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# Novobiocin Sensitivity of *Salmonella typhimurium dam* and/or *seqA* Mutants

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Abstract

This study was carried out to determine the effects of novobiocin, a gyrase inhibitor, on the growth, survival, motility and whole cell proteins of *S.* Typhimurium *dam* and/or *seqA* strains. Our results showed that the *dam* and *seqA*/*dam* mutants are the most sensitive to novobiocin, compared to wild type and *seqA* strains. Surprisingly, the motility of *seqA* mutants increased after exposure to novobiocin only in stationary phase cells. All the other strains showed a significant decrease in their motility. The analysis of protein profiles of all strains demonstrated several modifications as manifested by the alteration of the expression levels of certain bands. Our work is therefore of great interest in understanding the effects of novobiocin on *S.* Typhimurium and the involvement of DNA methylation.

Key words: *Salmonella*, DNA methylation, *seqA*, motility, novobiocin, proteins

## Introduction

DNA methylation is a mechanism by which bacteria regulate gene expression and control several cellular processes such as transposition, DNA replication, segregation of chromosomal DNA and mismatch repair. It has been proved that Dam protein regulates gene expression and virulence of *S.* Typhimurium (Heithoff *et al.*, 1999; Balbontin *et al.*, 2006). Indeed, lack of Dam methylation disturbs the expression of *std* fimbriae. Heithoff *et al.* (Heithoff *et al.*, 1999) have demonstrated that methylation at specific GATC sequences of promoters is crucial for transcription and alterations in the degree of methylation at promoter sequences may influence gene expression. Dam methyltransferase and regulatory proteins, such as Cap, Lrp, or OxyR compete for overlapping sites in or near promoters (Chatti and Landoulsi, 2008). Oshima *et al.* (2002) demonstrated that the promoters of most Dam controlled genes contained GATC sequences that overlap with recognition sites for fumarate nitrate reduction (Fnr) and catabolite activator protein (CAP) regulators. These authors suggested that the GATC network regulation takes place upstream of the coding sequences and that it is the consequence of an interaction with a regulatory protein like Fnr or CAP.

Like the Dam methyltransferase, it has been demonstrated that *seqA* also profoundly affects the transcription of various genes (Lobner-Olesen *et al.*, 2003). In *Escherichia coli*, the product of the *seqA* gene is the main negative regulator of chromosome replication initiation (Lu *et al.*, 1994, Slater *et al.*, 1995). SeqA protein binds preferentially to hemi-methylated GATC sites and sequesters the *oriC* region immediately after replication (Boye *et al.*, 1996). SeqA can also specifically bind to fully-methylated GATC sequences, not only hemimethylated, if regions other than *oriC* are considered. Our previous results have demonstrated that lack of *seqA* attenuates the virulence of *S.* Typhimurium in the mouse model (Chatti *et al.*, 2007). Jakomin *et al.* (2008) proved that *std* operon is regulated by SeqA protein. The effect of SeqA on plasmid topology has been also demonstrated (Norunn and Skarstad, 1999). Indeed, it has been shown that the *seqA* mutation increases negative superhelicity of chromosomal and plasmid DNA (Klungsøyr and Skarstad, 2004). So, these findings may reflect a similar effect of SeqA on chromosome topology.

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It was demonstrated that novobiocin (a member of the coumermycin family of antibiotics) inhibits bacterial DNA replication and transcription. Novobiocin inhibits the DNA gyrase (Gellert *et al.*, 1976), an enzyme that catalyzes the ATP-dependent introduction of negative superhelical turns into circular double-stranded DNA. Gyrase is needed for processes that require negatively superhelical DNA, such as DNA replication, transcription, DNA repair, and recombination (Chatterji *et al.*, 2001). Also, it has been reported that novobiocin affects membrane integrity, nucleic acid synthesis, and cell wall synthesis (Smith and Davis, 1967).

In this study, we investigated the effects of novobiocin treatment on the survival, motility and whole cell proteins of *seqA* and/or *dam* mutants of *S.* Typhimurium.

