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Dam methylation is required for efficient biofilm production in *Salmonella enterica* serovar Enteritidis

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ABSTRACT

The ecological success of *Salmonella enterica* to survive in different environments is due, in part, to the ability to form biofilms, something which is especially important for food industry. The aim of the current study was to evaluate the involvement of Dam methylation in biofilm production in *S.* Enteritidis strains. The ability to generate biofilms was analyzed in wild type and *dam* mutant strains. In *S.* Enteritidis, the absence of Dam affected the capacity to develop pellicles at the air-liquid interface and reduced the ability to form biofilm on polystyrene surfaces. Curli and cellulose production, determined by Congo red and calcofluor assays, were affected in *dam* mutant strains. Relative quantitative real-time PCR experiments showed that the expression of *csgD* and *csgA* genes is reduced in mutants lacking *dam* gene with respect to the wild type strains, whereas transcript levels of *bcsA* are not affected in the absence of Dam. To our knowledge, this is the first report on the participation of Dam methylation on biofilm production in Enteritidis or any other serovar of *S. enterica*. Results presented here suggest that changes in gene expression required for biofilm production are finely regulated by Dam methylation. Thus, Dam methylation could modulate *csgD* expression and upregulate the expression of factors related with biofilm production, including curli and cellulose. This study contributes to the understanding of biofilm regulation in *Salmonella* spp. and to the design of new strategies to prevent food contamination and humans and animals infections.

1. Introduction

The Centers for Disease Control and Prevention estimates that among nontyphoidal *Salmonella* species, *Salmonella enterica* causes 1 million cases of foodborne disease in the United States of America annually. *S.* Enteritidis is the most common serovar, presumably as a consequence of frequent chicken and egg contamination (CDC, 2011; EFSA, 2012).

In the bacterial world, existence within surface-associated structured multicellular communities may be the rule, rather than the exception. It has been demonstrated that several serovars of *S. enterica*, including Enteritidis, are able to attach to and form biofilms on a variety of surfaces (Chia et al., 2009; Giaouris and Nychas, 2006; Joseph et al., 2001; Ledeboer et al., 2006).

The extracellular matrix of the biofilm produced by *Salmonella* has curli (formerly, tafi, thin aggregative fimbriae) as the major proteinaceous component (Austin et al., 1998; Barnhart and Chapman, 2006) and cellulose as the main biofilm-associated exopolysaccharide fraction (Steenackers et al., 2012). The co-expression of curli and cellulose leads to the formation of a highly hydrophobic network with tightly packed cells aligned in parallel in a rigid matrix (Speranza et al., 2011).

It is generally accepted that bacteria in biofilms are more resistant to antimicrobial agents than their planktonic counterparts (Møretrø et al., 2009; Steenackers et al., 2012). Therefore, biofilms are hard to eradicate and can cause serious problems not only in medicine but also in the food industry. For instance, serious contamination of raw meat can happen primarily because decontamination procedures are either missing or ineffective (Poulsen, 1999). Failure in biofilm eradication can be due, in part, to differences in phenotypic responses among strains of the same microbial species; representing an important source of variability in microbiological studies intended for biofilm eradication (Lianou and Koutsoumanis, 2012, 2013). This situation emphasized the problems that food industry has in preventing the formation of biofilm.

The formation of bacterial subpopulations is often controlled by epigenetic mechanisms that generate inheritable phenotypic diversity without altering the DNA sequence. It has been shown in certain bacteria that biofilm formation is under epigenetic regulation (Dubnau and Losick, 2006; Rumbo-Feal et al., 2013). Epigenetic mechanisms are diverse, ranging from relatively simple feedback loops to complex self-perpetuating DNA methylation patterns (Casadesús and Low, 2013).

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DNA adenine methyltransferase (Dam) enzyme, which methylates N-6 of adenine in GATC sequences is required for the expression of virulence genes in certain bacteria (Julio et al., 2001), including *S. enterica* (Balbontín et al., 2006; Heithoff et al., 1999). Until now, the participation of Dam methylation in the regulation of genes involved in biofilm production, such as *csgD*, has not been studied in *Salmonella*. The regulation of biofilm production in this bacterium is very complex and involves the binding of several regulatory proteins, such as RpoS, OmpR, H-NS, CpxR, I-HF and MlrA to *csgDEFG* operon (Davidson et al., 2008; Gerstel and Römling, 2003; Prigent-Combaret et al., 2001; Römling et al., 1998a, 1998b). The first gene of this operon, *csgD*, is the key gene of *Salmonella* biofilm formation and regulates the production of certain components of the extracellular matrix, including curli and cellulose (Brombacher et al., 2006; Gerstel and Römling, 2003; Latasa et al., 2005; Römling et al., 1998b, 2000; Zakikhany et al., 2010). The aim of this study was to investigate the impact of Dam methylation on the biofilm production in *S.* Enteritidis.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

