipids and fatty acids.

Deoxyribonucleic acid (DNA) contains all the information required to build the cells and tissues of a prokaryotic or an eukaryotic organism. The exact replication of this information in any species assures its genetic continuity from generation to generation and is critical to the normal development of an individual. The information stored in DNA is arranged in hereditary units, known as genes, that control identifiable traits of an organism. Discovery of the structure of DNA and subsequent elucidation of how DNA directs synthesis of RNA, which then directs assembly of proteins—the so-called central dogma—were monumental achievements marking the early days of molecular biology. However, the simplified representation of the central dogma as DNA → RNA → protein does not reflect the role of proteins in the synthesis of nucleic acids. Moreover, proteins are largely responsible for regulating DNA replication and gene expression, the entire process whereby the information encoded in DNA is decoded into the proteins that characterize various cell types. One of these proteins is the DNA-Binding Protein SeqA.

1.1 SeqA: the DNA-binding protein

SeqA was discovered in some prokaryotes as a protein involved in the methylation / hemimethylation cycle of *Escherichia coli* DNA (Lu et al., 1994). SeqA binds to hemimethylated GATC sites formed by DNA replication and regulates activation of the *Escherichia coli* chromosome replication origin (Lu et al. 1994). Proper chromosome segregation also requires SeqA (Bach et al., 2003). Furthermore, SeqA trails the DNA replication fork and may contribute to nucleoid organization in newly replicated DNA (Brendler et al., 2000; Klungseyr & Skarstad, 2004; Løbner-Olesen et al., 2003; Yamazoe et al.,...
Aside from its roles in chromosome replication and nucleoid segregation, SeqA is known to regulate the transcription of certain genes. In bacteriophage lambda, SeqA activates the pr promoter in a GATC methylation-dependent fashion. SeqA also acts as a transcriptional coactivator by facilitating binding of the clI transcription factor to the lambda pI and pQ promoters. Competition between SeqA and the OxyR repressor for hemimethylated GATC sites has been shown to regulate phase variation in the Escherichia coli agr43 gene (Prieto et al., 2007). These examples raised the possibility that SeqA binding to critical GATC sites might likewise regulate the expression of prokaryotic genes like in Salmonella, which is a member of the Enterobacteriaceae family.

1.2 The genus Salmonella
Much of recent research is focused on Salmonella (figure 1) which causes diseases ranging from food and blood poisoning to typhoid fever and heart disease (Marinus, 1996).

Fig. 1. Salmonella typhimurium

Unlike its close relative Escherichia coli, Salmonella enterica is never encountered as a commensal in humans but is always associated with disease. Most Salmonella serovars capable of infecting humans cause gastro-intestinal disease and are spread via contaminated food and water. The serovar Typhi is unique in that, it is a strict human pathogen which gives rise to a severe systemic disease called typhoid fever. Salmonella enterica serovar Typhimurium (which will be referred to as Salmonella typhimurium throughout this chapter) causes a systemic disease in certain inbred mouse strains that share many similarities to typhoid fever in humans. The Salmonella infection is initiated upon ingestion of the bacteria. A small fraction of the bacteria survive the acidic environment in the stomach and establish an infection in the small intestine, where the Salmonella multiply and displace the normal flora bacteria. The bacteria then cross the intestinal epithelium by invading the M cells of the Peyer’s patches and enter the blood circulation. Systemic disease in both humans and mice is associated with the capacity of the bacteria to survive and replicate in macrophages and in the late stages of the disease, Salmonella can be found in large numbers in the liver and spleen. The murine typhoid fever model has been used extensively to study the interactions between pathogenic bacteria and their host.
Salmonella typhimurium represents a concern with regard to food safety due to its ability to grow in a wide range of adverse environmental conditions. Moreover, Salmonella typhimurium which contaminates foods may be derived from environments in which its previous growth occurs at moderate acidic conditions and, consequently, it may develop adaptive responses which enhance its resistance to other stress conditions occurring during food processing (Foster & Hall, 1990; Leyer & Johnson, 1993; Tosun & Gonul, 2003). One of the most important and intensively studied stress responses in Salmonella typhimurium is the acid tolerance response which increases its subsequent ability to survive in high acid foods, as well as in the extreme acid conditions of the gastrointestinal tract, increasing the risk of illness (Greenacre et al., 2003; Leyer & Johnson, 1992; Waterman & Small, 1998; Yuk & Schneider, 2006).

