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Phospholipid changes in *seqA* and *dam* mutants of *Escherichia coli*

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Abstract

SeqA and Dam proteins were known to be responsible for regulating the initiation of replication and to affect the expression of many genes and metabolisms. We have examined here the fatty acids composition and phospholipids membrane in *dam* and/or *seqA* mutants. The *dam* mutant showed an accumulation of the acidic phospholipids cardiolipin, whereas, the *seqA* mutant showed a higher proportion of phosphatidylglycerol compared with the wild-type strain. The *seqA dam* double mutant showed an intermediate proportion of acidic phospholipids compared with the wild-type strain. Based on these observations, we discuss the role of Dam and SeqA proteins in the regulation of phospholipids synthesis. ***To cite this article: D. Daghfous et al., C. R. Biologies 329 (2006).***

Keywords: Dam methylase; SeqA protein; Phospholipids; *Escherichia coli*; Membrane

1. Introduction

The initiation of replication of chromosomal DNA is coordinated with cell division. It has been proposed that DNA replication in bacterial cells is initiated on membranes and that the activities of replication proteins are regulated by membrane components [1].

Indeed, initiation of replication of the *Escherichia coli* chromosome is precisely regulated in the cell cycles [2]. The SeqA protein seems to be one of the key proteins in the control of this process [3–5]. It is an inhibitor of the onset of *E. coli* chromosome replication *in vivo* [6,7] and, at high concentrations, of the replication initiator protein, DnaA, in vitro, but it may stimulate replication at low DnaA concentrations in vitro [5]. It affects DNA topology and inhibits open complex formation at the replication origin [8,9]. It was demonstrated in vivo that SeqA limits DnaA activity in replication from the chromosomal origin *oriC* [4]. Moreover, SeqA protein is essential for sequestration, which affects *oriC* in the newly replicated hemimethylated state [3,6].

Until methylated by Dam methyltransferase [10,11], the newly replicated GATC sequences exist in a hemimethylated state that in the parental strand is methylated and in the nascent strand is not. Methylation of GATC sites by Dam methylase is implicated in

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regulation of *E. coli* chromosomal replication. In fact, the *seqA* gene was found to be responsible for the sequestration of hemimethylated *oriC* [6].

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 [12], the protein Dam methyltransferase [13] and the SeqA protein [14].

Acidic phospholipids, such as cardiolipin (CL) and phosphatidylglycerol (PG), decrease the affinity of adenine nucleotide for DNA protein [15,16]. Thus, it has been proposed that phospholipids regulate the activity of DnaA protein in cells and in vitro [15,17].

It has been reported recently that the transcription of genes implicated in lipid and phosphatidic acid biosynthesis was increased in *dam* mutant [18]. Therefore, Dam methyltransferase appears to be involved in the transcriptional regulation of the metabolism of fatty acids and phospholipids.

In addition, it has also 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 [19]. The mutation in the *seqA* gene allows efficient transformation of fully methylated minichromosomes into *dam* mutant cells [3,4] and the accumulation of *seqA* and *dam* double mutation can re-establish partially the replication asynchrony observed at every *seqA* and *dam* mutant.

We can suggest a possible interaction between the activities of SeqA and Dam methyltransferase proteins and membrane phospholipids composition.

We speculated that examination of fatty acid composition and phospholipids fractions in *dam* and/or *seqA* mutants would provide useful information for understanding the influence of mutations in *dam* and *seqA* genes on the fatty acid and phospholipids metabolisms in *E. coli*.

2. Experimental procedures

2.1. Bacterial strains used

The *E. coli* strains and their genotypes used in this study are listed in Table 1. Pre-incubated suspensions of *E. coli* cells were diluted 100-fold with Luria-Bertani (LB) medium and cultured at 30 °C. Exponentially growing *E. coli* cells in LB medium were harvested by centrifugation when the optical density at 600 nm reached 0.5.

Table 1
Bacterial strains used in this work

Strains	Genotypes	References
C600	LacY1, leuB6, supE44, thr-1, tonA21	[20]
C600 *dam13*	LacY1, leuB6, supE44, thr-1, tonA21, *dam13::Tn9*	[10]
C600 Δ*seqA*	LacY1, leuB6, supE44, thr-1, tonA21, Δ*seqA*	[3]
C600 *dam*$^-$ *seqA*$^-$	LacY1, leuB6, supE44, thr-1, tonA21, *dam13::Tn9, seqA::Tn3*	[4]

2.2. Analysis of fatty acids in total lipids and phospholipids

The different bacterial strains were cultured at 30 °C and cells were harvested from 1000-ml cultures in exponential phase by centrifugation (4000 g, 10 min, 4 °C), washed with 1% NaCl and total lipids were extracted by the method of Bligh and Dyer [21].

