

JB01156-07, revised

## The GATC-binding protein SeqA is required for bile resistance and virulence in *Salmonella enterica* serovar Typhimurium

Ana I. Prieto<sup>1,†</sup>, Marcello Jakomin<sup>1</sup>, Ignacio Segura<sup>1</sup>, M. Graciela Pucciarelli<sup>2</sup>,  
Francisco Ramos-Morales<sup>1</sup>, Francisco García-del Portillo<sup>2</sup>, and Josep Casadesús<sup>1,\*</sup>

<sup>1</sup> Departamento de Genética, Facultad de Biología,  
Universidad de Sevilla, Apartado 1095, Sevilla 41080

and

<sup>2</sup> Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, CSIC,  
Campus de Cantoblanco, 28049 Madrid, Spain

<sup>†</sup> Present address:

Department of Pathology, Cambridge University, Tennis Court Road,  
Cambridge CB2 1QP, England

Running title:

Lack of SeqA attenuates *Salmonella* virulence

\* Corresponding author: Josep Casadesús, Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Apartado 1095, Sevilla 41080, Spain. Telephone +34 95 455 7105. Fax +34 95 455 7104. E-mail: casadesus@us.es

## ABSTRACT

Disruption of the *seqA* gene of *Salmonella enterica* serovar Typhimurium causes defects similar to those described in *E. coli*: filament formation, aberrant nucleoid segregation, induction of the SOS response, envelope instability, and increased sensitivity to membrane-damaging agents. Differences between SeqA<sup>-</sup> mutants of *E. coli* and *S. enterica* are however found: SeqA<sup>-</sup> mutants of *S. enterica* form normal colonies and do not exhibit alterations in phage plaquing morphology. Lack of SeqA causes attenuation of *S. enterica* virulence by the oral route but not by the intraperitoneal route, suggesting a virulence defect in the intestinal stage of infection. However, SeqA<sup>-</sup> mutants are fully proficient in the invasion of epithelial cells. We hypothesize that attenuation of SeqA<sup>-</sup> mutants by the oral route may be caused by bile sensitivity, which in turn may be a consequence of envelope instability.

## INTRODUCTION

The ability of *Salmonella enterica* to induce disease in animals relies on the possession of virulence genes, some of which are clustered in pathogenicity islands while others are scattered in the chromosome (16). In *S. enterica* subspecies I, some virulence genes are plasmid-borne (33). In addition to virulence genes, whose products are only required for animal infection, certain housekeeping genes play relevant roles in the interaction of *Salmonella* with its animal host (15). Relevant examples are two-component systems such as PhoPQ and RcsBCD, which regulate virulence gene expression in specific stages of the infection process but also play relevant physiological roles outside the animal (34). Another example of a housekeeping function with a role in host-pathogen interactions is DNA adenine (Dam) methylation, which is essential for virulence in *Salmonella* and other bacterial pathogens (6, 21, 28, 47).

Mutants of *S. enterica* serovar Typhimurium lacking Dam methylation are severely attenuated in the mouse model, especially by the oral route (12, 18). Virulence attenuation caused by *dam* mutations is pleiotropic, and involves reduced invasion of epithelial cells (12), lowered cytotoxicity after infection of M cells (12), inefficient colonization of Peyer's patches and mesenteric lymph nodes (12, 18), sensitivity to bile and to other DNA-damaging agents produced inside the animal (19, 31), and envelope instability accompanied by leakage of proteins (32). A transcriptomic study of Dam methylation in *S. enterica* serovar Typhimurium has provided evidence that some of these virulence defects are caused by altered patterns of gene expression (2). However, Dam methylation is not a regulator of gene expression *per se* but a signaling system for DNA-protein interactions (47), and the proteins that regulate Dam-dependent expression of *Salmonella* virulence genes remain to be identified.

SeqA was discovered as a protein involved in the methylation/hemimethylation cycle of *E. coli* DNA (25). SeqA binds to hemimethylated GATC sites formed by DNA replication, and regulates activation of the *E. coli* chromosome replication origin (25). Proper chromosome segregation also requires SeqA (1). Furthermore, SeqA trails the DNA replication fork and may contribute to nucleoid organization in newly replicated DNA (4, 24, 27, 48). Aside from its roles in chromosome replication and nucleoid segregation, SeqA is known to regulate transcription of certain genes. In bacteriophage lambda, SeqA activates the p<sub>R</sub> promoter in a GATC methylation-dependent fashion (41). SeqA also acts as a transcriptional co-activator by

facilitating binding of the cII transcription factor to the lambda p<sub>I</sub> and p<sub>aQ</sub> promoters (40). Competition between SeqA and the OxyR repressor for hemimethylated GATC sites has been shown to regulate phase variation in the *E. coli agn43* gene (9). These examples raised the possibility that SeqA binding to critical GATC sites might likewise regulate the expression of *S. enterica* genes.

Here, we examine the consequences of *seqA* gene disruption in *Salmonella enterica* serovar Typhimurium, and describe analogies and differences between *E. coli* and *S. enterica* SeqA<sup>-</sup> mutants. Of special interest is the observation that disruption of the *seqA* gene in *S. enterica* serovar Typhimurium causes attenuation of virulence by the oral route, a trait that may be correlated with sensitivity to bile salts encountered in the host intestine. Transcriptomic analysis does not provide evidence for SeqA-mediated regulation of known virulence genes in *Salmonella*. In fact, lack of SeqA causes minor changes in the global transcriptional profile of the cell, as previously described in *E. coli* (27). However, an intriguing observation is that the relatively short list of SeqA-regulated loci in *S. enterica* includes a significant number of *Salmonella*-specific genes.

## MATERIALS AND METHODS

**Bacterial strains, plasmids and phages:** The strains of *Salmonella enterica* used in this study (Table 1) belong to serovar Typhimurium, and derive from the mouse-virulent strain ATCC 14028. An exception is DA6522, an LT2 derivative kindly provided by Dan I. Andersson, University of Uppsala, Uppsala, Sweden. *E. coli* DH5 $\alpha$  (49) was the standard host for recombinant plasmids. *E. coli* CC118 (20) was kindly provided by Victor de Lorenzo, CNB, CSIC, Cantoblanco, Spain. Plasmid pGE108 (Km<sup>r</sup>) is a ColE1 derivative carrying a *cea::lacZ* fusion (35). Plasmid pIZ1574, constructed for this study, is a pBluescript II SK+ derivative containing the *seqA* coding sequence from strain ATCC 14028, previously amplified by the polymerase chain reaction (see below). Plasmid pIZ1588 is a pBAD18 derivative expressing the *S. enterica seqA* gene from the arabinose-dependent p<sub>BAD</sub> promoter (17), and was constructed by cloning an *Xba*I-*Hind*III fragment from pIZ1574 onto pBAD18. Transductional crosses using phage P22 HT 105/1 *int201* [(36) and G. Roberts, unpublished] were used for strain construction operations involving chromosomal markers, and for transfer of plasmids among *Salmonella* strains. The transduction protocol was described elsewhere (13). To obtain phage-free isolates, transductants were purified by streaking on green plates (7). Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. Strain SV4861 was constructed by transducing the *invJ::Km<sup>r</sup>* allele from SB302 to ATCC 14028. SB302 is a derivative of strain SL1344 of *S. enterica* serovar Typhimurium (23), obtained from J. E. Galán, Yale University, New Haven, CT.

Table 1

**Media and chemicals:** Luria-Bertani broth (LB) was used as rich medium. The E medium of Vogel and Bonner (45) was used as minimal medium. Unless otherwise indicated, the carbon source for both LB and E was 0.2% glucose. Solid media contained agar at 1.5% final concentration. Deoxycholic acid (sodium salt) and sodium choleate (ox bile extract) were both from Sigma Chemical Co., St. Louis, MO. Antibiotics were used at the final concentrations described previously (44). Green plates were prepared according to Chan et al. (7), except that methyl blue (Sigma) substituted for aniline blue.

**Minimal inhibitory concentrations (MIC) of sodium deoxycholate, ox bile extract, and ethanol:** Exponential cultures in LB broth were prepared as previously described (31).