S. Enteritidis 5694 was kindly given by Dr. Anne Morris Hooke, Miami University; originally from Dr. F. Collins' collection, Trudeau Institute, Saranac Lake, New York. *S.* Enteritidis ArJEG was kindly given by Dr. Mariana Pichel, Instituto Nacional de Enfermedades Infecciosas "Carlos G. Malbrán", Buenos Aires, Argentina. *S.* Enteritidis ArJEG has been analyzed by pulsed-field gel electrophoresis and multi locus variable number tandem repeat analysis; it is the genetic subtype more frequently associated with salmonellosis outbreaks in Argentina (Dr. Pichel, personal communication). *S.* Enteritidis 5694 and *S.* Enteritidis ArJEG were used to construct *dam* mutant strains. The strains and plasmids used in this study are listed in Table 1. For transformation experiments bacteria were grown in Luria–Bertani (LB) broth (Sambrook et al., 1989) and on LB agar. When required, antibiotics were utilized at the following final concentrations: ampicillin (Sigma), 100 μg/ml; chloramphenicol (Sigma), 30 μg/ml; and kanamycin (Sigma), 40 μg/ml. For biofilm culture conditions bacteria were grown in LB broth without salt (LBNaCl$^-$), pH 7.0 at 28 °C for 48 h without agitation. For planktonic culture conditions bacteria were grown to an optical density (OD) of 0.6 at 600 nm in LB broth, pH 7.0 at 37 °C with agitation of 200 rpm.

Table 1
Bacterial strains and plasmid used in this study.

	Relevant characteristic	Reference/source
Strains		
S. enterica serovar Enteritidis (*S.*E)		
5694	Wild type	Dr. F. Collins collection.
5694 Δ*dam*	5694Δ*dam*	Sarnacki et al. (2009)
ArJEG	Wild type	INEI-"Carlos G. Malbrán" collection. Clinical isolate.
ArJEG Δ*dam*	ArJEGΔ*dam*	This work.
Escherichia coli K-12		
DH5α	F-ϕ80*lacZ*M15 *endA recA hsdR*($r_K^- m_K^-$) *supE thi gyrA relA* Δ(*lacZ*YA-argF) U169	Laboratory stock.
Plasmids		
pCP20	FLP$^+$, λcI857$^+$, λp_R Rep^{ts}, Amp^r, Cm^r	Datsenko and Wanner (2000)
pKD3	Template plasmid for mutagenesis, Amp^r, Cm^r	Datsenko and Wanner (2000)
pKD4	Template plasmid for mutagenesis, Amp^r, Km^r	Datsenko and Wanner (2000)
pKD46	γ, β, and *exo* from λ phage, *araC*-P_{araB}, Amp^r	Datsenko and Wanner (2000)

2.2. Bacterial transformation

Gene deletion was performed as described by Datsenko and Wanner (2000). The mutagenic primers are listed in Table 2. Gene deletion was confirmed by sequencing (Cromátida, Argentina) and analyzed with Sequencher (Gene Codes Corporation) and Vector NTI software. Dam mutants were evaluated phenotypically by determining DNA methylation status and sensitivity to 2-aminopurine as described by Sarnacki et al. (2009). *S.* Enteritidis was transformed by electroporation as previously described (Dower et al., 1988). Briefly, bacteria grown for 24 h were subcultured in 5 ml LB broth for 2 h, harvested into 10% glycerol, and washed twice. 45 μl of bacteria was mixed with 5 to 10 μl of plasmid or PCR product and subjected to electroporation in a 0.2-cm electroporation cuvette (Bio-Rad) at a voltage of 2.5 kV, a resistance of 200 Ω, and a capacitance of 25 μF. After this, for recovery, 800 μl of SOC broth (Sambrook et al., 1989) was added. Bacteria transformed with a plasmid or with a PCR product were incubated at the adequate temperature for 2 and 18 h, respectively. Then, 100 μl of bacteria was spread onto selective plates containing the appropriate antibiotic.

2.3. Phenotypic assays for biofilm formation

To assess the biofilm formation ability of the strains we used the visual pellicle formation assay described by Weiss-Muszkat et al. (2010) with some modifications. Briefly, 1 colony-forming unit of *Salmonella* was grown in 5 ml of LBNaCl−, pH 7.0, at 28 °C and 200 rpm for 12 h, in 15 ml glass tubes. Agitation was stopped and static cultures were tested visually and photographed by a digital camera every 24 h for 72 h. Biofilm development was visualized as a floating pellicle at the air–broth interface (Latasa et al., 2012). The following scoring scheme was first described by Cisar et al. (1979) and adapted for this work: 0, no pellicle formation; 1, very small and non-uniform pellicle; 2, easily visible pellicle that can be dispersed by shaking; 3, clearly visible pellicle non-dense which cannot be dispersed by shaking; and 4, dense pellicle which totally blocks the surface of the culture and cannot be dispersed by shaking. Control tubes without bacteria were also included in all observations (Fig. 1A).

Table 2
Primers used in this study.

Primers Gene Targeted	Primer[a]	Sequence[b] (5 → 3′)
Gene deletion		
dam	dam:: Cm (F)	ATGTGTGTAGGCTGGAGCTGCTTC
dam	dam:: Cm (R)	GGCAATCAAATACTGTTTCATCCGCTTCTCCTTGAGAA TTACATATGAATATCCTCCTTAG
Verification of predicted construction		
dam	Rpe	TACGACAACCTGAACGGTTG
dam	damX	GCAGCGTGCGGTCAACATG
Real-time PCR		
16S rRNA	q-16 s-F	GCCGCAAGGTTAAAACTCAA
	q-16 s-R	AAGGCACCAATCCATCTCTG
csgD	q-csgD-F	GCCTCATATTAACGGCGTG
	q-csgD-R	AGCGGTAATTTCCTGAGTGC
csgA	q-csgA-F	AATGCCACCATCGACCAGTG
	q-csgA-R	CAAAACCAACCTGACGCACC
bcsA	q-bcsA-F	GCCCAGCTTCAGAATATCCA
	q-bcsA-R	TGGAAGGGCAGAAAGTGAAT

Primers were purchased from Invitrogen Inc. and were designed using the DNA sequence information available for the *Salmonella enterica* serovar Enteritidis strain (*Salmonella* sp. comparative sequencing blast server BLAST Server Database at www.sanger.ac.uk).
[a] F, forward primer; R, reverse primer.
[b] Underlining indicates a sequence homologous to pKD3 or pKD4.