1.3 DNA replication in Salmonella typhimurium

In Salmonella typhimurium, the initiation of replication of chromosomal DNA is coordinated with cell division. It was demonstrated that DNA replication in bacterial cells is initiated on membranes and the activities of replication proteins are regulated by membrane components (Jacob et al., 1963). Indeed, initiation of DNA replication is precisely regulated in the cell cycles (Messer & Weigel, 1996). The SeqA protein seems to be one of the key proteins in the control of this process (Lu et al., 1994; Wold et al., 1998). It is an inhibitor of the onset of Escherichia coli chromosome replication in vivo (Boye et al., 1996; Slater et al., 1995) and, at high concentrations, of the replication initiator protein, DnaA in vitro, but it may stimulate replication at low DnaA concentrations in vitro (Wold et al., 1998). It affects DNA topology and inhibits open complex formation at the replication origin (Kang et al., 2003; Torheim & Skarstad, 1999). It was demonstrated that SeqA limits in vivo DnaA activity in replication from the chromosomal origin oriC (Von Freiesleben et al., 1994). Moreover, SeqA protein is essential for sequestration, which affects oriC in the newly replicated hemimethylated state (Lu et al., 1994; Slater et al., 1995). Disruption of the seqA gene of Salmonella typhimurium causes filament formation, aberrant nucleoid segregation, induction of the SOS response, envelope instability, and increased sensitivity to membrane-damaging agents like bile salt. These defects are similar to those described in Escherichia coli. Recent results (Prieto et al., 2007) indicated that lack of SeqA renders Salmonella enterica sensitive to sodium cholate (ox bile extract) but the cause of this sensitivity remains unknown. In the next sections, we show the importance of DNA sequestration by describing in detail the implication of SeqA in the replication synchrony. Then, we demonstrate the changes in the lipid profile of the membrane cell after seqA mutation and we expose explanations of increasing bile sensitivity of Salmonella typhimurium, in seqA mutant. In final section, we summarize our paper and present the future directions of our research.

2. DNA replication and membrane sequestration

The initiation of chromosomal replication occurs only once during the cell cycle in both prokaryotes and eukaryotes. This initiation is the first and tightly controlled step of a DNA synthesis. Because much of what is known about the regulation of the initiation of bacterial chromosomal replication comes from studies of Escherichia coli, this review focuses mainly on regulatory mechanisms in Salmonella typhimurium.
2.1 What is DNA sequestration by SeqA protein?

Replication of the bacterial chromosomal DNA initiates only once, at a specific region known as the origin of chromosomal replication oriC, by the initiator protein DnaA. This protein interacts specifically with 9-bp non-palindromic sequences (DnaA boxes) that exists at oriC. To ensure that initiation at an origin occurs only once per cell cycle, specific mechanisms exist to control chromosomal replication. In one mechanism, the SeqA protein that is tightly bound to hemimethylated DNA by a mechanism known as sequestration and which recognizes GATC sequences overrepresented within oriC and prefers binding to hemimethylated over binding to fully or unmethylated oriC (Figure 2).

Fig. 2. DNA sequestration by SeqA

The chromosomal DNA is methylated at adenine residues in GATC sequences by Dam methylase. Following passage of the DNA replication fork, GATC sites methylated on the top and bottom strands in a mother cell (denoted as fully methylated) are converted into two hemimethylated DNA duplexes: one methylated on the top strand and nonmethylated on the bottom strand and one methylated on the bottom strand and nonmethylated on the top strand due to semi-conservative replication. Most GATC sites are rapidly remethylated by the enzyme DNA methyltransferase (Dam methylase or Dam) and exist in the hemimethylated state for only a fraction of the cell cycle (Figure 3).

Exceptions are the DNA replication origin of Salmonella typhimurium, the dnaA promoter, and possibly additional GATC sites in the chromosome which bind SeqA. SeqA preferentially binds to clusters of two or more hemimethylated GATC sites spaced one to two helical turns apart (Figure 4).

In the case of oriC, sequestration delays remethylation and prevents binding of the DnaA protein, which controls the initiation of DNA replication. At other sites, binding of SeqA tetramers to hemimethylated GATC sites may organize nucleoid domains. Notably, the transcription profile of a Salmonella typhimurium SeqA mutant was found to be similar to that of a Dam overproducer strain. Based on this observation, a model was developed in which Dam and SeqA compete for binding to hemimethylated DNA generated at the replication fork.
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2.2 Effects of *seqA* mutation on DNA replication

As we said before, following the replication fork progression and the nascent strand synthesis, the daughter DNA becomes hemimethylated. SeqA protein binds to the hemimethylated GATC sequences (hemi-sites) and performs various roles to control the cell cycle progression. Immediately after the initiation of replication SeqA binds to the replicated oriC and sequesters it from remethylation and reinitiation of replication at the replicated oriC. SeqA tracks replication forks as a multiprotein complex and contributes to the maintenance of superhelicity and decatenation of daughter chromosomes through the stimulation of topoisomerase IV and results in a synchronous replication.