## Experimental

### Materials and Methods

**Bacterial strains and growth conditions.** Bacterial strains used in this study, derived from the wild type strain *S.* Typhimurium 14028 are SV1610 (*dam-228::MudJ*) SV4752 (*ΔseqA1*) and SV4784 (*dam-225::MudJ/ΔseqA1*) (Jakomin *et al.*, 2008). Overnight cultures of *Salmonella* were grown in nutrient broth (NB) medium and diluted into 50 ml of fresh sterile broth medium. Bacteria were routinely incubated in nutrient broth (NB) (Pronadisa, Spain) at 37°C overnight with shaking (200 rpm). Novobiocin was added at various concentrations directly to the flask and turbidity was monitored by measuring the optical density at 600 nm of the medium.

***Survival study.*** The strains to be tested were incubated overnight at 37°C in NB. Appropriate dilutions of bacteria ($10^8$ cfu/ml) were made in sterile PBS solution buffer containing various concentration of novobiocin or no drug (0, 80, 200 and 400 μg/ml) and incubated for 1 to 4 hours at room temperature. At the given time points, 100 μL of cultures were plated on Nutrient agar, after which the resulting colonies were counted and the fractional survival was calculated.

**Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) determination by broth dilution.** For MIC and MBC determinations, serial dilutions of novobiocin were prepared. The tubes were incubated at 37°C overnight with shaking and the highest dilution in which there were no growth was recorded as the MIC. For MBC testing, aliquots (20 μl) of broth from tubes containing no growth were plated onto solid medium and again incubated overnight at 37°C. The highest dilution in which there were no survivors was recorded as the MBC. In the above method, controls for each strain were performed using the sterile liquid medium without novobiocin. All MICs and MBCs were confirmed by triplicate assays.

**Motility assays.** Bacterial strains were grown in NB broth at 37°C overnight with agitation. The bacteria were then diluted 1:100 with fresh NB and incubated at 37°C with shaking. Cells were considered to be in exponential and stationary phases when they reach an optical density of 0.5 and 1, respectively. Bacterial culture (50 μl) was spotted onto an NB plate with 0.3% agar and incubated at 37°C. The concentration of novobiocin used to study the motility of all strains was 200 μg/ml. The swimming motility was estimated by measuring the diameter of the bacterial zone after incubation at 37°C during 24 hours. Media used for swarming consisted of 0.5% bacto-agar with 8 g/liter nutrient broth, to which 5 g/liter glucose was added. Swarm plates were typically allowed to dry at room temperature overnight before being used.

**Whole cell proteins extraction.** Pellets were resuspended in TRIS buffer (20 mM, pH 7.5) containing EDTA (5 mM) and $MgCl_2$ (5 mM). The cells were lysed by sonication. The cell debris was removed by centrifugation at 14 000 × g for 10 min at 4°C. The supernatant containing extracted proteins was stored at –20°C until further analysis. Protein concentration was determined according to the method of Lowry (1959).

**Statistical.** The experiment was repeated three times, and statistical significance was calculated using Student's *t* test.

## Results

**Effect of novobiocin on the growth of mutants.** To study the effect of the novobiocin on *Salmonella* growth, different concentrations ranging from zero to 400 μg/ml were tested. Compared with the control cell suspensions without novobiocin, we observed a modification of the classical cell growth curves of the different strains. Fig. 1 shows the basic phenomena observed in the inhibition of cell growth by novobiocin. The results showed that the antimicrobial activity of novobiocin increases with concentration. These two mutants do not reach the stationary phase after treatment with novobiocin at a concentration of 400 μg/ml. As the novobiocin concentration was increased, so did the duration of the lag phase whereas the rate of the growth after inhibition decreased and the cell density at which stationary phase was entered also decreased. The duration of the lag phase was proportional to the novobiocin concentration and variable according to the different *Salmonella* strains tested.

