Envelope instability in DNA adenine methylase mutants of *Salmonella enterica*

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Mutants of *Salmonella enterica* serovar Typhimurium lacking DNA adenine (Dam) methylase show reduced secretion of invasion effectors encoded in the *Salmonella*-pathogenicity island 1 (SPI-1). Concomitant with this alteration, a high number and quantity of extracellular proteins are detected in cultures of Dam[−] mutants. This study shows by subcellular fractionation analysis that the presence of numerous extracellular proteins in cultures of Dam[−] mutants is linked to an exacerbated release of membrane particulate material. The membrane 'leaky' phenotype and the impaired functionality of type III secretion systems were, however, unrelated since exacerbated release of proteins to the medium was evident in Dam[−] strains carrying mutations in either SPI-1 (*invA*, *invJ*) or flagellar (*flhD*) genes. This result supports the view that Dam methylation controls a plethora of cellular processes. Electron microscopy analysis demonstrated that the accumulation of membrane particulate material occurs preferentially as vesicles in stationary cultures of Dam[−] strains. In addition, a reduction in the relative amount of peptidoglycan-associated lipoprotein (PAL), TolB, OmpA and murein lipoprotein (Lpp) bound to peptidoglycan was observed in actively growing Dam[−] mutants. The existence of an envelope defect was further confirmed by the increased sensitivity to deoxycholate exhibited by Dam[−] mutants, mostly during exponential growth. Unexpectedly, lack of Dam methylation neither increased envelope instability nor impaired the association of PAL-Tol-Lpp proteins to the peptidoglycan in *Escherichia coli*. Accordingly, *E. coli* Dam[−] mutants did not show sensitivity to deoxycholate. Altogether, these results indicate that, besides its role in modulating the secretion of effectors by the SPI-1-encoded type III apparatus, Dam methylation controls cell envelope integrity in *S. enterica*.

Keywords: Dam methylation, membrane vesicle, protein secretion

INTRODUCTION

Methylation of deoxyadenosine residues is found in DNAs from diverse groups of Proteobacteria (Barras & Marinus, 1989; Marinus, 1996; Casadesús & Torreblanca, 1996; Reisenauer *et al.*, 1999). In *Escherichia coli* and *Salmonella enterica*, the enzyme DNA adenine methyltransferase (or Dam methylase) methylates aden-

osine residues embedded in 5'-GATC-3' sites, using S-adenosylmethionine as the methyl donor (Lacks & Greenberg, 1977; Hattman *et al.*, 1978). Dam methylation is post-replicative and causes changes in DNA structure that can influence protein binding (Sternberg, 1985; Bolker & Kahmann, 1989; Campbell & Kleckner, 1990). The methylation state of one or more critical GATC sites can serve as a signal to modulate cellular processes such as DNA replication (Boye & Lobner-Olesen, 1990; Campbell & Kleckner, 1990), mismatch repair (Radman & Wagner, 1986), chromosome segregation (Ogden *et al.*, 1988), transposition of insertion elements (Roberts *et al.*, 1985), synthesis of fimbriae

Abbreviations: Dam, DNA adenine methylase; PAL, peptidoglycan-associated lipoprotein; PG, peptidoglycan (fraction); SP, secreted protein; SPI-1, *Salmonella*-pathogenicity island 1; TP, total protein; VES, vesicle; VSN, vesicle supernatant.

(Braaten *et al.*, 1994; van der Woude & Low, 1994; van der Woude *et al.*, 1996; Nicholson & Low, 2000), and bacterial conjugation (Torreblanca & Casades, 1996; Torreblanca *et al.*, 1999). A subset of these phenomena involves transcriptional gene regulation by Dam methylation (Roberts *et al.*, 1985; Bolker & Kahmann, 1989; van der Woude & Low, 1994; Heithoff *et al.*, 1999; Nicholson & Low, 2000).

Recent studies have shown that mutants of *S. enterica* serovar Typhimurium lacking Dam methylase are highly attenuated for virulence in the murine typhoid model (Heithoff *et al.*, 1999; Garcia-del Portillo *et al.*, 1999; Low *et al.*, 2001). DNA methylation is thus essential for virulence. Current evidence suggests that the role of Dam methylation in *Salmonella* virulence is multifactorial: Dam⁻ mutants show defects in several virulence-related traits such as invasion of the intestinal epithelium and cytotoxicity to M cells (Garcia-del Portillo *et al.*, 1999). *In vitro* infection assays have also shown that Dam⁻ mutants survive within professional phagocytic cells while having reduced capacity to invade cultured non-phagocytic cells (Garcia-del Portillo *et al.*, 1999). This partial inability to invade epithelial cells was tentatively related to reduced secretion of InvJ and SipC, two invasion proteins encoded in the *Salmonella*-pathogenicity island 1 (SPI-1) (Garcia-del Portillo *et al.*, 1999). Besides the impaired secretion of invasion proteins, *dam* mutations also cause a marked increase in the amount and number of proteins that are present in the extracellular medium (Garcia-del Portillo *et al.*, 1999).