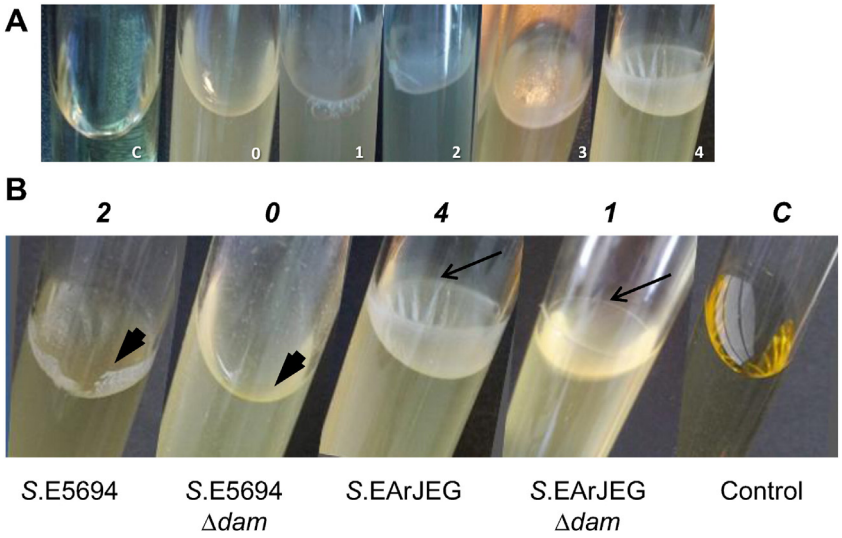

Fig. 1. Pellicle formation at the air-liquid interface. A) Scoring criteria: 0, no pellicle formation; 1, very small and non-uniform pellicle; 2, easily visible pellicle that is dispersed by shaking; 3, clearly visible pellicle non-dense which is not dispersed by shaking; 4, dense pellicle which totally blocks the surface of the culture and cannot be dispersed by shaking; and C, control tube without bacteria. B) Wild type and *dam* mutant strains were incubated statically for 48 h in LBNaCL^{-} at 28 °C. Wild type strains — but not *dam* mutants — form a visible pellicle at the liquid–air interface. Scores values of pellicle formation for each strain are indicated at the top of the photographs. Head arrows and arrows indicate the pellicle at air–liquid interface. The photographs are representative of 3 independent experiments.

2.4. Biofilm formation on polystyrene

A quantitative assay using crystal violet (CV) (Britania S.A., Argentina) staining was adapted from the method previously described by Fletcher (1977); recommendations made by Merritt et al. (2005) were also taken into account. Briefly, bacterial cultures were grown overnight in LB broth pH 7.0 at 28 °C and 200 rpm, bacteria were subsequently subcultured in 10 ml of the same broth and at similar conditions to an OD of 0.6 at 600 nm. 96-well polystyrene flat bottom microtiter trays (Kartell, Italy) were inoculated with 200 μl of the culture and incubated at 28 °C without agitation. 48 and 72 h afterwards, OD was measured at 600 nm using a microplate photometer Multiskan EX (Thermo Scientific) and the absorbance was registered before coloration (ODb). Following, liquid was removed and the wells were washed with sterile distilled water to remove any unbound cells. Biofilms were fixed by adding 250 μl of pure methanol for 15 min, dried at 50 °C and finally stained for 20 min with CV in water (1% v/v). The stained biofilms were thoroughly washed with water and then dried. CV staining was visually assessed and the microtiter plates were photographed. For quantitative determination of biofilms, CV-stained cells were suspended in 250 μl of 95% ethanol. The OD of the suspended CV (ODa) was determined and normalized to the OD of the corresponding grown cell density, is to say ODb. Thus, ODa/ODb × 100 represents the value defined by Gualdi et al. (2008) as 'adhesion unit'. Assays were performed five times with six technical replicates per repeat. As a control, two wells with LB, without bacteria, were included for each replicate. Student's *t* test was used to determine whether the observed differences were statistically significant.

2.5. Cellulose and curli production

The production of cellulose and curli was determined as described previously by Römling et al. (2003). The production of curli was characterized using LBNaCL^{-} agar supplemented with 40 mg/l of Congo red (Sigma, St. Louis, Mo.) and 20 mg/l of brilliant blue (Sigma). 10 μl of bacterial culture with an OD of 0.6 at 600 nm was dropped on Congo red plates and incubated for 48 h at 28 °C, the morphology of the colonies was examined afterwards. Three distinct morphotypes were considered: (i) "*rdar*" (red, dry, and rough) indicating curli and cellulose production; (ii) "*pdar*" (pink, dry and rough) indicating cellulose synthesis but lack of curli production; and (iii) "*saw*" (smooth and white) indicating the lack of both curli and cellulose production. In a separate experiment, the production of cellulose was characterized by inoculating bacteria onto LBNaCl^{-} plates containing 200 mg per liter of calcofluor (fluorescent brightener 28, Sigma) and incubating at 28 °C for 48 h (Zogaj et al., 2001). Cellulose production was judged by comparing the fluorescence of wild type strains with that of mutant strains under UV light (366 nm). All assays were repeated three times with independent bacterial cultures. For microscopic analysis of cellulose production, the assay described by Castelijn et al. (2012) was adapted. Biofilms were grown on glass coupons (18 mm × 18 mm) placed in 10 ml of LBNaCL^{-} broth pH 7.0 inoculated with 500 μl of an overnight grown culture. To analyze the cellulose content of the biofilms the broth was supplemented with 40 μg/ml of calcofluor. After 48 h incubation at 28 °C the coupons were washed with peptone buffered saline (PBS) and examined to visualize the calcofluor stain. At least 15 random microscopic fields (1000×) per sample, were acquired using a Spot RT digital camera attached to an Eclipse 600 fluorescence microscope (Nikon Inc., USA).