Samples containing around  $3 \times 10^2$  colony-forming-units (CFU) were transferred to polypropylene microtiter plates (Soria Genlab, Valdemoro, Spain) containing known amounts of sodium deoxycholate (DOC), ox bile extract or 96% ethanol. After 12 h incubation at 37°C, growth was visually monitored. Assays were carried out in triplicate.

**Construction of a *S. enterica* SeqA<sup>-</sup> mutant:** The *seqA* gene from strain ATCC 14028 of *Salmonella enterica* ser. Typhimurium was amplified by the polymerase chain reaction, using two 30-nucleotide primers: 5'ATG CGA ATT CAG GGC GGA ATG GTT TCC CAG 3' and 5' ATG CGA ATT CAG CGC ATT GGC GCG ATC TTC 3'. The resulting 1.8 kb fragment, which contained both the *seqA* coding sequence and the promoter region, was digested with *EcoRI* and ligated to a dephosphorylated pBluescript II SK+ vector. Transformation of *E. coli* DH5 $\alpha$  and search for Lac<sup>-</sup> colonies permitted the detection of pBluescript derivatives carrying the *seqA* insert. One such clone was then used to generate a deletion in the *seqA* coding sequence. Because *seqA* is the upstream gene in a two-cistron transcriptional unit that includes also the *pgm* gene (26), an in-frame deletion, 300 bp long, was generated to prevent polarity. The deletion was obtained by PCR, using primers 5' CGT AAG ATC TCG CAC GCG CGT CTA TTT TG 3' and 5' GCT GAA GAT CTG CTA TAG AGT CTA TCA TCA AC 3'. The resulting fragment was digested with *BglII*, and ligated to obtain a deleted pBluescript derivative that contained only 243 bp (81 codons) of the *seqA* coding sequence. The deleted pBluescript derivative was introduced into *E. coli* DH5 $\alpha$  by transformation. Its insert was then excised with *KpnI* and *SacI*, cloned onto pDMS197 (11), and propagated in *E. coli* CC118  $\lambda$  *pir* (20). One pMDS197 derivative containing the  $\Delta$ *seqA* allele was transformed into *E. coli* S17-1  $\lambda$  *pir* (38). The resulting strain was used as donor in matings with *S. enterica* ATCC 14028, selecting Tc<sup>r</sup> transconjugants on E plates supplemented with tetracycline 30 mg/l. Several Tc<sup>r</sup> transconjugants were grown in nutrient broth (without NaCl) containing 5% sucrose. Individual tetracycline-sensitive segregants were then examined for the incorporation of the  $\Delta$ *seqA* allele. Replacement of the chromosomal *seqA* gene by the  $\Delta$ *seqA* construct was detected by PCR amplification, using the same pair of oligonucleotides employed to generate the deletion. One such isolate was propagated as strain SV4752 ( $\Delta$ *seqA1*).

**RNA isolation, microarray procedures and data analysis:** To prepare cells for RNA extraction, 25 ml of fresh LB was inoculated with a 1:100 dilution from an overnight bacterial culture and grown in a 250 ml flask incubated with shaking at 250 rpm in a New Brunswick

Innova 3100 waterbath at 37°C. Three biological replicates were performed for each strain, and RNA was extracted at an optical density (OD<sub>600</sub>) ~ 0.5-0.6 (exponential phase) and ~3 (stationary phase). Specifically, the OD's<sub>600</sub> for RNA extractions from exponential cultures of the wild type were 0.53, 0.52 and 0.53; for exponential cultures of SV4752, 0.60, 0.64 and 0.62; for stationary cultures of the wild type, 3.10, 3.21 and 3.19; and for stationary cultures of SV4752, 3.18, 3.20 and 3.15. RNA extractions were performed as described by Mangan et al. (29), and their quality was assessed on an Agilent 2100 Bioanalyzer. Transcriptomic analyses were performed on a DNA microarray engineered by the Salgenomics consortium of research groups (grant GEN2003-20234-CO6 from the Spanish Ministry of Education and Science, co-ordinated by F. Garcia-del Portillo). The Salgenomics microarray contains 6,992 probes (including open-reading frames, RNA genes and intergenic regions) from the genome sequence of *S. enterica* SL1344, and was developed using genome sequences from the Wellcome Trust Sanger Institute (<ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella>). Use of an SL1344 microarray was justified because the ATCC 14028 genome is not completely sequenced; however, pairwise comparisons of sequenced genes indicate that SL1344 and ATCC 14028 are closely related strains (data not shown). Hybridization, microarray scanning and data analysis were performed as described elsewhere [(2); J. Mariscotti and F. Garcia-del Portillo, submitted]. Raw transcriptomic data are provided as supplementary material (Tables S1 and S2).

**Preparation of protein extracts:** Bacteria were grown overnight in LB medium at 37°C in non-shaking, static cultures. Under these growth conditions, the final OD<sub>600</sub> reached a value of ~1.0. Bacteria contained in 10 ml of the culture were centrifuged at 15,000 g, 10 min, 4°C. The supernatant was filtered with a 0.45 µm-pore Millipore device, and the extracellular proteins precipitated with 10 % trichloroacetic acid (TCA), washed in acetone, and resuspended in cold PBS pH7.4 as previously described (32). Bacteria present in the pellet were directly suspended in cold PBS pH 7.4. An appropriate volume of SDS-Laemmli buffer was finally added to both fractions, extracellular soluble proteins (SP) and total bacteria-associated protein (TP), and finally boiled for 5 min at 95°C. Upon centrifugation (15,000 g, 5 min, room temperature), the volumes to be load into the gels were adjusted to 1x10<sup>8</sup> bacterial cells (TP) or 5x10<sup>9</sup> bacterial cells (SP).

**Competitive index virulence assays:** Eight-week-old female BALB/c mice (Charles River Laboratories, Santa Perpetua de Mogoda, Spain) were used for virulence tests. Groups of 3-4

animals were inoculated with a 1:1 ratio of two strains. Bacteria were grown overnight at 37°C in LB without shaking. Oral inoculation was performed by feeding the mice with 25  $\mu$ l of saline (0.9% NaCl) containing 0.1% lactose and  $10^8$  bacterial colony-forming-units (CFU). Bacteria were recovered from the mouse spleen 6 days after inoculation, and CFU were enumerated on appropriate media. A competitive index (CI) for each mutant was calculated as the ratio between the wild type and the mutant strain in the output divided by their ratio in the input (3). To compare the virulence of a double mutant with that of a single mutant, a “cancelled out” competitive index (COI) was calculated. A COI is the the ratio between the double mutant and the single mutant in the output divided by their ratio in the input (3). Assays were carried out by triplicate. Student's *t*-test was used to analyze CI's and COI's. The null hypothesis was that CI's were not significantly different from 1. COI's were analyzed with two null hypotheses: (i) mean COI is not significantly different from 1; (ii) mean COI is not significantly different from the CI of the corresponding single mutant. *P* values of 0.01 or less were considered significant.

**$\beta$ -galactosidase assays:** Levels of  $\beta$ -galactosidase activity were assayed as described by Miller (30), using the  $\text{CHCl}_3$ -sodium dodecyl sulfate permeabilization procedure.

**Bacterial infection of epithelial cells:** Bacteria were grown overnight in LB medium at 37°C in static, non shaking conditions. HeLa epithelial cells were seeded the day before the infection in 24-well plates and grown at 37°C, 5%  $\text{CO}_2$  to obtain 80% confluency. Bacteria were added to reach a multiplicity of infection of 10 bacteria/eukaryotic cell. Simultaneous infection with two strains permitted the calculation of a competitive index for invasion, CII (37). HeLa cells were infected for 30 min to obtain an infection rate of 70-80% of the cell population. Infected cells were washed three times with phosphate buffered saline, and incubated in fresh tissue culture medium containing 100 mg/l gentamicin. After 2 hours post-infection, the concentration of gentamicin was lowered to 10 mg/l. Numbers of viable intracellular bacteria were obtained by lysing infected cells with 1% Triton X-100 and subsequent plating. CII calculations and statistic analysis were as described previously (37).