When rounds of replication are allowed to run to completion, the number of chromosomes per cell is 2n (n = 0, 1, 2, 3, etc). When initiations are asynchronous, as in *dnaA* (Ts) initiation mutants at the permissive temperature and in the *Escherichia coli* dam mutant (Boye &
Lebner-Olesen, 1990; Skarstad et al., 1988), the presence of a different number of chromosome equivalents (three, five, six, etc.) was detected by flow cytometry. The presence of cells containing a number of chromosomes different from 2n suggests that the seqA mutant has a defect in the synchrony of replication initiation. Wild type and seqA mutant of *Salmonella typhimurium* growing exponentially in glucose-casamino acid medium were treated with rifampicin and cephalaxin, which block initiation of replication and cell division respectively. Wild-type cells initiated replication synchronously (number of chromosomes per cell is 2n). The appearance of cells with chromosome numbers other than 2n indicates a moderate asynchrony of initiation. So, flow cytometer analysis of our seqA mutants has shown that replication initiation is asynchronous and can occur throughout the cell cycle, not only at the normal cell age for initiation. The most likely reason for this asynchrony phenotype is that secondary initiations occurred at newly replicated origins in seqA mutants, due to lack of sequestration and inadequate methylation. We showed that initiation synchrony was dependent on intact GATC methylation sites. This loss of synchrony affected culture growth rates and cell size distributions only slightly and suggest that seqA mutants have a slight defect in synchronizing replication initiation. All these results suggest that DNA sequestration plays a role in preventing the occurrence of multiple initiations at a single origin in the same replication cycle. However, using flow cytometry, we found that the asynchrony of initiation, which is one of the phenotypes of the seqA mutation, was returned to almost normal in a seqA null mutant harboring the wild-type seqA gene under the control of a tac promoter.

The OFF- to ON-phase rate was reduced in a seqA mutant, but much of this effect could be accounted for by a reduction in the Dam/DNA ratio caused by increased asynchronous initiation of DNA replication that occurs in the absence of SeqA, which normally sequesters oriC and plays a critical role in timing of DNA replication (Bogan & Helmsstetter, 1997).

3. Membrane instability after seqA disruption

The origin of replication, oriC, is highly enriched in GATC sequences, which are sites for methylation by Dam methylase. Semi-conservative replication of fully methylated DNA generates hemimethylated oriC sites.

3.1 Membrane sequestration hemimethylated of oriC

Early studies demonstrated that membranes are capable of binding to hemimethylated oriC in vitro and in vivo, but not to fully methylated or unmethylated oriC (Ogden et al., 1988). While they are sequestered at the membrane, the recently replicated origins are unavailable for reinitiation and are protected from methylation by Dam methylase for an extended period. The origins remain sequestered until conditions in the cell are no longer in a state supportive for initiation (Figure 5).

Prior to initiation of DNA replication, Dam methylase sites are fully methylated. Immediately following replication, the newly synthesized strand is unmethylated, and the resulting hemimethylated origin is sequestered at the at lipid bilayer of membrane by SeqA. This is not accessible to replatively active ATP–DnaA. After approximately one-third of the cell cycle, the sequestered origin is released and methylated by Dam methylase. At this point in the cell cycle, the levels of ATP–DnaA are not sufficient to catalyze a new round of replication. As such, sequestration serves as a mechanism to prevent secondary initiations. Subsequent work identified SeqA protein to be an essential factor for oriC sequestration.
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Fig. 5. Membrane sequestration of recently replicated origins.

Even though the first steps of SeqA purification involve liberating SeqA from the membrane fraction of cell lysates by treatment with high concentrations of salt and sonication, the primary sequence for SeqA protein does not suggest any obvious membrane-associating domains. This is supported by the crystal structure of the C-terminal DNA-binding domain, and by biochemical studies that show that the N-terminal domain serves in the aggregation of SeqA protein into functional homotetramers (Guarné et al., 2002). Yet, there is some evidence that SeqA has an association with membranes (d’Alencon et al., 1999; Wegrzyn et al., 1999). The original data that newly replicated, hemimethylated origins are
whether membrane sequestration of oriC occurs directly through the SeqA protein or through an as yet unidentified factor remains unclear. We speculated that the examination of fatty acids composition and phospholipids fractions in wild type and seqA mutants would provide useful information to understand the interaction between SeqA protein and bacterial membrane in Salmonella typhimurium.

3.2 Effects of seqA mutation on membrane lipids

The coordination of the synchronization of the replication initiation, the activation of the DnaA protein at oriC, and the cellular cycle suggested the existence of a very narrow interaction between the bacterial membrane lipids and the SeqA protein (Landoulsi et al., 1990). Acidic phospholipids, such as cardiolipin and phosphatidylglycerol, decrease the affinity of adenine nucleotide for DnaA protein (Mizushima et al., 1997; Sekimizu & Kornberg, 1988). Thus, it has been proposed that phospholipids regulate the activity of DnaA protein in cells and in vitro (Makise et al., 2002; Sekimizu & Kornberg, 1988). It has been demonstrated that the seqA mutation can overcome the incompatibility phenotype observed between the chromosomal oriC and minichromosomal oriC copies in the dam mutant strain (Lobner-Olesen & Von Freiesleben, 1996). The mutation in the seqA gene allows efficient transformation of fully methylated minichromosomes into dam mutant cells (Lu et al., 1994; Von Freiesleben et al., 1994). We can suggest a possible interaction between the activities of SeqA protein and membrane lipids. We analyzed the phospholipids and the fatty acids composition of the bacterial membrane with the aim of correlating the membrane structure variation in this lipids with seqA gene mutation.