Fatty acids were converted to their methyl esters (FAMEs) according to the method described by Cecchi and collaborators [22]. An aliquot of the solution was evaporated; then 2 ml of hexane, a known quantity of heneicosanoic acid methyl ester [C21:0] as an internal standard and 0.5 ml of sodium methylate (1%) were added. After stirring over 1 min and standing for 2 min, the mixture was neutralized by 0.2 ml of H_2SO_4 (1 N); then, the methyl esters were washed with 1.5 ml of distilled water. The upper phase solvent containing the FAMEs was removed under vacuum.

Fatty acids relative composition was subsequently determined as % of the total fatty acids using a HP 6890 chromatograph equipped with a flame ionisation detector (FID) and an electronic pressure control (EPC) injector. A polyethylene glycol fused silica capillary column (Innowax, 30 m × 0.25 mm × 0.25 μm film thickness) purchased from Agilent (Wilmington, Delaware, USA) was used.

The column was operated at 150 °C for 1 min, and the temperature was raised by 15 °C min^{-1} to 200 °C and finally held at 242 °C for 2 °C min^{-1}. N_2 was used as the carrier gas at a flow rate of 1.6 ml min^{-1} and the split ratio used was 60:1.

Total lipids were placed as thin stripes on thin-layer silica gel plates and resolved by ascending chromatography using chloroform–acetone–methanol–acetic acid-water (50:20:10:10:5, vol:vol:vol:vol:vol) [23]. Standards of various commercial phospholipids (Sigma) were run simultaneously. The phospholipids were visualized with I_2 vapours and their bands were marked and scrapped off the plates; the phospholipids were eluted

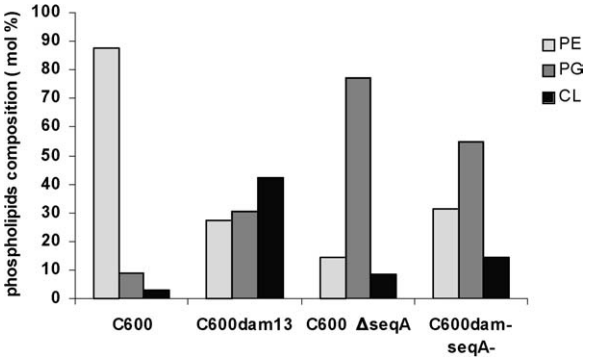

Fig. 1. Comparative analysis of phospholipids level in four isogenic strains for *E. coli*. Percentage of phospholipids analysis from C600 (wild type), C600 *dam13* (*dam*13::Tn9), C600 Δ*seqA* and C600 $dam^{-}seqA^{-}$ (*dam13*::Tn9, *seqA*::Tn3). Exponentially growing C600 (wild type), C600 *dam13*, C600 Δ*seqA* and C600 $dam^{-}seqA^{-}$ were incubated at 30 °C, centrifuged when the optical density at 600 nm reached 0.5. After extraction of the total phospholipids, individual phospholipids were separated by solid-phase extraction. Their contents were then calculated from the fatty acid contents measured by the capillary GC method, as described in the § *Experimental procedures*. Average values of duplicates are given, and the deviation was less than 5% of each value.

and their transmethylated fatty acids analysed as described above.

3. Results

3.1. Phospholipid composition of the seqA mutant membrane

In this study, we analysed phospholipid compositions of the bacterial membrane with the aim of correlating the membrane structure variation in phospholipids and in fatty acids, already suspected by Wegrzyn and collaborators [24], with *seqA* gene mutation.

Indeed, the phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) and cardiolipin (CL) proportions were affected by the *seqA* mutation while comparing them with the wild-type strain that have been incubated at 30 °C.

The major phospholipids present in *E. coli* wild-type strain membrane were phosphatidylethanolamine (PE), accounting for about 78% of total phospholipids, followed by phosphatidylglycerol (PG) and cardiolipin (CL) (9% and 3% of total phospholipids, respectively) (Fig. 1).