**Microscopic observation of cells and nucleoids:** Cells were harvested by centrifugation, washed and resuspended in saline. A small aliquot was spread on a slide glass and dried at

room temperature. Nucleoid staining with 4',6-diamino-2-phenyl-indole (DAPI) and cell visualization followed Hiraga's "fluo-phase" combined method (22).

ACCEPTED

## RESULTS

**Phenotypes of *S. enterica* SeqA<sup>-</sup> mutants:** In *E. coli*, SeqA<sup>-</sup> mutants form very small colonies (25). In contrast, the  $\Delta seqA1$  allele did not alter the morphology of *S. enterica* colonies. The absence of a growth difference associated to a *seqA* mutation was also observed in liquid cultures: the growth rate of a *S. enterica* SeqA<sup>-</sup> strain in both LB and minimal E medium remained largely unaltered (data not shown). Another difference between SeqA<sup>-</sup> mutants of *E. coli* and *Salmonella* was found in phage plaquing experiments. While phage lambda has been shown to form large plaques on *E. coli* (46), the lambdoid phage P22 HT and its clear-plaque derivative H5 formed plaques of similar sizes on both 14028 (*seqA*<sup>+</sup>) and SV4752 ( $\Delta seqA1$ ). An effect of SeqA on lysogenization, as described for lambda (46), was also ruled out for P22: the plaques formed by P22 HT on strains ATCC 14028 (*seqA*<sup>+</sup>) and SV4752 ( $\Delta seqA1$ ) of *S. enterica* were identical (small and turbid), and the number of clear plaques was below 0.01% in both cases (data not shown). Aside from these differences, SeqA<sup>-</sup> mutants of *S. enterica* resemble those of *E. coli* in the following traits:

(i) Nucleoid staining with 4',6-diamino-2-phenyl-indole (DAPI) and cell visualization following Hiraga's "fluo-phase" combined method showed filament formation and aberrant nucleoid segregation (Figure 1). Similar phenotypes have been described in *E. coli* SeqA<sup>-</sup> mutants (25).

Figure 1

(ii) A pBluescript derivative carrying the wild type *seqA* gene (pIZ1574) could not be maintained in a Dam<sup>-</sup> strain (SV4698). The same construct was easily introduced in a Dam<sup>+</sup> isogenic strain (ATCC 14028). In contrast, expression of *seqA* from an arabinose-driven pBAD promoter permitted the introduction of a pBAD-*seqA* plasmid (pIZ1588) in both Dam<sup>+</sup> and Dam<sup>-</sup> hosts. However, a Dam<sup>-</sup> host carrying pIZ1588 formed normal colonies only in the absence of arabinose (and at low arabinose concentrations). From these observations, we concluded that overproduction of *Salmonella* SeqA is lethal in a Dam<sup>-</sup> background, as described in *E. coli* (25).

(iii) A SeqA<sup>-</sup> mutant of *S. enterica* (strain SV4972) showed moderate SOS induction, as previously described in *E. coli* (25). Induction of the *S. enterica* SOS system was monitored using a *cea::lac* fusion carried on plasmid pGE108 (35). This fusion has been extensively used in our laboratory to monitor SOS induction in *Salmonella* (14, 31). The presence of a *seqA* mutation in the background increased *cea::lac* expression 3-4 fold: 40 ± 16 Miller units in ATCC 14028 / pGE108 vs 188 ± 23 Miller units in its isogenic derivative SV4972 (averages of 4 independent experiments).

(iv) In *E. coli*, the viability of SeqA<sup>-</sup> mutants requires induction of the SOS system, since the cell division inhibitor Sula is necessary for survival (25). In an analogous fashion, we were unable to transduce the *lexA*(Ind<sup>-</sup>) allele of DA6522 to SV4972, suggesting that a *S. enterica* SeqA<sup>-</sup> LexA(Ind<sup>-</sup>) mutant is inviable.

(v) The antecedent that *seqA* mutations cause sensitivity to sodium deoxycholate in *E. coli* (46) suggested the possibility that SeqA<sup>-</sup> mutants of *Salmonella* might be likewise sensitive to bile salts. Bile salt sensitivity was tested as previously described (31). The results, summarized in Table 2, indicated that lack of SeqA renders *S. enterica* sensitive to both sodium deoxycholate (DOC) and ox bile extract. The minimal inhibitory concentrations of both DOC and bile were similar to those previously described for Dam<sup>-</sup> strains (31, 32).

(vi) Because *E. coli* SeqA<sup>-</sup> mutants are sensitive to ethanol (46), we tested whether their *Salmonella* counterparts had a similar phenotype. The minimal inhibitory concentration of ethanol was found to be 20-fold reduced in a SeqA<sup>-</sup> background (Table 2).

Table 2

**Envelope instability in *S. enterica* SeqA<sup>-</sup> mutants:** In previous studies we showed that DNA adenine methylase mutants of *S. enterica* secrete high amounts of proteins to the culture medium, a trait that can be easily observed in SDS-PAGE gels from culture supernatants (32). The extreme sensitivity to bile and to membrane-damaging agents shown by SeqA<sup>-</sup> mutants raised the possibility that they could have a similar phenotype. Fig. 3 shows an SDS-PAGE gel in which the profile of extracellular proteins present in supernatants of SeqA<sup>-</sup>, Dam<sup>-</sup>, and SeqA<sup>-</sup> Dam<sup>-</sup> strains were compared with that of the wild type. Lack of SeqA seems to cause

exacerbated release of proteins to the extracellular milieu, to a degree similar to that of  $Dam^-$  mutants.

Figure 2

**Virulence assays with *S. enterica*  $SeqA^-$  mutants:** The observation that a  $SeqA^-$  mutant of *S. enterica* was sensitive to bile raised the possibility that a *seqA* mutation might impair *Salmonella* virulence. BALB/c mice were subjected to mixed infections, inoculating groups of 3-4 animals with a 1:1 ratio of  $SeqA^+$  and  $SeqA^-$  isogenic strains. Competitive index (CI) analysis indicated that a *seqA* mutation caused moderate but significant attenuation of virulence by the oral route but had no effect by the intraperitoneal route (Figure 3). This observation suggests that the  $SeqA^-$  mutant is impaired for virulence at the intestinal stage of infection, and can be tentatively correlated with the sensitivity to bile salts conferred by a *seqA* mutation. Our finding that lack of  $SeqA$  does not attenuate *Salmonella* virulence by the intraperitoneal route disagrees with a previous report (8). However, it must be noted that the *seqA::MudI1734* allele used by Chatti et al. (8) is known to be polar on the downstream gene *pgm* (5), while we employed a non-polar, in-frame deletion allele of *seqA*. Lack of phosphoglucomutase, the product of the *pgm* gene, has in fact a mild attenuating effect on *Salmonella* virulence, both orally and intraperitoneally (D. A. Cano and J. Casadesús, unpublished). This may also explain why the *seqA::MudI1734* allele causes a stronger attenuation by the oral route than the  $\Delta seqA1$  allele (8).

COI analysis with isogenic  $SeqA^-$  and  $SeqA^- Dam^-$  strains indicated that a *dam* mutation was epistatic over a *seqA* mutation, both orally and intraperitoneally. The latter observation can be explained by the well known fact that the  $SeqA$  protein does not bind to unmethylated DNA (47). Hence, whatever the function of  $SeqA$  in the infection process, it cannot be exerted in the absence of methylated or hemimethylated GATCs. COI analysis confirmed the attenuation of  $SeqA^-$  mutants by the oral route, since the COI  $SeqA^- / Dam^- SeqA^-$  was >10 fold lower than the CI wild type /  $Dam^-$  (Fig. 3).

The observation that *S. enterica*  $SeqA^-$  mutants are less virulent upon oral inoculation raised the possibility that lack of  $SeqA$  might impair invasion of the intestinal epithelium. This possibility was investigated by comparing invasion of cultured HeLa cells by  $SeqA^+$  and  $SeqA^-$  isogenic strains. A non-invading  $InvJ^-$  mutant (SV4861) was included in these

experiments as an internal control. The average invasion rate obtained in standard 2 h infection assays of HeLa cells was similar for both SeqA<sup>+</sup> and SeqA<sup>-</sup> strains (CII = 0.911), and >50 fold lower (CII = 0.019) in SV4861 (InvJ<sup>-</sup>), indicating that a *seqA* mutation does not impair invasion of epithelial cells.