**Effects of novobiocin on the survival of the mutants.** The percentage of strains' survival was fol-

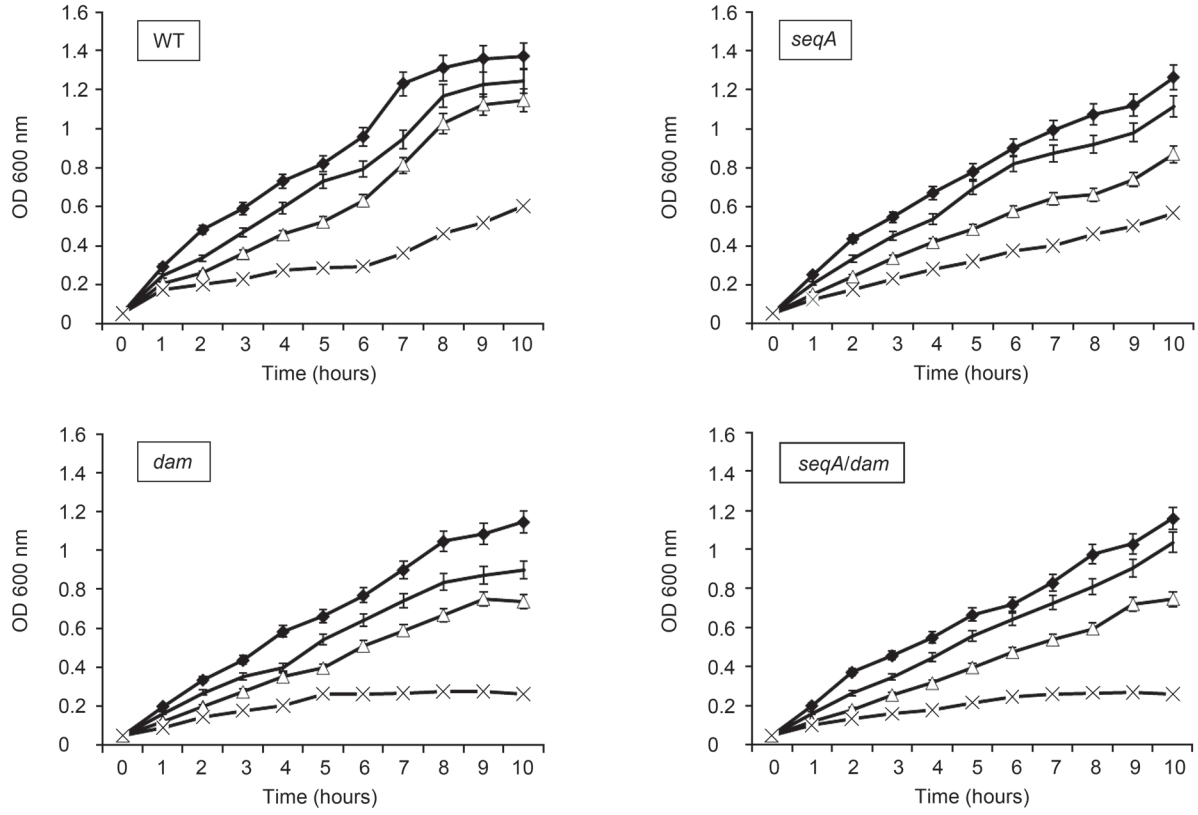


Fig. 1. Growth kinetics of *Salmonella typhimurium* and its isogenic mutants (*dam*, *seqA* and *dam*/*seqA*) under different concentrations of novobiocin: 0 μg/ml (black square), 80 (without marker), 200 μg/ml (white triangle) and 400 μg/ml (X).

lowed for 4 hours. Control strains were used for comparative study. Significant differences between all strains were mainly observed after treatment during 1 hour. The results obtained showed that the *dam* and *seqA*/*dam* strains are significantly more sensitive to novobiocin, compared to the other strains (Fig. 2). *seqA* mutant was significantly more resistant than *dam* and *seqA*/*dam* strains.