2.6. Expression of biofilm production related genes

The expression of *csgD*, *csgA* and *bcsA* was determined for all strains. Relative quantitative real-time PCR (qPCR) was used to determine the average expression from RNA harvested from at least four independent replica cultures of each strain. RNA extraction, cDNA preparation and PCR quantification were performed as described previously (Sarnacki et al., 2009, 2013). Primers used are listed in Table 2. qPCR was performed with an appropriate primer set, cDNAs, and EvaGreen (Solis BioDyne) that contained nucleotides, polymerase, reaction buffer, and Green dye, using a 7500 Real-Time PCR Systems machine (Applied Biosystems®). Melting curve analysis verified that each reaction contained a single PCR product. For the relative gene expression analysis, a comparative cycle threshold method ($\Delta\Delta C_T$) was used (Livak and Schmittgen, 2001). The number of copies of each sample transcript was determined with the aid of the software. Briefly, the amplification efficiencies of the genes of interest and the 16S rRNA gene used for normalization were tested. Then each sample was first normalized for the amount of template added by comparison to the 16S rRNA gene (endogenous control). The normalized values were further normalized using the wild-type sample (calibrator treatment).

Hence, the results were expressed relative to the value for the calibrator sample, which was 1. Student's *t* test was used to determine if the differences observed in retrotranscribed mRNA content were statistically significant.

3. Results

3.1. Dam enzyme participates in Salmonella pellicle development at the air–liquid interface

To gain insight about the involvement of Dam methylation on the biofilm formation process in *Salmonella* we constructed and analyzed *dam* mutants from two different wild type strains: *S.* Enteritidis 5694, a wild type reference strain and *S.* Enteritidis ArJEG, the genetic subtype more frequently associated with salmonellosis outbreaks in Argentina.

The absence of Dam diminishes the ability to develop a pellicle at air–liquid interface in the reference strain (Fig. 1B; head arrows) as well as in the clinical strain (Fig. 1B; arrows) of *S.* Enteritidis. These results could indicate that Dam methylation is involved in the regulation of *Salmonella* capacity to adhere and/or to form biofilm. The clinical isolate of *S.* Enteritidis ArJEG forms thicker pellicles at the air–liquid interface (score = 4) than the collection strain *S.*E5694 (score = 2) (Fig. 1A and B).

3.2. Diminished biofilm forming capacity of Salmonella dam mutants

It is well documented that *Salmonella* spp. attach and form biofilms on a wide variety of food contact surfaces, including polystyrene (PS) (Chia et al., 2009; Joseph et al., 2001; Lapidot et al., 2006; Solomon et al., 2005; Wang et al., 2013). Because of its shock absorbing properties, lightness and thermal isolation PS is used for packaging food products such as vegetables, meats and eggs (ANAPE, 2014; Alliance of Foam Packaging Recyclers, 2003). Thus, we next investigated the ability of *dam* mutants and wild type strains of *S.* Enteritidis to form biofilm on PS utilizing flat bottom microtiter plates. Biofilm formation on the microplates was detected by CV staining. Unlike *S.* Enteritidis *dam* mutants, wild type strains clearly expressed their capacity to form biofilm at the interface between air and liquid media (Fig. 2A). Differences in the biofilm mass adhered to PS between wild type and *dam* mutants of *Salmonella* were quantified. To this purpose, the surface-associated dye was solubilized (Fig. 2B) and the optical density was measured. *Salmonella dam* mutants showed a significant reduction in the ability to generate biofilm on PS respect to the wild type strains (Fig. 2C). At 48 h the biofilm forming capacity of *S.*E5694Δ*dam* dropped to ~44% of that shown by its parental strain (74 units for wild type vs. 32 units for *dam* mutant). For the *dam* mutant generated from the clinical strain (*S.*EArJEGΔ*dam*) the reduction in the ability to adhere was even more dramatic (~30% of that shown by the wild type strain; 92 units for wild type vs. 31 units for *dam* mutant). Similar results were found at 72 h (Fig. 2C). These results are consistent with the defect in the pellicle formation shown by the mutants at the air–liquid interface (Fig. 1) and demonstrate that the absence of *dam* affects the ability of *S.* Enteritidis to form biofilm on inert surfaces.