Figure 3

**Transcriptomic analysis of SeqA-dependent gene expression:** Wild type ATCC 14028 and its isogenic SeqA<sup>-</sup> derivative SV4752 were grown in LB medium, and RNA extraction for microarray analysis was carried out in exponential and stationary cultures. Use of these two conditions was justified by the fact that SeqA binds to methylated DNA, found in both exponential and stationary phase cells, and hemimethylated DNA, present in dividing cells only (48). The experiments were based on the rationale that higher RNA content in a SeqA<sup>-</sup> background would indicate that SeqA represses gene expression in the wild type. In turn, lower RNA content in a SeqA<sup>-</sup> background would indicate that SeqA activates gene expression in the wild type. As shown in the computer image diagrams of Fig. 4, the RNA content for most *S. enterica* loci was similar in SeqA<sup>+</sup> and SeqA<sup>-</sup> hosts both in exponential and stationary cultures, suggesting that SeqA-regulated genes are rare in *Salmonella*. A similar conclusion was drawn from a SeqA transcriptomic study in *E. coli* (27). A number of SOS genes showed moderately increased expression in a SeqA<sup>-</sup> background as previously described in *E. coli* (27). Aside from SOS genes, a small number of genes showed differences higher than the two fold cutoff arbitrarily chosen as threshold. In exponential cultures (Table 3), 2 SeqA-repressed genes and 4 SeqA-activated genes were found. In stationary cultures (Table 4), 4 SeqA-repressed genes and 32 SeqA-activated genes were found. A noteworthy observation is that a significant proportion (16/42) of such genes are *Salmonella*-specific and in most cases have unknown function. The existence of six SeqA-regulated genes with unknown function that are not *Salmonella*-specific is likewise intriguing, since it raises the putative number of SeqA-regulated genes with unknown function to about half of the total detected.

Figure 4

## DISCUSSION

The cell division abnormalities observed in SeqA<sup>-</sup> mutants of *Salmonella enterica* closely resemble those previously described in their *E. coli* counterparts, which have been the subject of more numerous and deeper studies (1, 4, 10, 25, 27, 39, 46, 48). Other analogies between *E. coli* and *Salmonella* SeqA<sup>-</sup> mutants include SOS induction, requirement of the SOS regulon for viability, envelope instability and sensitivity to bile salts and other envelope-damaging agents. Given these analogies, the observation that a *seqA* mutation does not alter the morphology of P22 plaques is anecdotal, and should not blur the tentative conclusion that the well known roles of SeqA in *E. coli* chromosome replication and nucleoid organization (1, 4, 25, 27, 39, 48) can be tentatively extended to a relatively close bacterial genus like *Salmonella*. This view is consistent with the 87% amino acid identity found between the *E. coli* and *Salmonella* SeqA proteins (data not shown).

An interesting trait of *Salmonella* SeqA<sup>-</sup> mutants is their virulence attenuation by the oral route. *In vitro* assays have indicated that SeqA<sup>-</sup> mutants are not impaired for invasion of epithelial cells, suggesting that attenuation is not caused by deficient interaction with the intestinal epithelium. Furthermore, global gene expression (microarray) analysis has not unveiled altered expression of any known locus involved in *Salmonella* infection (neither at the intestinal stage nor during systemic infection). Hence, a tentative explanation for oral attenuation in SeqA<sup>-</sup> mutants may be their sensitivity to bile salts. During passage through the small intestine, *Salmonella* faces periodic release of bile, and the extreme sensitivity of SeqA<sup>-</sup> mutants to bile salts may cause massive cell killing in the *Salmonella* population, with concomitant reduction of virulence. In fact, SeqA<sup>-</sup> mutants are not attenuated upon intraperitoneal infection, which spares the bacterial population from exposure to bile in the host intestine.

The cause of bile sensitivity associated to *seqA* disruption remains unknown. In *E. coli*, SeqA<sup>-</sup> mutants show altered membrane permeability (46) and abnormal phospholipid composition (10), which may explain their increased sensitivity to a number of dyes. Our observation that the envelope of *S. enterica* SeqA<sup>-</sup> mutants is likewise unstable can be tentatively correlated with bile sensitivity, because unconjugated bile salts can enter the cell by diffusion (43). How can a *seqA* mutation impair membrane stability is an interesting enigma. In *E. coli*, SeqA has been shown to associate to the *E. coli* outer membrane in a replication cycle-dependent

manner (39). Thus, a structural role of SeqA in envelope stability cannot be discarded (46). An alternative explanation is that SeqA might regulate the expression of genes involved in the stability of the cell membranes, a possibility also considered in *E. coli* (42).

Our conclusion that SeqA does not play a major role in gene regulation in *S. enterica* is in agreement with a previous study carried out in *E. coli* (27). This view does not exclude the possibility that SeqA could play subtle roles, hardly visible by transcriptome analysis, in the expression of certain genes. For instance, the SeqA/OxyR competition described in the *E. coli* *agn43* gene (9) would likely be overlooked in a microarray study. Another possibility, not investigated here, is that SeqA might control bacteriophage P22 promoters, as shown for the  $p_R$ ,  $p_I$  and  $p_{aQ}$  promoters of bacteriophage lambda (40, 41). Despite these caveats, our study has detected a small number of *Salmonella* genes whose expression is impaired in a SeqA<sup>-</sup> background. The list includes both loci activated by SeqA and loci repressed by SeqA, and one third of such genes (16/42) are *Salmonella*-specific. However, impaired gene expression in a SeqA<sup>-</sup> background does not imply a role for SeqA in the control of transcription. A well documented example of this kind involves DNA adenine methylase mutants of *E. coli* and *Salmonella*, which show increased expression of the SOS regulon without any direct effect of Dam methylation on LexA-controlled transcription (27, 47). In a similar fashion, the existence of SeqA-regulated genes might reflect physiological changes occurring in response to a *seqA* mutation rather than a direct role of SeqA in transcriptional control.

## ACKNOWLEDGMENTS

This work was supported by grants BIO2004-3455-CO2-02 and GEN2003-20234-CO6-03 (to J. C.), BIO2004-3455-CO2-01 and GEN2003-20234-CO6-01 (to F. G. P.), all from the Spanish Ministry of Education and Science and the European Regional Fund. A. I. P. was supported by a predoctoral fellowship from the Fundación Ramón Areces. M. J. and I. S. were supported by FPI predoctoral fellowships from the Spanish Ministry of Education and Science. M. G. P. is an investigator supported by a contract of the 'Ramón y Cajal' program of the Spanish Ministry of Education and Science. We thank Dan I. Andersson, Victor de Lorenzo and Jorge Galan for providing bacterial strains, and Roberto Balbontín for advice in microarray experiments.

ACCEPTED

Downloaded from <http://jb.asm.org/> on September 23, 2017 by guest

**Table 1.** Strain list

Strain	Genotype	Reference or source <sup>a</sup>
DA6522	<i>lexA39</i> (Ind <sup>-</sup> ) Cm <sup>r</sup>	Dan I. Andersson
SV4698	<i>dam-225::MudJ</i>	Lab stock
SV4752	<i>ΔseqA1</i>	
SV4784	<i>dam-225::MudJ ΔseqA1</i>	
SV4861	<i>invJ::Km<sup>r</sup></i>	
SV4972	<i>ΔseqA1</i> / pGE108	

<sup>a</sup> Omitted for strains described in this study

**Table 2.** Minimal inhibitory concentrations of sodium deoxycholate, ox bile extract, and ethanol

Strain	Genotype	MIC (% w/v)		
		DOC	Ox bile extract	Ethanol
14028	Wild type	6	14	4
SV4752	$\Delta seqA1$	0.8	6	0.2
SV4698	<i>dam-225::MudJ</i>	0.4	5	N.T. <sup>a</sup>

<sup>a</sup> Not tested

ACCEPTED

Downloaded from <http://j.b.asm.org/> on September 23, 2017 by guest

**Table 3.** *Salmonella enterica* serovar Typhimurium genes with impaired expression in a SeqA<sup>-</sup> background identified by transcriptomic analysis in exponential cultures<sup>a</sup>

Gene	Protein function	SeqA <sup>-</sup> mutant/wild type expression ratio
<i>glnP</i>	glutamine ABC transporter permease component	5.53
STM4212	putative phage tail core protein	2.21
STM1329	putative inner membrane protein	0.43
STM2911	putative permease	0.38
STM0284	putative Shiga-like toxin A subunit	0.38
STM2137	putative cytoplasmic protein	0.38

<sup>a</sup> SeqA is an activator for genes with a relative expression value below 1.0, and a repressor for genes with values above 1.0.