The Phospholipids extracted from the bacterial membrane were separated and identified by thin layer chromatography. The content of each phospholipid was calculated from the fatty acids contents measured by the capillary gas chromatography method and is reported in the following section. The phospholipids found were phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin on the basis of the following criteria:

- Identity of chromatographic behavior in thin layer chromatography with synthetic and purified commercial phospholipids from various sources;
- The flow rate is the same as that of commercial phospholipids; and
- The phospholipids are the same as those reported by several authors and works (Ames, 1968).

**Phospholipids composition of Salmonella typhimurium wild type membrane**

The major phospholipids present in Salmonella typhimurium wild type strain membrane were phosphatidylethanolamine, accounting for about 75.2%, followed by phosphatidylglycerol and cardiolipin (19.4% and 5.3%, respectively) (Figure 6.a). These phospholipids distributions agreed very closely to those reported in the literature for Salmonella typhimurium (Ames, 1968).

**Phospholipids composition of the Salmonella typhimurium seqA mutant membrane**

Phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin proportions were affected by the seqA mutation while comparing them with the wild type strain. In the seqA mutant, the zwitterionic phosphatidylethanolamine fraction decreased from 75.2% to 20.53%. However, the acidic phospholipid fractions (phosphatidylglycerol and cardiolipin) becomes a majority of total phospholipids with 79.47%, distributed in 70.6% of phosphatidylglycerol and 8.8% of cardiolipin (Figure 6.b).
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**Fig. 6.** Comparative analysis of percentage of phospholipids levels in wild type (a) and seqA (b) *Salmonella typhimurium* strains. Their contents were calculated from the fatty acid contents measured by the capillary gas chromatography method. Average values of triplicates were given, and the deviation was less than 5% of each value (significance was assessed using the Student’s t-test).

The membrane fatty acid composition of the *Salmonella typhimurium* wild type strain was determined by the capillary gas chromatography method. Many fatty acids were found and seven main peaks were identified by comparing their retention times with those of known standards. Three saturated fatty acids were tetradecanoic (myristic) acid (C\(_{14:0}\)), hexadecanoic (palmitic) acid (C\(_{16:0}\)), and octadecanoic (stearic) acid (C\(_{18:0}\)), two monounsaturated fatty acids were hexadecenoic (palmitoleic) acid (C\(_{16:1\omega7}\)) and octadecenoic (oleic or vaccenic) acid (C\(_{18:1\omega9}\)), and two cyclic fatty acids were the cis-9,10-methylenehexadecanoic acid (cycC\(_{17}\)) and the cis-9,10-methyleneoctadecanoic (lactobacillic) acid (cycC\(_{19}\)). Their relative percentages were between 2% and 46% corresponding to more than 96% of all fatty acids observed. Some other minor fatty acids were also detected at lower relative concentrations: C\(_{17:0}\), C\(_{18:2\omega6}\), C\(_{18:3\omega6}\), C\(_{18:3\omega3}\), C\(_{19:0}\), and C\(_{20:0}\).

- **Fatty acid composition of Salmonella typhimurium wild type membrane**
  
  In the wild type strain, C\(_{16:0}\), C\(_{16:1\omega7}\), and C\(_{18:0}\) were the main constituents, representing about 60% of total fatty acids. The proportion of total lipid cyclic fatty acids obtained was about 25.79%. However, minimum cyclic fatty acids levels were observed for phosphatidylethanolamine and Cardiolipin (6.43% and 5.08%, respectively) and higher one for phosphatidylglycerol (38.35%). The unsaturated to saturated fatty acids ratio was in the majority with respect to between the phospholipid fractions (table 1).
Table 1. Membrane fatty acid composition (molar percent) in total lipids and different phospholipid classes in the Salmonella typhimurium wild type strain (MUFA: Monounsaturated fatty acids; SFA: Saturated fatty acids; UFA: Unsaturated fatty acids; CFA: Cyclic fatty acids; UFA/SFA: Unsaturated to saturated ratio).