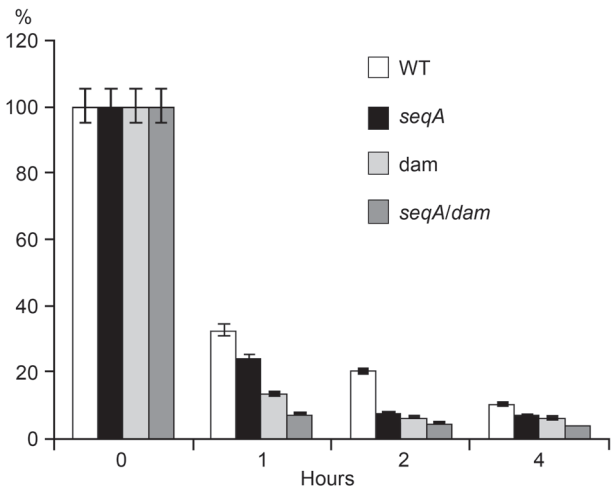


Fig. 2. Novobiocin sensitivity of *Salmonella typhimurium* and its isogenic *seqA* and/or *dam* mutants. Results mentioned are the means of three repetitions. The survival assay was estimated by counting the colony forming units (CFU). The experience was monitored for 4 hours.

**Determination of MIC and MBC.** The MIC and MBC values obtained are expressed in terms of the novobiocin concentration (Table I). The MIC values shown were determined by broth dilution. MICs range from 0.6 to 1.1 mg/ml of novobiocin. The MBC values range from 2 mg/ml to 5.6 mg/ml. The double mutant *dam*/*seqA* presents the lowest MBC value.

**Novobiocin alters the motility of mutants.** The motility of novobiocin-treated bacteria was investigated (Fig. 3A). Statistical analysis showed significant differences in the motility of wild type, *dam* and *seqA*/*dam* at exponential phase after drug exposure. At stationary phase, significant difference was observed only in *dam* and *dam*/*seqA* strains (Fig. 3A).

**Effect of novobiocin on the whole cell proteins.** The whole cell proteins of *S.* Typhimurium and its isogenic mutants were analyzed using SDS-PAGE. When comparing the whole cell preparations on SDS-PAGE,

Table I
MIC and MBC of novobiocin for Salmonella typhimurium (wild type) and isogenic *seqA* and/or *dam* mutants

| | MIC (mg/ml) | MBC (mg/ml) | MBC/MIC |
|---|---|---|---|
| WT | 1.1 ± 0.11 | 5.6 ± 0.21 | 5.09 |
| *seqA* | 1.1 ± 0.14 | 5.0 ± 0.13 | 4.54 |
| *dam* | 0.6 ± 0.09 | 2.5 ± 0.13 | 4.16 |
| *dam*/*seqA* | 0.6 ± 0.05 | 2.0 ± 0.08 | 3.33 |