The spontaneous loss of membrane material by Gram-negative enteric bacteria is known to increase in the absence of a specific subset of inner-membrane, periplasmic and outer-membrane proteins that maintain envelope integrity. That is the case for the Braun's (murein) lipoprotein (Lpp); the peptidoglycan-associated lipoprotein (PAL) (Lazzaroni & Portulier, 1992); the periplasmic protein TolB; and the inner-membrane complex formed by TolQ, TolR and TolA proteins (Lazzaroni *et al.*, 1999). A series of studies have provided evidence for the existence of large complexes containing these proteins that establish a physical link between the inner and the outer membrane (Bouveret *et al.*, 1995, 1999; Rigal *et al.*, 1997; Clavel *et al.*, 1998; Lazzaroni *et al.*, 1999). This function is essential for providing stability and integrity to the cell envelope. Thus, mutants defective in any of these proteins share distinct phenotypic traits such as shedding of membrane vesicles, release of periplasmic proteins and hypersensitivity to detergents (Lazzaroni *et al.*, 1999). Nevertheless, this so-called 'membrane-leakage' phenotype has been differentiated from the spontaneous shedding of outer-membrane vesicles that occurs, though to a much lesser extent, in actively growing bacteria (Kadurugamuwa & Beveridge, 1997).

We describe below a correlation between the accumulation of proteins in the extracellular medium of *S. enterica* Dam⁻ cultures and the disorganization of the

cell envelope. Dam⁻ mutants in exponential-growth phase are sensitive to deoxycholate and show reduced association to peptidoglycan of the envelope proteins PAL, TolB, OmpA and murein Lpp. As a result, lack of Dam methylation causes release of membrane vesicles to the extracellular medium of stationary cultures. Interestingly, these phenotypes occur concomitantly with the defect in secretion of SPI-1-encoded invasion proteins. Altogether, these data support the view that Dam methylation is necessary for both the proper activity of the SPI-1 type III secretion system and the maintenance of envelope integrity in *Salmonella*. We also describe that *E. coli* Dam⁻ mutants do not display an equivalent phenotype of envelope instability.

METHODS

Bacterial strains and growth conditions. The *Salmonella enterica* and *Escherichia coli* strains used in this study are listed in Table 1. All *S. enterica* strains belong to the serovar Typhimurium and derive from the virulent strains SL1344 and 14028s, or from the laboratory strain LT2. *E. coli* strains GM28 and GM33 were provided by M. G. Marinus, Department of Pharmacology, University of Massachusetts, Worcester, MA, USA. Cultures were prepared in Luria-Bertani (LB) broth and incubated overnight at 37 °C without shaking. When actively growing bacteria were required, the overnight culture was diluted 1:500 and growth was resumed to reach an OD₅₅₀ of 0.3.

Deoxycholate sensitivity assays. Deoxycholate sensitivity was tested on LB plates containing 1% sodium deoxycholate. Bacteria were grown in LB broth, centrifuged, suspended in PBS pH 7.4, and diluted as appropriate in the same buffer. Drops of 0.05 ml were then placed onto LB-deoxycholate agar, dried at room temperature, and incubated at 37 °C. Growth was monitored after 12–18 h. Assays were carried out in triplicate, using 10³, 10⁴ and 10⁵ bacterial cells per drop.

Preparation of protein extracts. Bacteria grown in LB medium to exponential (OD₅₅₀ 0.3) or stationary phase (OD₅₅₀ 1.0) were spun down by centrifugation at 10000 g, 15 min, 4 °C. The supernatant was filtered in a Millipore 0.22 µm pore size filter. Proteins were precipitated in 10% trichloroacetic acid (1 h, 4 °C), washed in acetone, suspended in PBS pH 7.4, and boiled in SDS-loading buffer as described by Kaniga *et al.* (1995). These proteins constituted the 'secreted protein' (SP) fraction. The bacterial pellet was washed twice in PBS pH 7.4 and boiled in SDS-loading buffer to obtain the cell-associated 'total protein' (TP) fraction. To determine the presence of particulate material in the supernatant, the 0.22 µm filtrate was subjected to high-speed centrifugation (300000 g, 4 °C, 20 min) and the pellet was boiled in SDS-loading buffer. The proteins present in this pellet constituted the 'vesicle' (VES) fraction. Proteins remaining in the supernatant after high-speed centrifugation were precipitated with the 10% trichloroacetic acid/acetone procedure as described above. This fraction was named 'vesicle-supernatant' (VSN), which contained soluble extracellular proteins. All subcellular fractions were analysed for protein content by SDS-PAGE in Tris/Tricine buffer by using 8% or 10% acrylamide gels (Shägger & von Jagow, 1987).