**Table 4.** *Salmonella enterica* serovar Typhimurium genes with impaired expression in a SeqA<sup>-</sup> background identified by transcriptomic analysis in stationary cultures<sup>a</sup>

Gene	Protein function	SeqA <sup>-</sup> mutant/wild type expression ratio
<i>rpsH</i>	30S ribosomal protein S8	2.94
<i>cbiT</i>	precorrin-8w decarboxylase	2.84
STM3098	putative transcriptional regulator	2.70
STM2133	putative cytoplasmic protein	2.69
<i>misL</i>	putative autotransporter	0.40
<i>kdpA</i>	potassium-transporting ATPase subunit A	0.40
<i>ydhL</i>	putative oxidoreductase	0.39
<i>glgP</i>	glycogen phosphorylase	0.39
STM2590	tail assembly protein I-like	0.39
<i>argE</i>	acetylornithine deacetylase	0.39
<i>gudT</i>	putative D-glucarate permease	0.39
<i>csgG</i>	putative curli operon transcriptional regulator	0.38
STM0053	putative transcriptional regulator	0.38
<i>yrfF</i>	putative inner membrane protein	0.38
<i>rpsA</i>	30S ribosomal protein S1	0.38
STM2609	DNA packaging-like protein	0.38
<i>yeaR</i>	putative cytoplasmic protein	0.38
<i>ygiN</i>	putative cytoplasmic protein	0.37
<i>menF</i>	menaquinone-specific isochorismate synthase	0.37
STM2743	putative cytoplasmic protein	0.37
STM1671	putative regulatory protein	0.37
STM4493	putative cytoplasmic protein	0.37
<i>chaB</i>	cation transport regulator	0.36
<i>yijP</i>	putative integral membrane protein	0.36
STM4192	putative cytoplasmic protein	0.36
<i>deoA</i>	thymidine phosphorylase	0.36
<i>yidE</i>	hypothetical protein	0.35

<i>avrA</i>	secreted effector protein	0.35
<i>sbcC</i>	ATP-dependent dsDNA exonuclease	0.35
<i>ybeU</i>	putative cytoplasmic protein	0.34
STM4493	putative cytoplasmic protein	0.34
<i>glgP</i>	glycogen phosphorylase	0.34
STM4015	putative cytoplasmic protein	0.33
<i>adi</i>	catabolic arginine decarboxylase	0.32
<i>yeeY</i>	putative transcriptional regulator	0.30
<i>narJ</i>	nitrate reductase 1 delta subunit	0.24

<sup>a</sup> SeqA is an activator for genes with a relative expression value below 1.0, and a repressor for genes with values above 1.0.

## FIGURE LEGENDS

**Figure 1.** Microscopic photographs of SeqA<sup>+</sup> and SeqA<sup>-</sup> *S. enterica* cells during exponential growth, visualized with Hiraga's fluo-phase combined method (22)

**Figure 2.** Lack of SeqA causes exacerbated release of proteins to the extracellular medium. (TP) bacteria-associated total protein; (SP) extracellular protein present in culture supernatant. Lanes correspond to the following strains: 1, wild type (ATCC 14028); 2, Dam<sup>-</sup> (SV4698); 3, SeqA<sup>-</sup> (SV4752); and, 4, Dam<sup>-</sup> SeqA<sup>-</sup> (SV4784). Samples loaded in the gel were adjusted to 1x10<sup>8</sup> bacterial cells (TP) or 5x10<sup>9</sup> bacterial cells (SP). Indicated are the molecular weight markers used (116, 83, 48.7, 33.4, 28.2, 20.7, and 7 kDa).

**Figure 3.** Graphical representation of competitive index analyses of SeqA<sup>-</sup> (SV4752), Dam<sup>-</sup> (SV4698) and SeqA<sup>-</sup> Dam<sup>-</sup> (SV4784) strains, after oral or intraperitoneal infections of BALB/c mice. The mixed infections performed were as follows: (a) wild type / SeqA<sup>-</sup>, oral infection; (b) wild type / Dam<sup>-</sup>, oral; (c) Dam<sup>-</sup> SeqA<sup>-</sup> / Dam<sup>-</sup>, oral; (d) SeqA<sup>-</sup> / Dam<sup>-</sup> SeqA<sup>-</sup>, oral; (e) wild type / SeqA<sup>-</sup>, intraperitoneal infection; (f) wild type / Dam<sup>-</sup>, intraperitoneal. The indices represented are the mean from ≥3 infections. Error bars represent the standard deviation.

**Figure 4.** Computer-generated, normalized representation of microarray data. The Cy5/Cy3 fluorescence intensity of each locus in the wild-type (horizontal axis) is plotted vs the intensity of the same locus in the mutant (vertical axis). Most genes fall near the imaginary bisectrix (slope = 1), indicating identical or similar expression. Genes above the bisectrix show higher expression in the mutant strain, indicating that SeqA represses their expression. Genes below the bisectrix show lower expression in the mutant strain, indicating that SeqA activates their expression.

## REFERENCES

1. **Bach, T., M. A. Krekling, and K. Skarstad.** 2003. Excess SeqA prolongs sequestration of *oriC* and delays nucleoid segregation and cell division. *EMBO J.* **22**:315-323.
2. **Balbontín, R., G. Rowley, M. G. Pucciarelli, J. López-Garrido, Y. Wormstone, S. Lucchini, F. García-del Portillo, J. C. D. Hinton, and J. Casadesús.** 2006. DNA adenine methylation regulates virulence gene expression in *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* **188**:8160-8168.
3. **Beuzón, C. R., and D. W. Holden.** 2001. Use of mixed infections with *Salmonella* strains to study virulence genes and their interactions in vivo. *Microbes Infect.* **3**:1345-1352.
4. **Brendler, T., J. Sawitzke, K. Seerguev, and S. Austin.** 2000. A case for sliding SeqA tracts at anchored replication forks during *Escherichia coli* chromosome replication and segregation. *EMBO J.* **22**:315-323.
5. **Cánovas, D., S. A. Fletcher, M. Hayashi, and L. Csonka.** 2001. Role of trehalose in growth at high temperature of *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* **183**:3365-3371.
6. **Casadesús, J., and D. A. Low.** 2006. Epigenetic gene regulation in the bacterial world. *Microbiol. Mol. Biol. Rev.* **70**:830-856.
7. **Chan, R. K., D. Botstein, T. Watanabe, and Y. Ogata.** 1972. Specialized transduction of tetracycline by phage P22 in *Salmonella typhimurium*. II. Properties of a high frequency transducing lysate. *Virology* **50**:883-898.
8. **Chatti, A., D. Daghfous, and A. Landoulsi.** 2007. Effect of seqA mutation on *Salmonella typhimurium* virulence. *J. Infect.* **54**:241-245.
9. **Correnti, J., M. Munster, T. Chan, and M. van der Woude.** 2002. Dam-dependent phase variation of Ag43 in *Escherichia coli* is altered in a seqA mutant. *Molecular Microbiology* **44**:521-532.
10. **Daghfous, D., A. Chatti, B. Marzouk, and A. Landoulsi.** 2006. Phospholipid changes in seqA and dam mutants of *Escherichia coli*. *C. R. Biologies* **329**:271-276.