In the *seqA* mutant, the acidic phospholipid fraction (PG and CL) becomes majority of total phospholipids with 85.7%, distributes in 77% of PG and 8.7% of CL (Fig. 1).

This result corroborates the cross-feedback model proposed by Shibuya and colleagues [25].

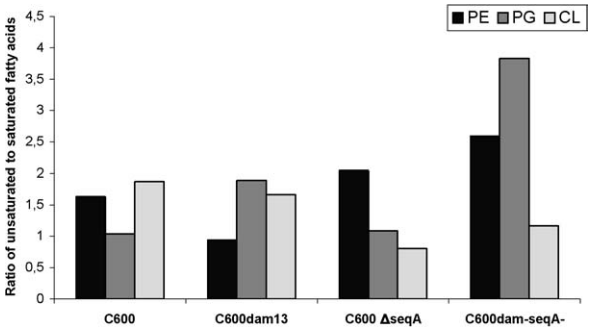

Fig. 2. The ratio of acyl chain-unsaturated fatty acids in different phospholipids classes in the *dam* mutant, *seqA* mutant and in the *dam seqA* double mutant. The ratio of acyl-chain-unsaturated to acyl-chain-saturated fatty acids (mol mol^{-1}) was calculated using the data in Table 2.

3.2. Fatty acid compositions of the seqA mutant phospholipids

In the wild-type strain (C600) palmitic acid (16:0), palmitoleic acid (16:1), and oleic acid (18:1) were the main constituents, representing 90.1% of the total fatty acids (Table 2).

In the *seqA* mutant (C600Δ*seqA*), the accumulation of the PG is accompanied with a weak variation of the Ratio of unsaturated to saturated fatty acids (Fig. 2). Indeed, the PG relative proportion of saturated C16:0 acid and unsaturated C16:1 and C18:1 acids appeared to be unaffected by the *seqA* mutation (Table 2).

Compared to the isogenic wild-type strain, the *seqA* mutant increases slightly the membrane CL proportion and decreases its level of acyl chain unsaturation of fatty acids (Table 2). This is due to the accumulation of the saturated C16:0 acid and to the reduction of the unsaturated C16:1 acid in the membrane CL proportion.

3.3. Effect of the dam mutation on phospholipids composition

Compared to the isogenic wild-type strain, the PE and PG and CL proportions were strongly affected by the *dam* mutation. In fact, the acidic phospholipids fraction remains majority with a high increase of the CL fraction (42.2%) of total phospholipids (Fig. 1).

We analysed the fatty acids composition of the *dam* mutant cardiolipin (Table 2). We notice that the level of acyl chain unsaturation of fatty acids in the CL phospholipid decreases slightly in relation to the wild-type strain. This diminution reflects a reduction of the unsaturated C16:1 and C18:1 fatty acids to the profile of the saturated C16:0 fatty acid (Table 2). We can also detect

Table 2
Fatty acid composition in total lipid and different phospholipid classes in the *dam* mutant, *seqA* mutant and in the *dam seqA* double mutant