3.3. Dam methylation is required for the adequate production of cellulose and curli in S. Enteritidis

Solomon et al. (2005) described the relationship between CV binding (assay used to quantify biofilm formation) and bacterial morphotype on Congo red agar (an assay to reveal curli and cellulose production). Therefore, morphotyping was used to investigate whether the impaired biofilm formation found in *S.* Enteritidis *dam* mutants is related to a diminished production of cellulose and/or curli. Results are depicted in Fig. 3. Both wild type strains display "*rdar*" (red, dry, and rough) morphotype, which reflects the coexpression of curli fimbriae and cellulose (Collinson et al., 1991; Römling et al., 1998a). On the contrary, the *dam*-defective strain *S.*E5694Δ*dam* showed "*saw*" (smooth and white) colony morphology, indicating the absence of curli and cellulose production. Interestingly, the *dam* mutant generated from the clinical isolate (*S.*EArJEGΔ*dam*) did not show "*saw*" but "*pdar*" (pink, dry and rouge) morphotype indicating that, in this strain, curli production is affected whereas cellulose production is not.

Congo red assay provides a convenient method to identify curliated bacteria (Barnhart and Chapman, 2006), even though it is not considered as a conclusive procedure for establishing the absence of cellulose production (Kimizuka et al., 2009). For this reason, the calcofluor assay on plates was performed to determine cellulose production in wild type and *dam* mutant strains of *S.* Enteritidis; results are shown in Fig. 4. Interestingly, we found that *S.*E5694Δ*dam* strain completely lacks the ability to produce cellulose (seen as loss of fluorescence) whereas in *S.*EArJEGΔ*dam* strain fluorescence was observed mainly at the edge of the colonies (Fig. 4A). The cellulose production detected by

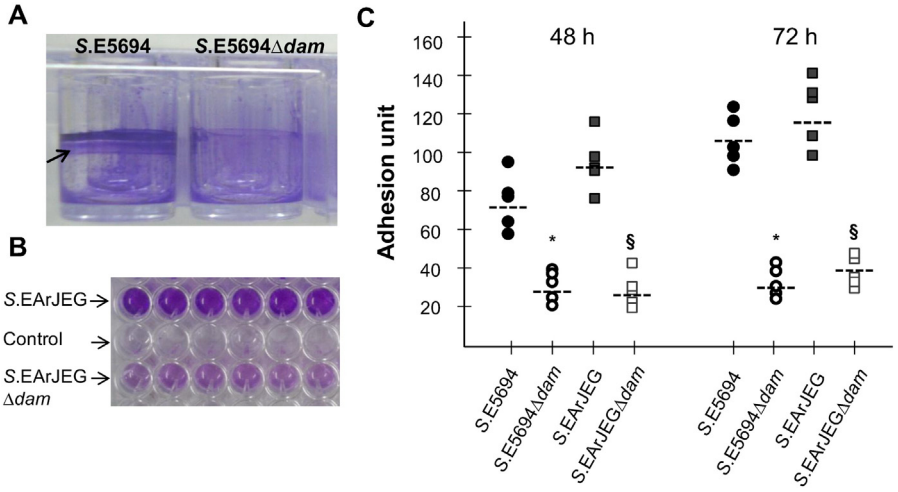

Fig. 2. Biofilm formation on polystyrene. Strains were incubated statically in LB broth for 48 and 72 h at 28 °C. A) Wild type *S.*E5694 strain forms biofilm predominantly near the surface of the liquid medium in the microtiter well (arrow). Bacterial biofilm was visible with crystal violet stain (CV). Note the lack of biofilm formation in *S.*E5694Δ*dam* strain. B) CV staining. Surface attached cells were revealed by solubilizing the dye with ethanol, visible differences are observed between wild type strain (*S.*EArJEG) and *dam* mutant strain (*S.*EArJEGΔ*dam*). Control: LB broth without bacteria. C) Quantification of biofilms formation on polystyrene plate. Adhesion units express the relation between optical density (OD) at 600 nm before and after CV staining ((ODa/ODb) * 100). Data were collected from five independent experiments with six technical replicates per repeat. Horizontal dashed lines, indicate the mean value. *S.*E5694 is represented with circles and *S.*EArJEG with square boxes, isogenic mutants are represented with empty symbols. *Significant difference ($p < 0.05$, *t*-test) respect to *S.*E5694. §Significant difference ($p < 0.05$, *t*-test) respect to *S.*EArJEG, at the same time point.

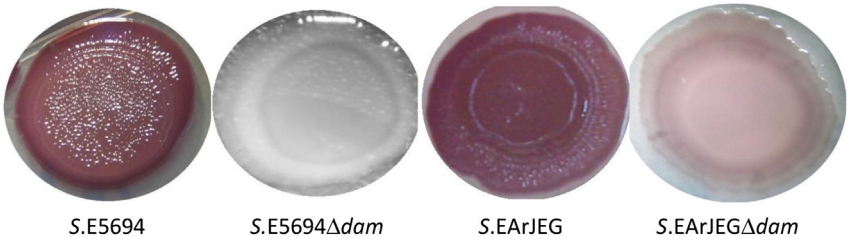

Fig. 3. Colony morphology on Congo red agar. 10 μl of bacterial culture was inoculated by triplicate on Congo red plates and incubated for 48 h at 28 °C. Differences in colony morphology were evident between wild type and *dam* mutant strains of *S.* Enteritidis. Note that only wild type strains display a characteristic "*rdar*" morphotype. Detailed images were taken with a stereoscopic microscope (Carl Zeiss-Germany) at a magnification of 4×. Picture is representative of three independent experiments.

calcofluor assay is consistent with the morphotypes presented on Congo red plates.