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Total lipids</th>
<th>Phosphatidylethanolamine</th>
<th>Phosphatidylglycerol</th>
<th>Cardiolipin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{14:0}</td>
<td>4.20 ± 0.09</td>
<td>4.75 ± 0.1</td>
<td>4.2 ± 0.005</td>
<td>11.27 ± 0.13</td>
</tr>
<tr>
<td>C_{16:0}</td>
<td>46.61 ± 0.22</td>
<td>53.16 ± 0.26</td>
<td>35.68 ± 0.61</td>
<td>65.2 ± 1.04</td>
</tr>
<tr>
<td>C_{16:1 w7}</td>
<td>7.16 ± 0.47</td>
<td>8.54 ± 0.31</td>
<td>5.43 ± 0.77</td>
<td>7.04 ± 0.06</td>
</tr>
<tr>
<td>cyc_{17}</td>
<td>7.53 ± 0.6</td>
<td>6.43 ± 0.24</td>
<td>10.70 ± 0.21</td>
<td>2 ± 0.51</td>
</tr>
<tr>
<td>C_{18:0}</td>
<td>8.51 ± 0.91</td>
<td>11.3 ± 0.04</td>
<td>8.80 ± 0.08</td>
<td>5.90 ± 0.09</td>
</tr>
<tr>
<td>C_{18:1 w9}</td>
<td>2.07 ± 0.01</td>
<td>2.45 ± 0.21</td>
<td>3.28 ± 0.31</td>
<td>2.24 ± 0.04</td>
</tr>
<tr>
<td>cyc_{19}</td>
<td>18.26 ± 0.38</td>
<td>19.17 ± 0.63</td>
<td>27.65 ± 0.59</td>
<td>3.08 ± 0.82</td>
</tr>
<tr>
<td>MUFA</td>
<td>5.66</td>
<td>4.37</td>
<td>4.26</td>
<td>3.27</td>
</tr>
<tr>
<td>∑SFA</td>
<td>59.32</td>
<td>59.04</td>
<td>48.68</td>
<td>82.37</td>
</tr>
<tr>
<td>∑UFA</td>
<td>9.23</td>
<td>10.99</td>
<td>8.71</td>
<td>9.28</td>
</tr>
<tr>
<td>∑CFA</td>
<td>25.79</td>
<td>25.6</td>
<td>38.35</td>
<td>5.08</td>
</tr>
<tr>
<td>UFA/SFA</td>
<td>0.155</td>
<td>0.186</td>
<td>0.179</td>
<td>0.113</td>
</tr>
</tbody>
</table>

To determine whether the mutation in the seqA gene affected membrane lipid components, fatty acid composition was quantified. Our results indicated that the fatty acid composition of the total lipids appeared to be unaffected by the seqA mutation (table 2). The loss of cardiolipin and phosphatidylethanolamine was accompanied with a decrease in the proportion of C_{14:0}, C_{16:0}, and C_{16:1 w7} and an increase in the proportion of C_{18:0} especially for the cardiolipin phospholipid (from 5.9% to 41.93%). Compared with the isogenic wild type strain, cardiolipin and phosphatidylethanolamine phospholipids showed an increase in the percentages of cyc_{19} and a decrease in their C_{18:1 w9}, which resulted in low level of acyl chain unsaturation of fatty acids (table 2). The phosphatidylglycerol fraction showed a great increase of both C_{16:0} and cyc_{17} and a decrease in C_{16:1 w7}, which resulted in a low unsaturated to saturated fatty acids ratio (table 2).

Various physiological and biochemical changes took place as a consequence of many gene mutations, which can lead to numerous damages in the structure and function of the membrane cells (Shibuya et al., 1985; Taylor & Cronan, 1976). The purpose of the work presented in this section was to investigate a possible connection between both the seqA gene (coding for the sequestration protein SeqA) and some membrane components in Salmonella typhimurium. The phospholipids and fatty acids were the object of attention because the membrane delimiting the cell, and presumably playing a key role in DNA replication, is supposed to be constituted largely of lipids. Interactions of SeqA protein with cellular membranes have been previously reported. However, although regulation of the activities of this protein by membranes or their components was reported (Oshima et al., 2002) or suggested (Slater et al., 1995; Wegrzyn et al., 1999), little is known about the influence of SeqA on the composition of Salmonella typhimurium cell membranes. So, we
suggest that in addition to its direct role in the sequestration of oriC region of the chromosome on the membrane, SeqA could activate or impair the expression of some genes (e.g., STM1329: putative inner membrane protein and yijP: putative integral membrane protein, respectively) that interact with lipid metabolism and regulate acidic phospholipids synthesis.