	C14:0	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3
C600 (total)	1.2 ± 0.21	35.7 ± 0.52	18.9 ± 1.00	0.9 ± 0.15	5.5 ± 0.91	37.6 ± 0.74	0.2 ± 0.05	0.2 ± 0.05
C600 (PE)	7.0 ± 0.35	28.3 ± 0.85	9.1 ± 0.48	0.8 ± 0.09	1.9 ± 0.03	46.3 ± 0.43	6.5 ± 0.53	0.0
C600 (PG)	0.0	41.6 ± 0.64	11.9 ± 1.02	3.7 ± 0.07	3.6 ± 0.25	29.8 ± 0.41	4.7 ± 0.29	4.7 ± 0.33
C600 (CL)	16.0 ± 0.65	1.3 ± 0.05	21.9 ± 0.70	8.9 ± 0.10	8.7 ± 0.27	26.1 ± 0.56	0.3 ± 0.10	16.9 ± 0.36
C600 *dam13* (total)	2.2 ± 0.09	32.1 ± 0.34	23.6 ± 0.53	2.2 ± 0.31	2.0 ± 0.22	35.5 ± 1.35	0.5 ± 0.11	1.9 ± 0.14
C600 *dam13* (PE)	3.4 ± 0.10	43.0 ± 0.42	19.9 ± 0.32	3.1 ± 0.15	1.9 ± 0.23	25.1 ± 0.49	2.2 ± 0.13	1.4 ± 0.03
C600 *dam13* (PG)	2.4 ± 0.10	25.5 ± 0.45	13.3 ± 0.12	5.4 ± 0.03	1.3 ± 0.15	40.2 ± 0.70	9.3 ± 0.26	2.6 ± 0.09
C600 *dam13* (CL)	0.0	23.6 ± 0.46	9.2 ± 0.26	10.0 ± 0.15	4.1 ± 0.22	21.9 ± 0.26	25.4 ± 0.87	5.9 ± 0.15
C600 Δ*seqA* (total)	0.6 ± 0.06	31.7 ± 0.44	19.6 ± 0.32	2.0 ± 0.03	3.8 ± 0.22	42.0 ± 0.38	0.2 ± 0.04	0.3 ± 0.01
C600Δ*seqA* (PE)	1.5 ± 0.06	20.7 ± 0.27	7.3 ± 0.21	3.8 ± 0.12	6.9 ± 0.13	32.3 ± 0.46	21.3 ± 0.21	6.3 ± 0.23
C600Δ*seqA* (PG)	0.9 ± 0.06	43.3 ± 0.47	13.3 ± 0.10	1.7 ± 0.06	1.9 ± 0.14	36.7 ± 0.35	1.1 ± 0.01	1.1 ± 0.07
C600Δ*seqA* (CL)	5.9 ± 0.20	43.0 ± 0.73	3.2 ± 0.12	1.5 ± 0.02	4.5 ± 0.10	36.0 ± 0.36	5.9 ± 0.41	0.0
C600 *dam*$^-$*seqA*$^-$ (total)	0.7 ± 0.11	39.3 ± 0.88	14.4 ± 0.36	6.7 ± 0.15	6.0 ± 0.14	32.4 ± 0.36	0.5 ± 0.03	0.1 ± 0.02
C600 *dam*$^-$*seqA*$^-$ (PE)	0.0	10.1 ± 0.11	3.5 ± 0.37	12.8 ± 0.27	5.0 ± 0.12	43.8 ± 0.33	24.8 ± 0.19	0.0
C600 *dam*$^-$*seqA*$^-$ (PG)	2.9 ± 0.04	6.1 ± 0.10	2.5 ± 0.17	1.4 ± 0.19	10.3 ± 0.16	51.5 ± 0.53	23.3 ± 0.68	2.0 ± 0.05
C600 *dam*$^-$*seqA*$^-$ (CL)	10.2 ± 0.87	24.7 ± 0.52	23.4 ± 0.32	0.0	11.0 ± 0.15	13.0 ± 0.25	9.8 ± 0.77	7.9 ± 0.40

Exponentially growing C600 (wild type), C600 *dam13*, C600 Δ*seqA* and C600 *dam*$^-$*seqA*$^-$ were incubated at 30 °C, centrifuged when the optical density at 600 nm reached 0.5. Extraction of the total phospholipids and purification of each phospholipid were performed as described in the § *Experimental procedures*. The contents of fatty acids in the total phospholipids (A), PE (B), PG (C) and CL (D) were determined as described in the § *Experimental procedures*. The composition of each fatty acid is shown as the relative value to the total content of fatty acids. Average values of triplicates were given, and the deviation was less than 5% of each value. Fatty acids: C14:0, myristic acid; C16:0, palmitic acid; C16:1, palmitoleic acid; ΔC17:0, cis-9–10-methylene-hexadecanoic acids; C18:0, stearic acid; C18:1cis-11, cis vaccenic acid; C18:2, linoleic acid; C18:3, linolenic acid.

an accumulation of the unsaturated linoleic acid (C18:2) (Table 2).

However, the PG fraction of the *dam* mutant membrane shows a meaningful increase of the level of acyl chain unsaturation of fatty acids (Fig. 2). This is due to the reduction of the saturated C16:0 fatty acid and to the slight increase of the unsaturated C18:1 fatty acid (Table 2).

3.4. Phospholipids and fatty acids compositions of the dam seqA double mutant

To evaluate the combined effect of the two mutations in the *seqA* and *dam* genes on the bacterial membrane integrity, we compared the phospholipids composition of the double mutant with the isogenic wild-type strain (Fig. 1).