The microscopic analysis of cellulose production using calcofluor assay was performed in bacteria grown on small glass sheets. As shown in Fig. 4B, fluorescence is not homogeneously distributed across the glass surface. This finding was particularly evident in the wild type strain *S.*E5694 and in agreement with a previous report by Castelijn et al. (2012). These authors observed that in *S.* Typhimurium grown under biofilm forming conditions cellulose is expressed only by some small clusters of cells. Also, it was noted that even in those strains with "*saw*" morphotype, as *S.*E5694Δ*dam*, a low number of fluorescent bacteria were observed. Interestingly, the cellulose production in *S.*EArJEGΔ*dam* seems to be unaffected.

3.4. The expression of genes associated with biofilm production decreases in the absence of Dam

To this point results suggest that in *S.* Enteritidis Dam methylation regulates biofilm formation. Therefore, the expression of *csgD* (the main transcriptional activator that regulates biofilm formation in *Salmonella* spp.) was evaluated in wild type and *dam* mutants. Results of qPCR experiments are depicted in Fig. 5A. The expression of *csgD* in *dam* mutants of *S.* Enteritidis was significantly reduced ($p < 0.05$) compared with their parental strains. In *S.*E5694Δ*dam csgD* was expressed at one third of the wild type level, whereas in *S.*EArJEGΔ*dam csgD* expression was one fifth of the parental strain level. As seen in Fig. 5A, the expression of *csgD* in the clinical strain *S.*EArJEG was at least four times higher than in the reference strain *S.*E5694 ($p < 0.05$). This finding is in agreement with the increased cellulose and curli expression, and biofilm formation shown by *S.*EArJEG strain. As a control, the expression of *csgD* was also determined in wild type strains grown under planktonic conditions. As expected, the expression of *csgD* in the free floating cells was negligible.

The biosynthesis of cellulose requires the *bcs* (bacterial cellulose synthesis) operon encoding structural genes, with the catalytic subunit of the cellulose synthase encoded by *bcsA* (Solano et al., 2002; Zogaj et al., 2001). Next, the expression of *bcsA* was tested in wild type and *dam* mutant strains. Despite the fact that *dam* mutants of *Salmonella* produce less cellulose (Fig. 4), no significant differences in *bcsA* expression were found between *dam* mutants and their parental strains (Fig. 5B). Clinical strain *S.*EArJEG presented a significant 4-fold increase in the expression of *bcsA* compared with the collection strain *S.*E5694 (Fig. 5B). As a control, the expression of *bcsA* was also determined in wild type strains grown in the planktonic life style and as expected the gene expression was minimal.

The expression *csgA*, the gene encoding the major structural subunit of curli fimbriae, was analyzed in *dam* mutants and wild type strains. As shown in Fig. 5C, *csgA* expression was significantly reduced in the absence of *dam*. The expression of this gene was reduced approximately 20 times in *Salmonella* strains lacking *dam* with respect to their parental strains. Results are in line with the observed decrease in curli production in these mutants (Fig. 3) and consequently with the decrease in biofilm formation on PS (Fig. 2) and in biofilm production on air–liquid interface (Fig. 1).

The expression of *csgA* was significantly increased in the clinical isolate compared with the reference wild type strain ($p < 0.05$). We found an 8-fold increase in *csgA* expression in *S.*EArJEG compared with the collection strain *S.*E5694. The expression of *csgA* in planktonic conditions, was significantly lower ($p < 0.05$) with respect to wild type strains cultured under biofilm conditions.

4. Discussion

S. Enteritidis is currently one of the most common serovars involved in human salmonellosis outbreaks associated with consumption of contaminated food. The ecological success of *S. enterica* to survive in

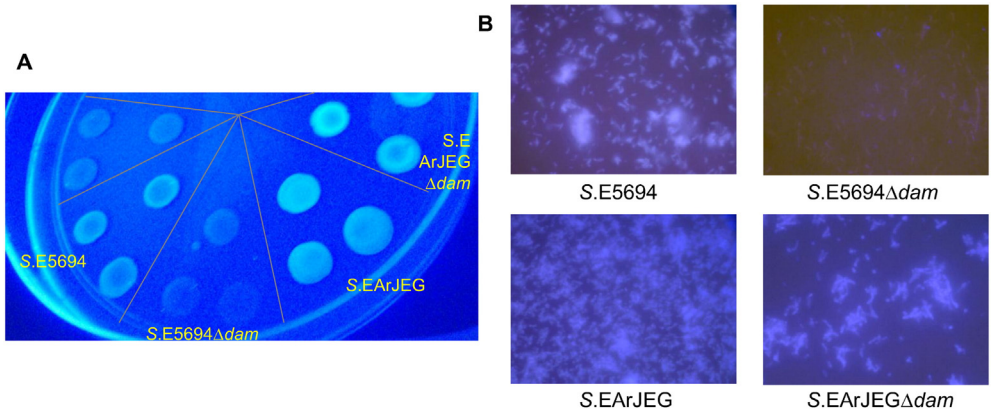

Fig. 4. Detection of cellulose production. A) Strains were grown on LB agar without salt supplemented with calcofluor at 28 °C for 48 h. Under UV light the calcofluor binding capability (indicative of cellulose expression) was observed. Strains lacking *dam* show less fluorescence respect to their parental strains. For *S.*EArJEGΔ*dam*, fluorescence was more intense at the edge of the colony than in the center. Picture is representative of three independent experiments. B) Microscopic analysis of cellulose production. Biofilms were grown on glass coupons for 48 h at 28 °C. Clusters of fluorescence bacterial cells are evident. Magnification 100×.