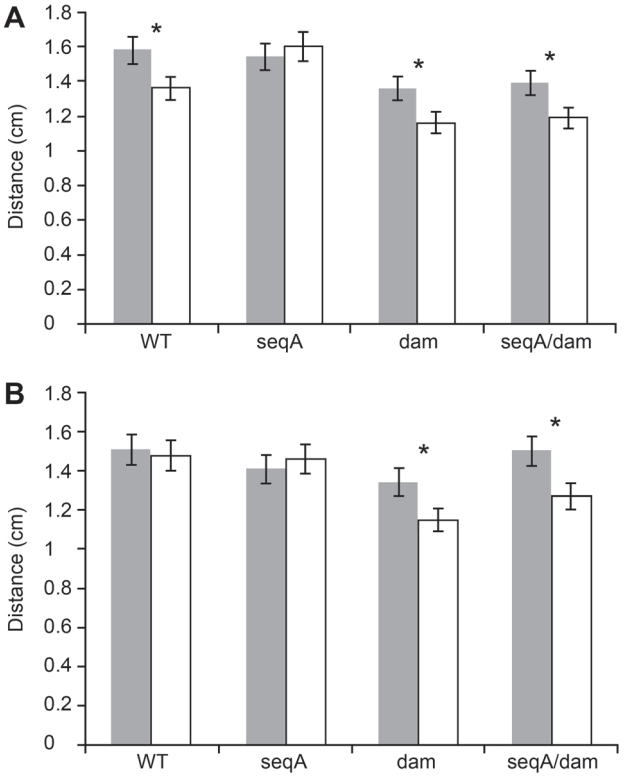


Fig. 3. The motility of exponential (A) and stationary phase (B) cells treated (grey) and untreated (white) strains.
* Significant difference ($p < 0.05$).

changes in protein profiles were observed under the two growth conditions. Therefore, as shown in Fig. 4, changes were manifested either by disappearance or modification of the expression level. In the absence of novobiocin, the alteration of protein profiles of *dam* and/or *seqA* was manifested by modification of the expression level of certain bands. After exposure to novobiocin, we noted changes of the expression level

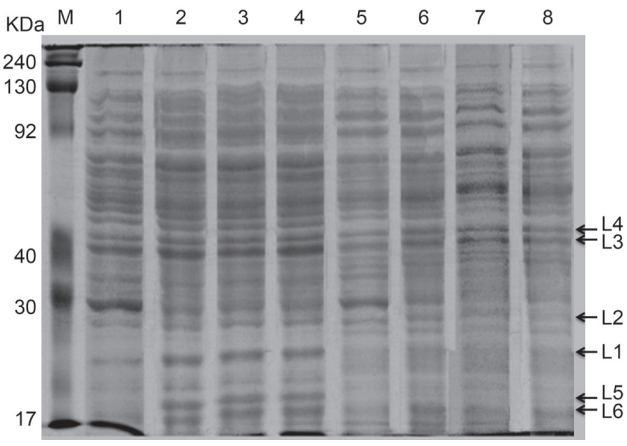


Fig. 4. The whole cell proteins of *Salmonella typhimurium* and its isogenic *dam* and/or *seqA* mutants in the absence (Column 1 to 4) or presence (Column 5 to 8) of novobiocin. Column 1 and 5: WT, Column 2 and 6: *dam*: Column 3 and 7: *seqA*, Column 4 and 8: *dam*/*seqA*. Arrows indicated the lines which show the most bands alterations.

Table II
Ratio of protein levels in each strain before and after drug treatment. Only bands in Line L1 to L6 are analyzed. B: band, the number refers to the column.

| | L1 | L2 | L3 | L4 | L5 | L6 |
|---|---|---|---|---|---|---|
| B1/B5 | 1.15 | 1.055 | 1.403 | 1.23 | 1.142 | 1.211 |
| B2/B6 | 1.49 | 1.0001 | 1.220 | 1.042 | 3.05 | 0.607 |
| B3/B7 | 1.27 | 1.0033 | 1.22 | 1.098 | 3.125 | 1.064 |
| B4/B8 | 1.31 | 1.22131 | 1.33 | 1.185 | 3.21 | 1.222 |

of some proteins for all tested strains. Changes of protein bands are estimated by calculating the ratio of band area before and after novobiocin treatment. Table II shows the changes observed after drug treatment.

## Discussion

It is well known that supercoiling can influence promoter activity (Wang and Lynch, 1993). Thus, DNA supercoiling affects binding of regulatory proteins, such as Dam and SeqA. In our previous work, we suggested that GATC sites could regulate virulence and stress response of *S.* Typhimurium (Chatti and Landoulsi, 2008). The growth of wild-type, *dam*, *seqA* and *dam*/*seqA* mutants were detected in the presence and absence of novobiocin. There was no difference in the growth rates between wild-type and *seqA* mutant str ains of *S.* Typhimurium in NB medium with or without novobiocin. However, our results showed that the growth of *dam* and *seqA*/*dam* was the most sensitive mutants. Soutourina *et al.* (2001) have demonstrated that novobiocin (200 and 400 μM) induces a small decrease of the growth rate for *Pseudomonas* and *Enterobacter*. *E. coli dam* mutant was previously shown to be more sensitive to novobiocin than the wild type strain (Onogi *et al.*, 2000).