The acidic membrane phospholipid proportion (PG and CL) is in the majority with respect to the zwitterionic phospholipid, PE, in the *dam seqA* double mutant with 68.8% of total phospholipids distributes in 54.6% of PG and 14.2% of CL (Fig. 1). We can also note that in the *dam seqA* double mutant, PG and CL acidic phospholipids have intermediate proportions in relation to the *seqA* and *dam* simple mutants (Fig. 1).

In the *dam seqA* double mutant, we observe an accumulation of acyl-chain-unsaturated fatty acids in the PG and PE fractions. Indeed, the PE fraction shows a reduction of C16:0, whereas the PG fraction accumulates about two-fold quantity of unsaturated C18:1 fatty acid in relation to the isogenic wild cells (Table 2).

4. Discussion

Interactions of *E. coli* Dam and SeqA proteins with cellular membranes have been reported previously [6,12,18,24,26]. However, although regulation of the activities of these proteins by membranes or their components was reported [18] or suggested [6,24], little is known about the influence of Dam methyltransferase and SeqA on the composition of cell membranes. In this report, we have shown that zwitterionic (PE) and acidic phospholipids (PG and CL) proportions as well as their acyl chain unsaturation of fatty acids levels were affected by different mutations in the *seqA* and *dam* genes in relation to wild-type isogenic bacteria membrane.

We observed that the *dam* mutant membrane accumulates a high fraction of the acidic phospholipid cardiolipin (42.2% of the total membrane phospholipid) containing saturated fatty acids. Thus, we might assume that the expression of genes coding for components of the membranes or for proteins involved in synthesis of such components might be impaired in the *dam* mutant.

This hypothesis is reinforced by the fact that Dam-mediated methylation is known to be responsible for regulating metabolism, replication and mismatch repair

in *E. coli* [11,18]. Since the Dam methyltransferase protein has also been proposed to form and/or maintain chromosome structure [27], it is likely that this protein could affect the expression of many genes, not only by direct activation or repression of particular promoters via interaction with their GATC sequences, but also by changing DNA topology.

Taken together with the earlier observation that *dam* mutant initiates chromosome replication asynchronously [26] and that this asynchrony mainly results from an inability to inactivate newly replicated origins that have been sequestrated in the membrane [12], we proposed that the initiation of the replication might be controlled by the Dam methyltransferase directly by affecting the methylation status of GATC sequences of *oriC* and indirectly by regulating the DnaA activation via the modification of the acidic phospholipids composition and their acyl chain saturation of fatty acids of the membrane.

Like the Dam methyltransferase, it has been demonstrated that SeqA also affects DNA topology and inhibits open complex formation at the replication origin [8]. Indeed, Weito and collaborators [28] have shown that the *seqA* mutation increases negative superhelicity of chromosomal and plasmid DNA and affects profoundly the transcription of various genes [24, 29–31]. In addition, SeqA is involved in translocating *oriC* into the membrane after initiation of DNA replication [3].

So, Dam and SeqA proteins participate in the movement of *oriC* into the membrane and may interact with the membrane phospholipids. To examine this hypothesis, we have extracted and analysed the phospholipids composition of the *seqA* mutant membrane as well as its level of unsaturation of fatty acids.

In this report, we have shown that in the absence of the SeqA protein, the bacteria membrane accumulates a high fraction of acidic phospholipids (85.7% of the total phospholipids with 77% the phosphatidylglycerol).

So, we suggest that in addition to its direct role in the sequestration of *oriC* on the membrane, SeqA can interact with the lipid metabolism and regulate the acidic phospholipids synthesis.

In the *dam seqA* double mutant, we observe an accumulation of acyl-chain-unsaturated fatty acids in the PG and PE fractions. However, Makise and collaborators [17] have reported that unsaturated fatty acids increase membrane fluidity that may be important for the control of the DnaA binding to *oriC* in vitro and can be related with the partial re-establishment of the replication asynchrony observed at every *seqA* and *dam* mutant [19].

This suggests that Dam methyltransferase and SeqA proteins might play direct roles in fatty acids' and phospholipids' metabolisms and that impairment of functions of both proteins results in less dramatic alterations in these metabolisms.

In conclusion, the data presented in this work indicate that the fatty acids and phospholipids metabolisms are significantly influenced in *E. coli dam* and/or *seqA* mutants and it is likely that several different molecular mechanisms are involved in the regulation of the composition of cellular membranes by these proteins.

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