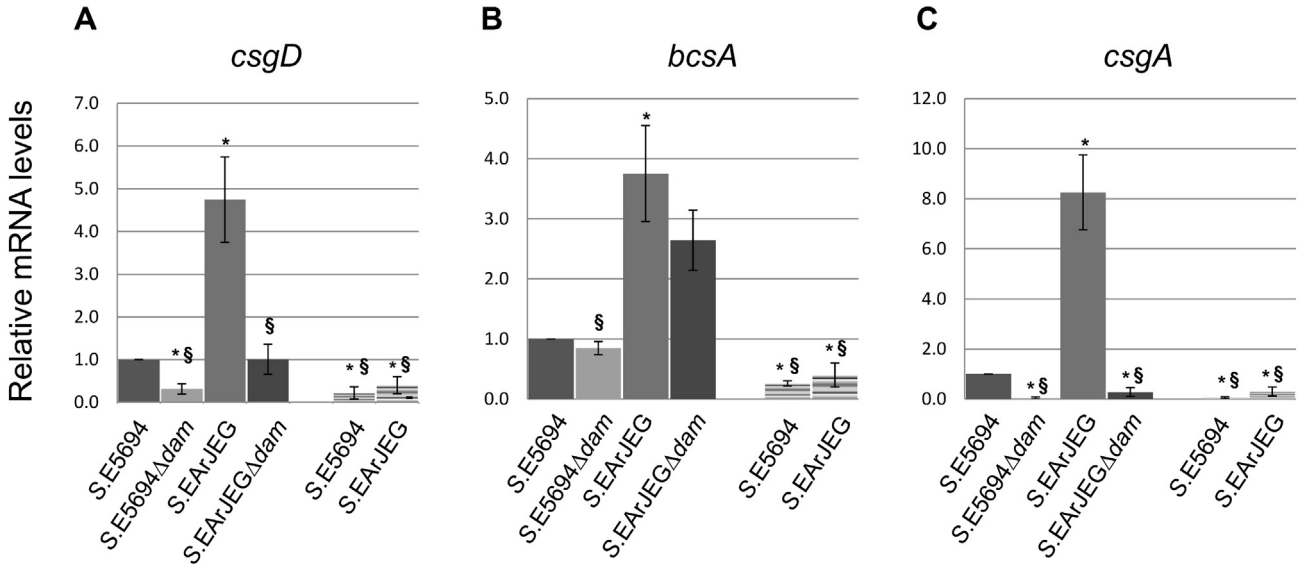

Fig. 5. Relative expression of genes related to biofilm production determined by qPCR. Total mRNA was harvested from all strains on biofilm conditions (solid bars). Also, wild type strains (*S*.E5694 and S.EArJEG) were analyzed on planktonic conditions (striped bars). The relative mRNA amount was determined by reverse transcription real-time quantitative PCR and related to mRNA levels of wild type strain *S*.E5694 cultured under biofilm conditions, set as 1. Values are means ± SD of four independent mRNA extractions performed in triplicates. *Significant difference ($p < 0.05$, *t*-test) respect to *S*.E5694 cultured under biofilm conditions. §Significant difference ($p < 0.05$, *t*-test) respect to *S*.EArJEG cultured under biofilm conditions.

different environments is due, in part, to the ability to form biofilms, something which is especially important for food industry. The present work aimed to evaluate the involvement of Dam methylation on biofilm formation in *S*. Enteritidis. Studies were performed in two wild type strains (the collection strain *S*. Enteritidis 5694 and the genetic subtype more frequently associated with salmonellosis outbreaks in Argentina *S*. Enteritidis ArJEG) and their isogenic *dam* mutants.

Throughout the experiments, *S*. Enteritidis ArJEG showed the highest capacity to produce biofilm. Earlier, Castelijn et al. (2012) postulated that clinical *Salmonella* isolates have adapted to and/or have been selected for their ability to produce dense biofilms under different conditions. Whether this provides a selective advantage for the clinical isolates in the colonization of the host remains to be elucidated. On the other hand, *dam* mutant strains were affected in their capacity to develop pellicles at the air-liquid interface and to form biofilm on PS. These findings indicate that in *S*. Enteritidis Dam methylation is required for efficient biofilm production.

The ability of foodborne pathogens like *S. enterica* to form biofilms on a range of surfaces has received great attention (Giaouris and Nychas, 2006; Joseph et al., 2001; Prouty and Gunn, 2003; Solano et al., 1998; Steenackers et al., 2012). Bacterial biofilm formation on surfaces is characterized by a sequential process of initial attachment, microcolony formation, production of extracellular substances and maturation. Several research groups pointed out the relationship between bacterial capacity to adhere to solid surfaces and the expression of cellulose and curli (Jain and Chen, 2007; Lapidot and Yaron, 2009; Macarisin et al., 2012; Patel et al., 2010, 2013; Patel and Sharma, 2010; Solomon et al., 2005). Solomon et al. (2005) showed that strains of *S. enterica* deficient in cellulose and curli are less effective to form biofilm on PS. This study shows that *dam* mutants of *S*. Enteritidis are defective in biofilm formation on PS and in curli production. Interestingly, the absence of Dam methylation does not inhibit the production of cellulose although, depending on the genetic background, cellulose production can be drastically reduced as observed in *S*.E5694Δ*dam* strain.