### Fatty acids

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Total lipids</th>
<th>Phosphatidylyethanolamine</th>
<th>Phosphatidylglycerol</th>
<th>Cardiolipin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{14:0}</td>
<td>3.74 ± 0.18</td>
<td>4.19 ± 0.25</td>
<td>2.77 ± 0.21</td>
<td>4.85 ± 0.87</td>
</tr>
<tr>
<td>C_{16:0}</td>
<td>42.81 ± 0.14</td>
<td>45.8 ± 0.32</td>
<td>54.25 ± 0.13</td>
<td>36.38 ± 0.38</td>
</tr>
<tr>
<td>C_{16:1 w7}</td>
<td>4.24 ± 0.37</td>
<td>2.53 ± 0.57</td>
<td>1.38 ± 0.02</td>
<td>4.13 ± 0.24</td>
</tr>
<tr>
<td>cyc_{17}</td>
<td>11.6 ± 0.11</td>
<td>20.63 ± 0.13</td>
<td>13.70 ± 0.08</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td>C_{18:0}</td>
<td>12.96 ± 1.06</td>
<td>1.15 ± 0.17</td>
<td>4.08 ± 0.89</td>
<td>41.93 ± 0.01</td>
</tr>
<tr>
<td>C_{18:1 w9}</td>
<td>1.5 ± 0.12</td>
<td>0.40 ± 0.18</td>
<td>0.45 ± 0.07</td>
<td>0.000</td>
</tr>
<tr>
<td>cyc_{19}</td>
<td>20.7 ± 0.27</td>
<td>24.11 ± 1.06</td>
<td>22.42 ± 0.98</td>
<td>9.77 ± 0.49</td>
</tr>
<tr>
<td>MUFA</td>
<td>2.45</td>
<td>1.19</td>
<td>0.95</td>
<td>2.59</td>
</tr>
<tr>
<td>(\sum) SFA</td>
<td>59.51</td>
<td>51.14</td>
<td>61.10</td>
<td>83.16</td>
</tr>
<tr>
<td>(\sum) UFA</td>
<td>5.74</td>
<td>2.93</td>
<td>1.83</td>
<td>4.13</td>
</tr>
<tr>
<td>(\sum) CFA</td>
<td>32.30</td>
<td>44.74</td>
<td>36.12</td>
<td>10.12</td>
</tr>
<tr>
<td>UFA/SFA</td>
<td>0.096</td>
<td>0.057</td>
<td>0.030</td>
<td>0.050</td>
</tr>
</tbody>
</table>

Table 2. Membrane fatty acid composition (molar percent) in total lipids and different phospholipid classes in the *Salmonella typhimurium seqA* mutant strain.

### 4. Increased sensitivity of membrane to bile salt after seqA disruption

The overall purpose of this last section was to study the modifications of the cell membrane compounds of *Salmonella typhimurium* during the growth in the presence of ox bile doses. The results obtained evidenced that the tested substances induced noticeable modifications of the phospholipids and fatty acids composition of cell membrane during bacterial growth.

#### 4.1 Phospholipids composition of bile treated seqA mutants

Exposed to the ox bile, and compared with the non treated cells, our results indicated that the phospholipids composition of the bile treated wild type strain was in the majority with respect to the non treated wild type strain. So it appeared to be unaffected by the ox bile stress. The acidic phospholipid fractions (phosphatidylglycerol and Cardiolipin) account for 25.4% of total Phls distributed in 20.7% of phosphatidylglycerol and 4.7% of Cardiolipin. A non significant decrease in the phosphatidylethanolamine fraction (74.6%) was observed. To evaluate the combined effects of the seqA mutation and the ox bile stress on the bacterial membrane integrity, we compared the phospholipids composition of the exposed seqA mutant with the non exposed seqA and wild type strains. Compared with these two *Salmonella typhimurium* strains, the acidic phospholipids (phosphatidylglycerol and Cardiolipin) showed a great increase with 81.7% and 14.1%, respectively. However, the phosphatidylethanolamine proportion decreased dramatically to 4.2% (Figure 7).
Fig. 7. Comparative analysis of phospholipid levels in *Salmonella typhimurium* wild type and seqA strains (control and exposed to ox bile). Exponentially growing wild type strain was incubated at 37°C with ox bile extract at a concentration of one percent. Their contents were also calculated from the fatty acids contents. Average values of triplicates were given, and the deviation was less than 5% of each value (significance was assessed using the Student’s *t*-test).

4.2 Effect of the ox bile combined to the seqA mutation on membrane fatty acids composition of *Salmonella typhimurium*

The membrane fatty acids composition (molar percent) of the wild type and seqA mutant strains exposed to ox bile is shown in table 3.

- **Membrane fatty acids composition of the wild type strains exposed to the ox bile.**
  
  For the wild type strain cultured with the ox bile, no significant changes were observed in both total lipids and phospholipids (cardiolipin, phosphatidylglycerol, and phosphatidylethanolamine). The fatty acids composition appeared to be unaffected by the ox bile stress with an unsaturated to saturated ratio, in the majority, with respect to that of wild type control strain.

- **Membrane fatty acids composition of the seqA mutant strains exposed to the ox bile.**
  
  To determine whether the mutation in the seqA gene added to the ox bile stress affected membrane lipid components, fatty acids composition was quantified. The membrane fatty acids composition of the total lipids was highly affected by the ox bile stress (table 3). The fatty acids were characterized by low level of cyclic fatty acids, representing about 22.06% of total content, and high level of unsaturated fatty acids, representing about 15.74% (25.79% / 9.23% and 32.30% / 5.74%, respectively, for the wild type and the seqA mutant strains). These changes were due to a decrease in the cyclopropane derivatives C17 and C19 cyclic fatty acids and a concomitant increase in the unsaturated fatty acids (C16:1w7 and C18:1w9) and resulted in a high unsaturated to saturated ratio (table 3). The accumulation of the cardiolipin fraction was accompanied with an increase in the C18:1w9 Composition, which rise up from 0.0% to 3.0% (table 3). In the phosphatidylglycerol fraction, data confirmed that also C18:1w9 becomes a prominent species accounting for about 4.25% (3.28% and 0.45% respectively for the wild type and seqA phosphatidylglycerol fractions). Finally, we noticed that the phosphatidylethanolamine phospholipids were characterized by a reduction in cyclic fatty acids (cyc17 and cyc19) to the profile of their unsaturated fatty acids derivatives (C16:1w7 and C18:1w9). These phospholipids changes resulted in a high unsaturated to saturated ratio (table 3).