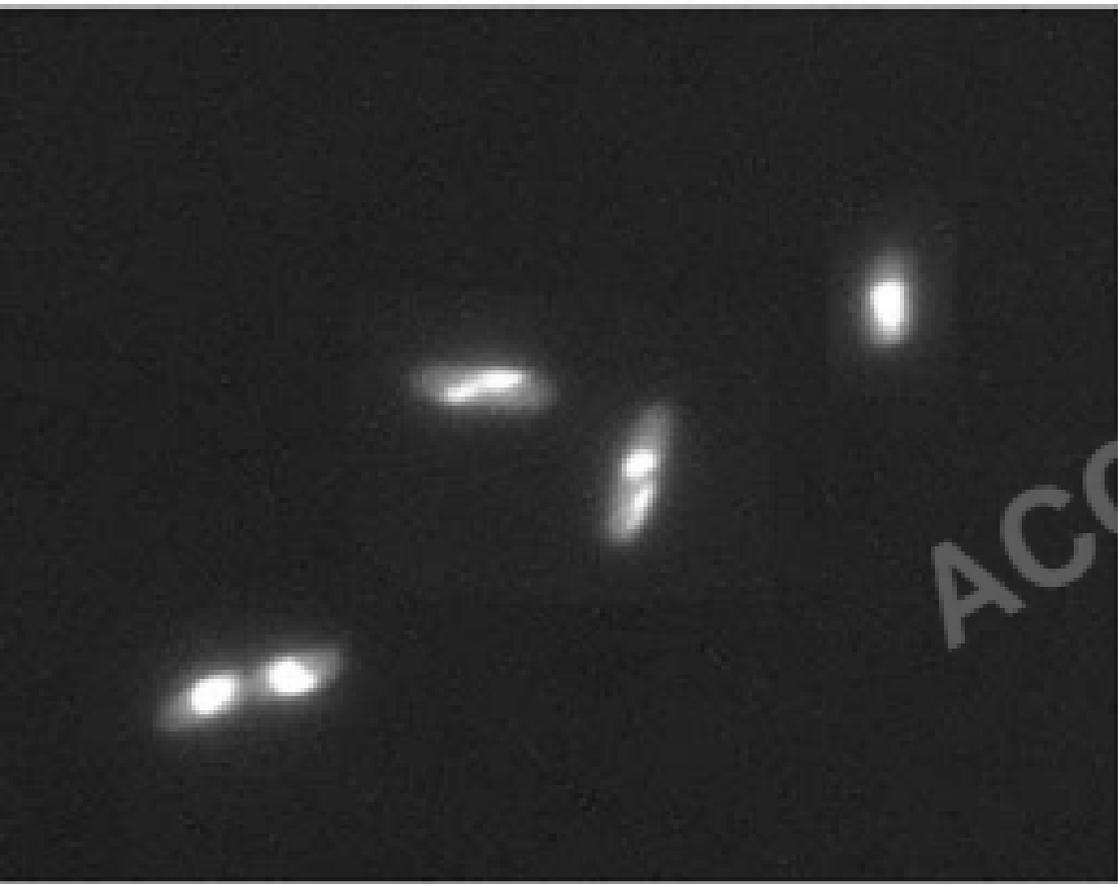
11. **Edwards, R. A., H. K. Linda, and D. M. Schifferli.** 1998. Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. *Gene* **207**:149-157.
12. **García-del Portillo, F., M. G. Pucciarelli, and J. Casadesús.** 1999. DNA adenine methylase mutants of *Salmonella typhimurium* are deficient in protein secretion, cell invasion and M cell cytotoxicity. *Proc. Natl. Acad. Sci. USA* **96**:11578-11583.
13. **Garzón, A., D. A. Cano, and J. Casadesús.** 1995. Role of Erf recombinase in P22-mediated plasmid transduction. *Genetics* **140**:427-434.
14. **Gibert, I., and J. Casadesús.** 1990. *sulA*-independent division inhibition in His-constitutive strains of *Salmonella typhimurium*. *FEMS Microbiol. Lett.* **69**:205-210.
15. **Groisman, E. A., and J. Casadesús.** 2005. The origin and evolution of human pathogens. *Mol. Microbiol.* **56**:1-7.
16. **Groisman, E. A., and H. Ochman.** 1997. How *Salmonella* became a pathogen. *Trends Microbiol.* **5**:343-349.
17. **Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J. Bacteriol.* **177**:4121-4130.
18. **Heithoff, D., R. L. Sinsheimer, D. A. Low, and M. J. Mahan.** 1999. An essential role for DNA adenine methylation in bacterial virulence. *Science* **284**:967-970.
19. **Heithoff, D. M., E. I. Enioutina, R. A. Daynes, R. L. Sinsheimer, D. A. Low, and M. J. Mahan.** 2001. *Salmonella* DNA adenine methylase mutants confer cross-protective immunity. *Infect. Immun.* **69**:6725-6730.
20. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1992. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* **172**:6557-6567.
21. **Heusipp, G., S. Fälker, and M. A. Schmidt.** 2007. DNA adenine methylation and bacterial pathogenesis. *Int. J. Med. Microbiol.* **297**:1-7.
22. **Hiraga, S., H. Niki, T. Ogura, C. Ichinose, H. Mori, B. Ezaki, and A. Jaffé.** 1989. Chromosome partitioning in *Escherichia coli*: novel mutants producing anucleate cells. *J. Bacteriol.* **171**:1496-1505.

23. **Hoiseith, S. K., and B. A. Stocker.** 1981. Aromatic-dependent *Salmonella enterica* are non-virulent and effective as live vaccines. *Nature* **291**:238-239.
24. **Klungsoyr, H. K., and K. Skarstad.** 2004. Positive supercoiling is generated in the presence of *Escherichia coli* SeqA protein. *Mol. Microbiol.* **54**:123-131.
25. **Lu, M., J. L. Campbell, E. Boye, and N. Kleckner.** 1994. SeqA: a negative modulator of replication initiation in *E. coli*. *Cell* **77**:413-426.
26. **Lu, M., and N. Kleckner.** 1994. Molecular cloning of the *pgm* gene encoding phosphoglucomutase of *Escherichia coli*. *J. Bacteriol.* **176**:5847-5851.
27. **Løbner-Olesen, A., M. G. Marinus, and F. G. Hanssen.** 2003. Role of SeqA and Dam in *Escherichia coli* gene expression: a global/microarray analysis. *Proc. Natl. Acad. Sci. USA* **100**:4672-4677.
28. **Løbner-Olesen, A., O. Skovgaard, and M. G. Marinus.** 2005. Dam methylation: coordinating cellular processes. *Curr. Op. Microbiol.* **8**:154-160.
29. **Mangan, M. W., S. Lucchini, V. Danino, T. O. Croinin, J. C. Hinton, and C. J. Dorman.** 2006. The integration host factor (IHF) integrates stationary-phase and virulence gene expression in *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.* **59**:1831-1847.
30. **Miller, J. H.** 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.
31. **Prieto, A. I., F. Ramos-Morales, and J. Casadesús.** 2004. Bile-induced DNA damage in *Salmonella enterica*. *Genetics* **168**:1787-1794.
32. **Pucciarelli, M. G., A. I. Prieto, J. Casadesús, and F. García-del Portillo.** 2002. Envelope instability in DNA adenine methylase mutants of *Salmonella enterica*. *Microbiology* **148**:1171-1182.
33. **Rotger, R., and J. Casadesús.** 1999. The virulence plasmids of *Salmonella*. *Int. Microbiol.* **2**:177-184.
34. **Rychlik, I., and P. A. Barrow.** 2005. Salmonella stress management and its relevance to behaviour during intestinal colonisation and infection. *FEMS Microbiol. Rev.* **29**:1021-1040.
35. **Salles, B., J. M. Weiseman, and G. Weinstock.** 1987. Temporal control of colicin E1 induction. *J. Bacteriol.* **169**:5028-5034.
36. **Schmieger, H.** 1972. Phage P22 mutants with increased or decreased transducing abilities. *Mol. Gen. Genet.* **119**:75-88.