On the other hand, in *S*.EArJEGΔ*dam* strain, fluorescence was more intense at the edge of the colony than in the center. It is worth noting that colonies grow by radial expansion of a drop of inoculum placed on agar; therefore, cells situated in the center of the colony are older than those situated on the edge. Thus, the observed results could indicate that cellulose is mainly produced in younger cells. This phenomenon is in agreement with the statement by Grantcharova et al. (2010), that a heterogeneous gene expression takes place in microcompartments formed within the complex biofilm structure and with the hypothesis of tasks distribution on bacterial biofilms (Castelijn et al., 2012; Davidson et al., 2008). An alternative explanation could be that cells in the edge produce cellulose because they are in a more aerobic environment than cells inside the colony.

The role of cellulose production in *Salmonella* biofilm is controversial. It is widely accepted that cellulose is one of the main components of the extracellular matrix (Jain and Chen, 2007; Solano et al., 2002; Zogaj et al., 2001). However, it has been recently shown that *S*. Agona and *S*. Typhimurium develop the liquid–air interface pellicle even when small amounts of cellulose are produced (Vestby et al., 2009). In addition, an alternative strategy of biofilm formation — dependent on an overproduction of capsular polysaccharide and independent of curli fimbriae and cellulose production — has been described (Malcova et al., 2008). Our results show that biofilm formation is affected in *dam* mutants regardless the amount of cellulose produced.

It has been postulated that survival of *Salmonella* Typhimurium on plants is more affected when curli genes are deleted that when cellulose gene (*bcsA*) is absent (Lapidot and Yaron, 2009). Moreover, the expression of curli fimbriae is closely related to the growth of *S. enterica* in hen eggs (Cogan et al., 2004). The fact that Dam methylation regulates the expression of curli is important because it contributes to explain the attenuation of *Salmonella dam* mutants, but it is even more relevant considering that *dam* mutants have been proposed as live vaccines against *Salmonella* in animals (Dueger et al., 2001, 2003; Mohler et al., 2011, 2012). Defects in biofilm formation and in curli production could imply reduced bacterial survival in different environments adding promising safety features to *Salmonella dam* vaccines. Moreover, limited persistence in the environment and minimal spread to non-target animals and humans are among the ideal characteristics for poultry live vaccines (Van Immerseel et al., 2005).

In *Salmonella* and in *Escherichia coli* the production of cellulose and curli is positively regulated by CsgD (Brombacher et al., 2006; Chapman et al., 2002; Gualdi et al., 2008; Latasa et al., 2005; Römling et al., 1998b; Zakikhany et al., 2010). The expression of *csgD* in *dam* mutants was significantly reduced indicating that, in *S*. Enteritidis, this key biofilm regulator gene is modulated by Dam methylation. It can be expected that further information in this direction will allow new

strategies on biofilm control to be developed. In theory, the expression of*csgD* could be regulated by Dam methylation since GATC motifs are present in coding DNA sequence as well as upstream and downstream of the gene (Fig. S1, supplementary file).

The regulatory role attributed to CsgD for curli production is supported by previous observations that inactivation of *csgD* prevents the transcription of the *csgBAC* operon (Hammar et al., 1995) and of *adrA*, a putative regulatory gene required for cellulose synthesis (Römling et al., 2000; Zogaj et al., 2001). In fact, it is known that *csgD* encodes a transcriptional regulator that positively regulates the expression of curli (Römling et al., 1998a), cellulose (Römling et al., 2000), O-Ag capsule (Gibson et al., 2006) and serine hydroxymethyltransferase (Chirwa and Herrington, 2003), and negatively regulates factors that inhibit biofilm formation (Prigent-Combaret et al., 2001). Therefore, it is reasonable to assume that a decrease in transcript levels of *csgD*, as occurs in the absence of Dam, would result in a decreased expression of *csgA*, as shown here. Again, the expression of *csgA* could be regulated by Dam methylation because GATC motifs are present in the operon *csgBAC* (Fig. S1, supplementary file). A decreased expression of *csgA* as a result of a diminished expression of *csgD*, has been reported earlier in *S.* Typhimurium (Baugh et al., 2012; Latasa et al., 2012). Additionally, the expression of *csgA* was significantly increased in the clinical isolate compared with the reference wild type strain. The increased curli expression in the clinical isolate strain is in line with its ability to produce a dense biofilm on air–liquid interface and with its morphotype in Congo red agar. It is likely that the augmented expression of *csgD* found in the clinical isolate leads to the increase in the expression of *csgA*. Similarly, the production of other components of *Salmonella* biofilm (e.g., BapA — Latasa et al., 2005) could be potentiated in clinical isolates or strains that have an abundant expression of *csgD*. Interestingly, although *dam* mutants produce less cellulose the expression of *bcsA* mRNA was not diminished in these strains. This fact may be explained taking into account that cellulose biosynthesis is mainly regulated at posttranscriptional level (Simm et al., 2004; Römling et al., 2000; Zogaj et al., 2001). Further studies are needed to elucidate how DNA methylation by Dam may affect the regulation of cellulose production in *S.* Enteritidis.

In summary, this study shows for the first time that DNA methylation by Dam enzyme modulates *csgD* expression and thereby upregulates the expression of factors related with biofilm production, including cellulose and curli. Also, we observed a strain-dependent biofilm forming behavior; this fact must be taken into account when selecting strains for any microbiological study. Finally, identification of genetic factors — as methylation — affecting the biofilm production in food pathogens, such as *Salmonella*, could be useful for the development of new methods for eradication and/or control of bacterial biofilms in food production environments.

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