It has been proposed that intracellular pathogens like *Salmonella* are exposed to several stressing agents such as bile during the infection process. Stress conditions which pathogenic pathogen encounter during infection course can affect membrane components.
The Absence of the “GATC - Binding Protein SeqA” Affects DNA Replication in *Salmonella enterica* Serovar Typhimurium

### Table 3. Membrane fatty acid composition (molar percent) in total lipids and different phospholipid classes in the *Salmonella typhimurium* *seqA* mutant strain exposed to ox bile.

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Total lipids</th>
<th>Phosphatidylethanolamine</th>
<th>Phosphatidylglycerol</th>
<th>Cardiolipin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>4.78 ± 0.04</td>
<td>5.15 ± 0.90</td>
<td>2.80 ± 0.21</td>
<td>5.42 ± 0.06</td>
</tr>
<tr>
<td>C16:0</td>
<td>44.31 ± 0.56</td>
<td>46.38 ± 0.22</td>
<td>56.01 ± 0.03</td>
<td>37.38 ± 0.03</td>
</tr>
<tr>
<td>C16:1 w7</td>
<td>9.89 ± 0.09</td>
<td>4.36 ± 0.07</td>
<td>3.18 ± 0.42</td>
<td>6.13 ± 0.24</td>
</tr>
<tr>
<td>cyc17</td>
<td>5.96 ± 0.44</td>
<td>17.03 ± 0.06</td>
<td>11.16 ± 0.18</td>
<td>0.09 ± 0.83</td>
</tr>
<tr>
<td>C18:0</td>
<td>13.11 ± 0.03</td>
<td>2.84 ± 0.83</td>
<td>4.38 ± 0.61</td>
<td>42 ± 0.01</td>
</tr>
<tr>
<td>C18:1 w9</td>
<td>5.85 ± 0.52</td>
<td>4.20 ± 0.03</td>
<td>4.25 ± 0.07</td>
<td>3 ± 0.54</td>
</tr>
<tr>
<td>cyc19</td>
<td>16.1 ± 0.07</td>
<td>20.04 ± 0.36</td>
<td>18.22 ± 0.46</td>
<td>5.98 ± 0.07</td>
</tr>
<tr>
<td>MUFA</td>
<td>3.06</td>
<td>2.74</td>
<td>1.75</td>
<td>3.43</td>
</tr>
<tr>
<td>∑SFA</td>
<td>62.2</td>
<td>54.37</td>
<td>63.19</td>
<td>84.8</td>
</tr>
<tr>
<td>∑UFA</td>
<td>15.74</td>
<td>8.56</td>
<td>7.43</td>
<td>9.13</td>
</tr>
<tr>
<td>∑CFA</td>
<td>22.06</td>
<td>37.07</td>
<td>29.38</td>
<td>6.07</td>
</tr>
<tr>
<td>UFA/SFA</td>
<td>0.253</td>
<td>0.157</td>
<td>0.117</td>
<td>0.107</td>
</tr>
</tbody>
</table>

The environmental control of regulatory mechanisms is mediated by complex processes. *Salmonella* comes in contact with bile salts in the intestine and it is able to resist the action of bile and respond to escalating bile concentrations by increasing mechanisms of resistance. Previous studies showed that bacteria with enhanced tolerance to acid, bile and blood serum survive (Morgan et al., 1986; Wilmes-Riesenber et al., 1996) and cause disease (Foster & Hall, 1990; Rowbury et al., 1989) better than sensitive bacteria and showed that *in vitro* acid adapted *Salmonella* were more resistant towards bile (Velkinburg & Gunn, 1999) and acids (Foster & Hall, 1990) in comparison to non-adapted cells. Results obtained in this study show that the ox bile stress added to the *seqA* mutation is an important factor affecting *Salmonella typhimurium* resistance and could contribute to find new strategies based on intelligent combinations of hurdles, which could prevent the development or survival of *Salmonella spp.* in gastrointestinal tract. *Salmonella typhimurium* cells have developed efficient protection systems to cope with a variety of physicochemical unfavorable conditions and to adapt to the environmental stresses. In particular, fundamental for the microbial cells is to maintain membrane integrity and functionality in response to environmental stresses encountered during infection. In response to stresses, the phospholipids can alter their acyl chain structure by changing the ratio of saturation to unsaturation, cis to trans unsaturation, branched to unbranched structure and type of
branching and acyl chain length (Russel, 1984). Different modulation mechanisms can be used in relation to the physiological state of the cells (Rock & Cronan, 1996).