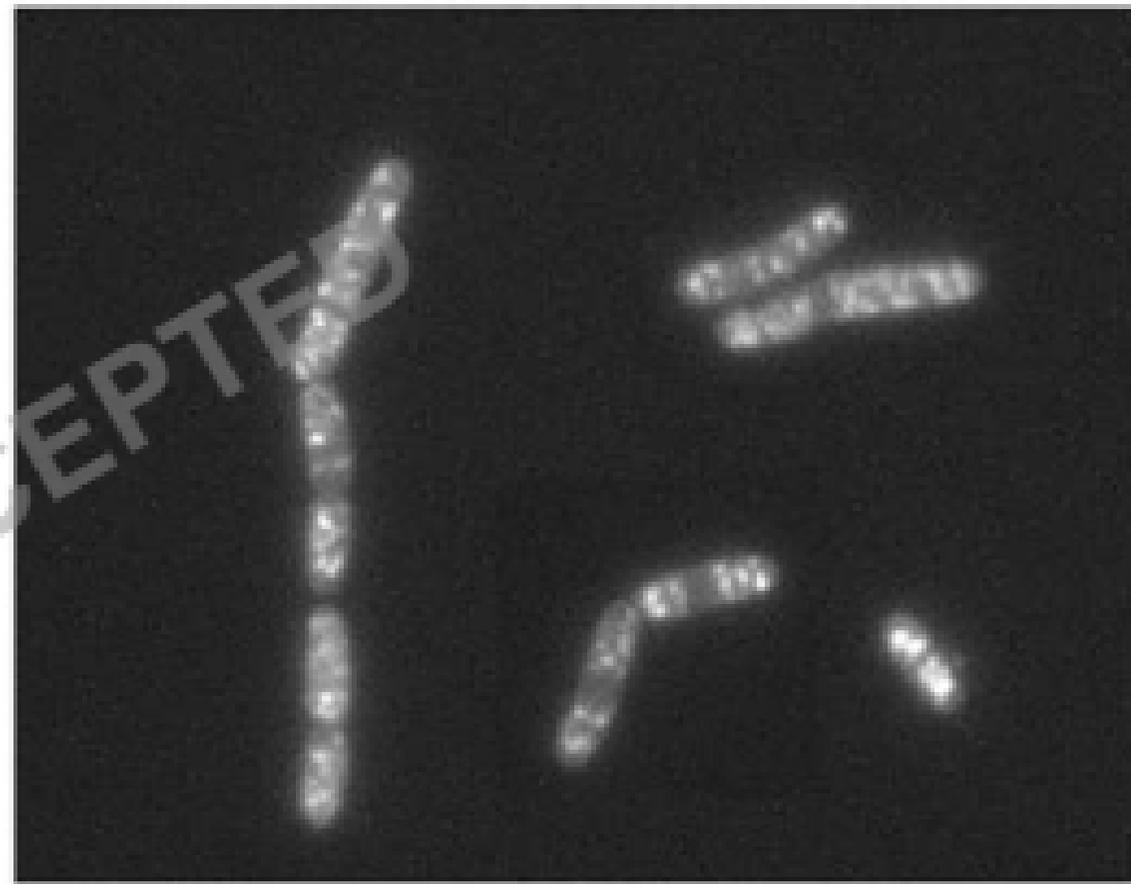
37. **Segura, I., J. Casadesús, and F. Ramos-Morales.** 2004. Use of mixed infections to study cell invasion and intracellular proliferation of *Salmonella enterica* in eukaryotic cell cultures. *J. Microbiol. Meth.* **56**:83-91.
38. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host-range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Technology* **1**:784-791.
39. **Slater, S., S. Wold, M. Lu, E. Boye, K. Skarstad, and N. Kleckner.** 1995. *E. coli* SeqA protein binds oriC in two different methyl-modulated reactions appropriate to its roles in DNA replication and origin sequestration. *Cell* **82**:927-936.
40. **Slominska, M., G. Konopa, J. Ostrowska, B. Kedzierska, G. Wegrzyn, and A. Wegrzyn.** 2003. SeqA-mediated stimulation of a promoter activity by facilitating functions of a transcription activator. *Mol. Microbiol.* **47**:1669-1679.
41. **Slominska, M., A. Wegrzyn, A. Konopa, K. Skarstad, and G. Wegrzyn.** 1999. SeqA, the *Escherichia coli* origin sequestration protein, is also a transcription factor. *Mol. Microbiol.* **40**:1371-1379.
42. **Strzelczyk, B., M. Slominska-Wojewodzka, G. Wegrzyn, and A. Wegrzyn.** 2003. Non-random distribution of GATC sequences in regions of promoters stimulated by the SeqA protein of *Escherichia coli*. *Acta Biochim. Pol.* **50**:941-945.
43. **Thanassi, D. G., L. W. Cheng, and H. Nikaido.** 1997. Active efflux of bile salts by *Escherichia coli*. *J. Bacteriol.* **179**:2512-2518.
44. **Torreblanca, J., and J. Casadesús.** 1996. DNA adenine methylase mutants of *Salmonella typhimurium* and a novel Dam-regulated locus. *Genetics* **144**:15-26.
45. **Vogel, H., and D. Bonner.** 1956. Acetylornithase of *Escherichia coli*: partial purification and some properties. *J. Biol. Chem.* **218**:97-106.
46. **Wegrzyn, A., B. Wrobel, and G. Wegrzyn.** 1999. Altered biological properties of cell membranes in *Escherichia coli* *dnaA* and *seqA* mutants. *Mol. Gen. Genet.* **261**:762-769.
47. **Wion, D., and J. Casadesús.** 2006. N<sup>6</sup>-methyl-adenine: an epigenetic signal for DNA-protein interactions. *Nat. Rev. Microbiol.* **4**:183-192.
48. **Yamazoe, M., S. Adachi, S. Kanava, K. Obsumi, and S. Hiraga.** Sequential binding of SeqA protein to nascent DNA segments at replication forks in synchronized cultures of *E. coli*. *Mol. Microbiol.* **55**:289-298.

49. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vector. *Gene* **33**:103-119.