To assess the effect of the *seqA* and/or *dam* deletions on the *in vitro* susceptibility to novobiocin, we determined the MICs and MBCs as well as the loss of viable counts after exposure to 400 μg/ml of novobiocin. The MICs of novobiocin were not affected by the *seqA* inactivation but decreased in *dam* and *seqA*/*dam* strains. The viable losses in time kill experiments confirm also that the *seqA* mutant is the most resistant compared to the *dam* and *dam*/*seqA*. Several studies have proved that *dam* strains are the most sensitive mutant toward many stresses such as hydrogen peroxide, bile (Chatti *et al.*, 2012; Badie *et al.*, 2007). However, all the mutants were more sensitive than the parent wild type strain. Therefore, novobiocin-induced gyrase inhibition can be expected to cause DNA strand breaks. Hence, it is not surprising that *dam* mutants, which suffer a basal level of MutHLS-induced DNA breaks, are more sensitive. Bacterial motility is a complex phenotype that is modu-

lated by many regulators. In this study, we found that *seqA* mutants did not show significant defects in motility, unlike WT, *dam* and *dam*/*seqA* strains under this drug during exponential phase. The stationary culture of these strains demonstrated a significant increase of the motility of *seqA* mutant and a significant decrease in the motility of both of *dam* and *seqA*/*dam* mutants. However, the motility of WT strain did not change in the stationary phase. These findings suggest the implication of the nucleoid-associated protein FIS which modulate the dynamics of DNA supercoiling during the growth phase (Traversa *et al.*, 2001). Fis promoter is activated by high negative superhelicity of the DNA *in vivo* and *in vitro* (Schneider *et al.*, 2000). In addition, it has been proved that CRP-cAMP modulates *fis* expression and the inhibition of DNA gyrase represses the expression of several CRP-cAMP sensitive genes. Taken together, we can suggest that phase dependant motility especially for *seqA* mutant could be the consequences of a direct or indirect action of FIS and/or CRP-cAMP (Soutourina *et al.*, 2002). The reduction in motility has been observed in *E. coli* in the presence of DNA gyrase inhibitors (Schneider *et al.*, 2000). In the presence of novobiocin, a more than two fold decrease in the *flhDC* activity was obtained in *E. coli* (Soutourina *et al.*, 2002). Also, inhibiting the DNA gyrase promotes the FimB-mediating inversion from OFF to ON and therefore it was concluded that DNA supercoiling determines the directionality of the FimB-mediated recombination (Dove and Dorman, 1994). The decrease of motility in *Salmonella* in the presence of novobiocin supports the link between the DNA supercoiling and motility regulation. Therefore, motility could be modulated by alteration of DNA topology, resulting from interactions between Dam or SeqA and the regulatory regions.

Whole cell proteins were investigated in the presence or absence of novobiocin. Our results showed that this drug alters the protein profiles of all strains. These changes as manifested by disappearance or modification of the expression levels of several bands. These data suggest that proteins of these strains are associated with growth and survival in the presence of novobiocin. The function of these proteins is subject to further investigation. However, changed proteins could be under the control of CRP-cAMP regulon. Further analysis by 2-DE would be needed for a better separation of proteins and more accurate estimation of sizes and to elucidate their role in bacterial response to novobiocin.

In conclusion, we suggest the involvement of DNA supercoiling on the DNA methylation control of several cellular processes. The difference in the sensitivity toward novobiocin may be due to the fatty acid composition of the membrane of each strain. Further studies on the effects of gyrase inhibitors on virulence genes expression and the deletion of *gyrA* and/or *gyrB* in *seqA* and/or *dam* mutants could help researchers to understand the relationship between DNA methylation and DNA supercoiling.

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