In a previous work Prieto et al., (2007) have shown that the seqA mutation renders *Salmonella enterica* sensitive to agents known to be antimicrobially active in the host like sodium choleate (ox bile extract) but the cause of this sensitivity remains unknown. In the present study we tried to explain the reasons and to investigate a possible connection between both the seqA gene (coding for the sequestration protein SeqA) and some membrane components and the sensitivity to the ox bile observed in *Salmonella typhimurium*.

In *Escherichia coli*, seqA mutants show altered membrane permeability (Wegrzyn et al., 1999) and abnormal phospholipids composition, which may explain their increased sensitivity to a number of dyes. The observation that the envelope of *Salmonella enterica* seqA mutants is likewise unstable (Aloui et al., 2010) can be tentatively correlated with bile sensitivity, because unconjugated bile salts can enter the cell by diffusion (Thanassi et al., 1997). Thus, a structural role of SeqA in envelope stability cannot be discounted (Wegrzyn et al., 1999). An alternative explanation is that SeqA might regulate the expression of genes involved in the stability and the integrity of the cell membrane against bile salts during infection process, a possibility also considered in *Escherichia coli* (Strzelczyk et al., 2003).

In summary, *Salmonella typhimurium* SeqA protein is required for maintenance of membrane integrity against the ox bile. Mutation in the seqA gene causes envelope defects and enhances sensitivity to the ox bile which together may contribute to the attenuation of virulence and may induce strong immune responses in infected animals. Recently, it has been demonstrated that *Salmonella typhimurium* lacking seqA gene exhibit a decrease of virulence in mice perhaps due to the bile sensitivity during the infection process. So we suggest that this mutant may be applied to the design of a live vaccine.

### 5. Conclusion

In the *Salmonella typhimurium* cell, DNA sequestration modulates a variety of processes such as DNA replication and transcription of certain genes. Deletion of the seqA gene produces a variety of phenotypes ranging from replication asynchrony to virulence attenuation, indicating multiple functions for the GATC-binding protein in modulating gene expression, proper chromosome segregation, initiation of chromosome replication, and nucleoid stabilization. Given these multiple roles, it is not surprising that seqA mutation is highly pleiotropic. However, the lack of SeqA protein does not impair viability. *Salmonella typhimurium* seqA− strain described here lacks binding of SeqA to GATC sequences and is more sensitive to this mutation than the wild type which shows the inverse. In addition, no great difference between the seqA mutant of *Salmonella typhimurium* and those of some enterobacterial species such as *Escherichia coli* was observed with replication asynchrony or alteration membrane. In conclusion, the role of SeqA in the prokaryotic cellular processes such as the DNA replication and lipid membrane metabolism is clear. So it may rely on its capacity as a global regulator of the gene expression during bacterial life, *in vitro*, in a similar manner as it does *in vivo*.

### 6. Future research

Our knowledge on the effects of SeqA protein in *Salmonella typhimurium* has considerably improved in the last decade. This fundamental research has several implications that will
prove to be useful for the development of novel therapeutic approaches. But, to date, therapeutic applications are still in their early experimental phases, but several recent studies provide promising results for future clinical developments. Over the last few years, many studies have demonstrated that \emph{Salmonella typhimurium} seqA mutants exhibit asynchronous DNA replication and are highly attenuated for virulence in mice and have been proposed as live vaccines. These results prove that GATC-binding sites might have a role in regulating virulence of \emph{Salmonella typhimurium} and perhaps in other related bacteria.

In addition, future research must focus on the study of the decreasing virulence and the proteomic and enzymatic activities of a seqA mutant strain. So this perspectives can be useful to more fully understand the significance of the results obtained above. Of special interests are: firstly, the growing list of genes governed by DNA sequestration in bacterial pathogens ; secondly, the finding of novel genes regulated by SeqA protein using high throughput analysis, and, thirdly, the evidence that this protein may regulate the expression of many unidentified genes involved in DNA replication and membrane metabolism.

Finally, the way in which SeqA participates clearly in the DNA replication and in the membrane integrity is a critical question that deserves further investigation in the near future, and may be research studies will have to identify explanations.

7. Acknowledgment

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- Mr Bchini Zin for proofreading and correcting this manuscript.

8. References


DNA Replication and Related Cellular Processes

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The Absence of the “GATC-Binding Protein SeqA” Affects DNA Replication in *Salmonella enterica* Serovar Typhimurium


Since the discovery of the DNA structure researchers have been highly interested in the molecular basis of genome inheritance. This book covers a wide range of aspects and issues related to the field of DNA replication. The association between genome replication, repair and recombination is also addressed, as well as summaries of recent work of the replication cycles of prokaryotic and eukaryotic viruses. The reader will gain an overview of our current understanding of DNA replication and related cellular processes, and useful resources for further reading.

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