ACCEPTED



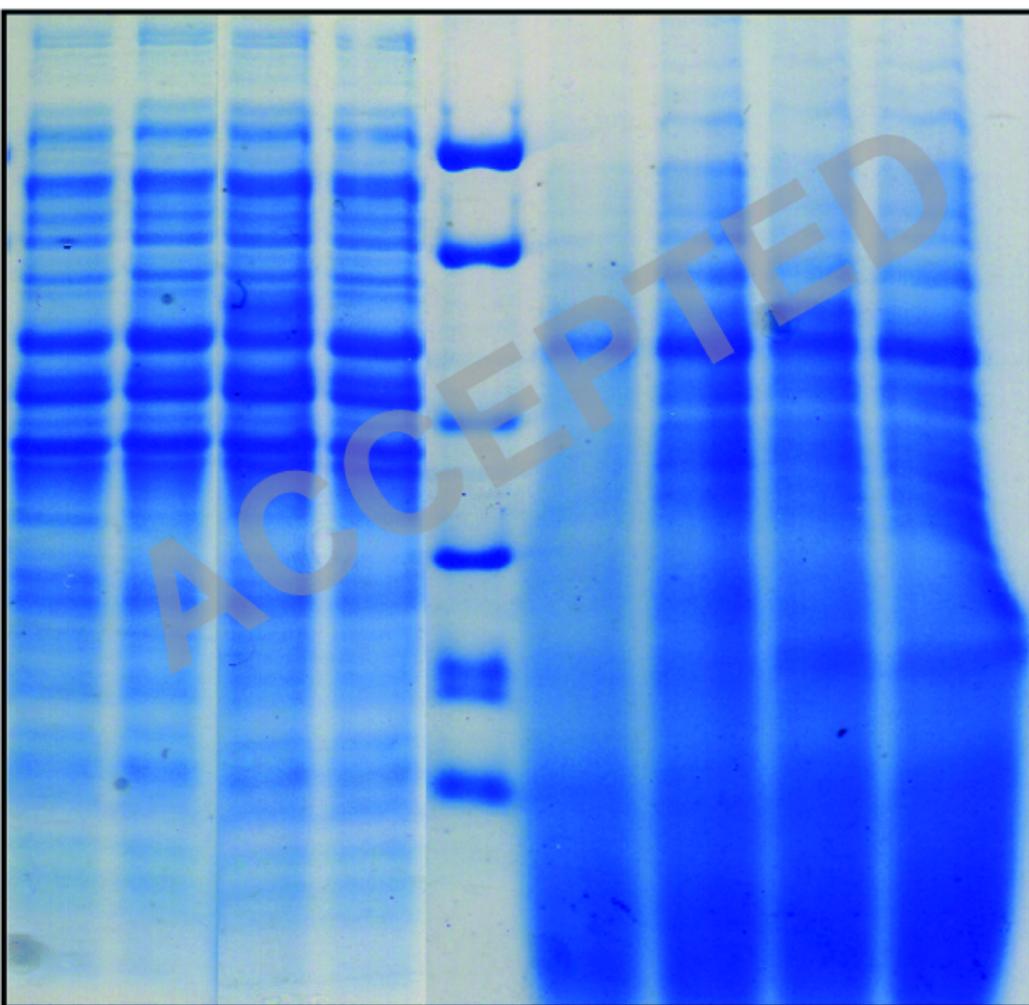
wild type



SeqA<sup>-</sup>

TP

SP



1

2

3

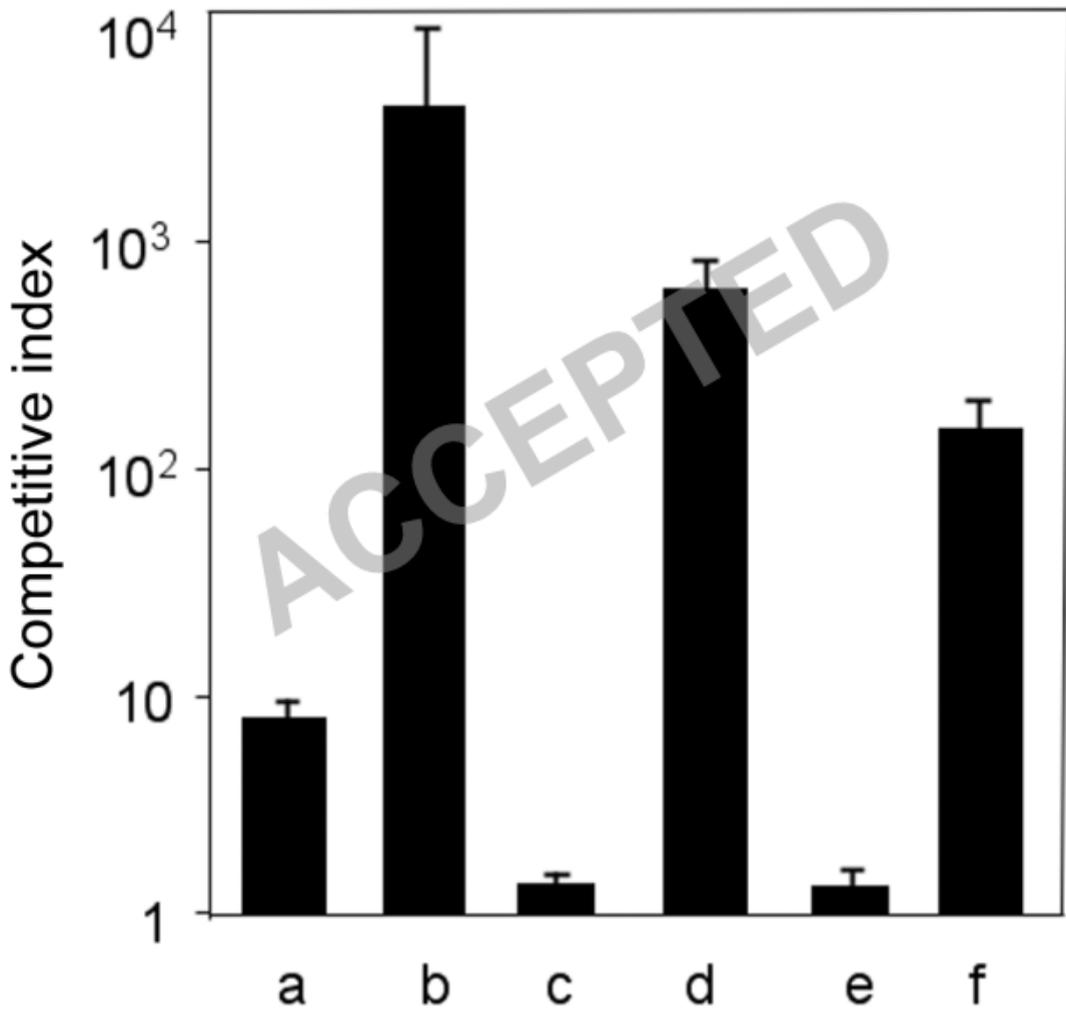
4

1

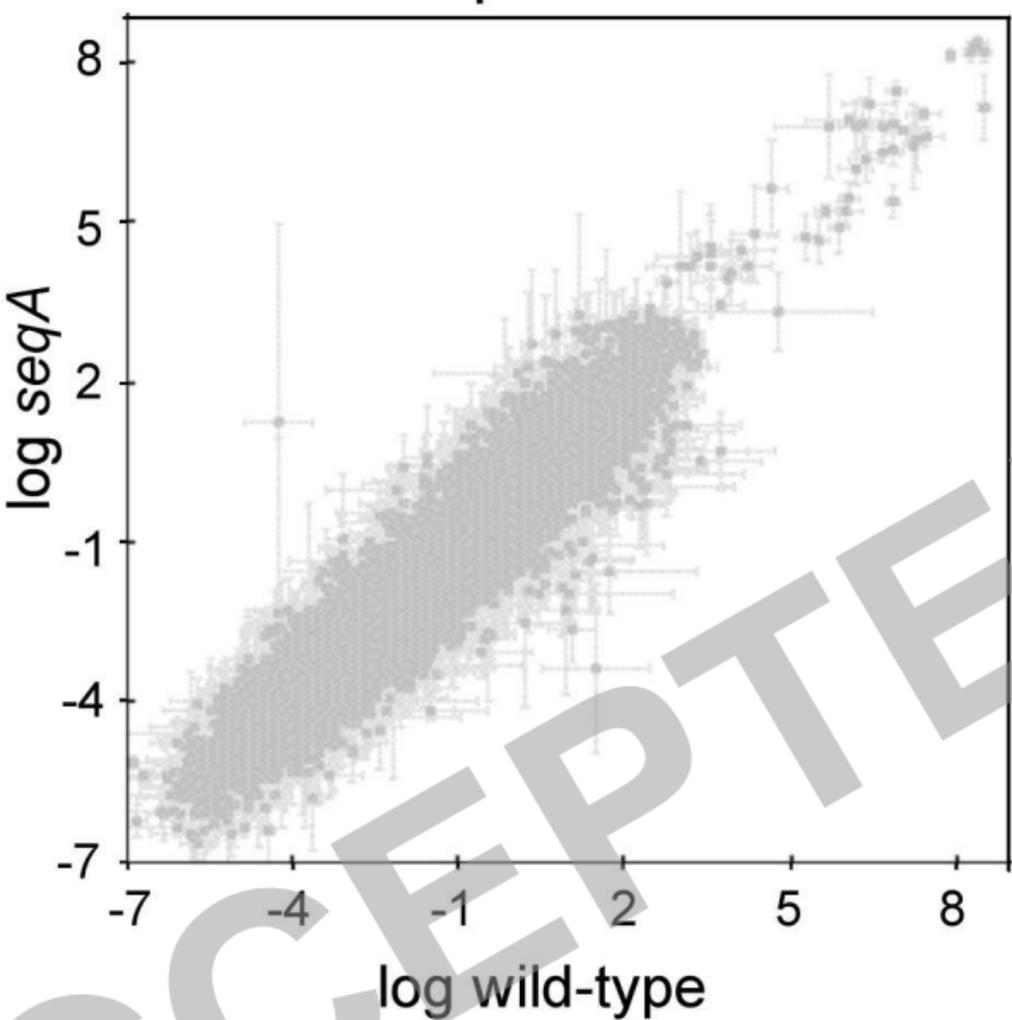
2

3

4



# Exponential



# Stationary