Analysis of membrane and peptidoglycan-associated proteins. A subcellular fractionation method was followed to determine the relative amount of envelope proteins that were

Table 1. Bacterial strains used in this study

Strain	Relevant genotype	Source/reference*
<i>S. enterica</i>		
LT2	Wild-type	Laboratory stock
SV3000	LT2 <i>dam-201::Tn10dTet</i>	Torreblanca & Casadesus (1996)
14028s	Wild-type, mouse virulent	E. A. Groisman
SV4392	14028s <i>dam-201::Tn10dTet</i>	This study
SL1344	<i>hisG rpsL</i> , mouse virulent	Hoiseth & Stocker (1981)
SV1610	SL1344 <i>dam-228::MudJ</i>	Garcia-del Portillo <i>et al.</i> (1999)
SB136	SL1344 <i>invA::aphT</i>	Jorge E. Galán
SB302	SL1344 <i>invJ::aphT</i>	Jorge E. Galán
SV4184	SL1344 <i>dam-228::MudJ invA::aphT</i>	This study
SV4186	SL1344 <i>dam-228::MudJ invJ::aphT</i>	This study
SV4316	SL1344 <i>flhDC::MudA</i>	This study
SV4331	SL1344 <i>flhDC::MudA dam-228::MudJ</i>	This study
<i>E. coli</i>		
GM28	F ⁻ <i>sup-85</i> (Am)	M. G. Marinus
GM33	F ⁻ <i>sup-85</i> (Am) <i>dam-3</i>	M. G. Marinus

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present in inner/outer membranes or firmly associated with peptidoglycan. Thus, $\sim 10^{10}$ bacteria were spun down (15 000 g, 15 min, 4 °C), washed and suspended in PBS pH 7.4 buffer. The cells were disrupted by sonication. Unbroken cells were removed by low-speed centrifugation, 5000 g, 5 min, 4 °C. The supernatant was centrifuged at high speed (200 000 g, 20 min, 4 °C) to obtain a pellet containing the envelope material, which was then treated with 0.4% Triton X-100 (3 h, 4 °C) and centrifuged at 15 000 g, 30 min, 4 °C in a microfuge. The 0.4% Triton X-100 soluble material corresponded to the 'Sol' fraction, enriched in inner-membrane proteins. The insoluble pellet was suspended in PBS pH 7.4 and corresponded to the fraction enriched in outer-membrane proteins ('Ins' fraction). Both the 'Sol' and 'Ins' fractions were finally suspended in SDS-sample buffer, boiled for 5 min and cleared by centrifugation before loading the samples in gels. To analyse proteins covalently bound or strongly associated with peptidoglycan, $\sim 2 \times 10^{11}$ bacteria were used to purify peptidoglycan. Bacteria were spun down and washed as described above. The pellet was then suspended in 1.5 ml PBS pH 7.4 buffer, and slowly mixed with 1.5 ml boiling 8% SDS. Boiling at 100 °C was carried out for 3 h and then continued overnight at 80 °C. Macromolecular peptidoglycan was recovered by high-speed centrifugation (300 000 g, 20 min, 30 °C). After four washing steps with warm (60 °C) distilled water, the pellet was suspended in 50 mM phosphate buffer pH 4.9 and digested overnight at 37 °C with *N*,*O*-diacetyl-muramidase (Cellosyl, Hoescht) (20 μ g ml⁻¹, 37 °C, 18 h). Samples were incubated at 100 °C for 20 min to inactivate the enzyme. Proteins released to the supernatant by peptidoglycan digestion were precipitated by simultaneous treatment with acid pH (pH 4.6 of muramidase buffer) and heat (100 °C, 10 min), a step included in the standard procedure to prepare mucopeptides for HPLC analysis (Glauner, 1988). After centrifugation at 15 000 g, 15 min, 4 °C, the pellet containing the precipitated proteins and undigested

peptidoglycan was suspended in 40 μ l PBS pH 7.4. After addition of SDS-sample buffer, the sample was centrifuged (15 000 g, 5 min, 4 °C) to remove undigested macromolecular peptidoglycan. The supernatant, containing proteins firmly associated with peptidoglycan (PG fraction) was then subjected to electrophoretic protein analysis. In all cases, the sample volume to load in the gels was adjusted by the optical density value of the culture at the time at which bacteria were collected.

Western analysis and antibodies. Western analysis was performed using a Bio-Rad blotting system for protein transfer to nitrocellulose membranes. Specific proteins present in the different subcellular fractions were detected with the following primary antibodies: polyclonal rabbit anti-PAL and anti-TolB antibodies (a gift of Professor J.-C. Lazzaroni, CNRS-INSU-Université de Lyon, France), polyclonal rabbit anti-murein lipoprotein (Lpp) (a gift from M. Inouye, Robert Wood Johnson Medical School, Piscataway, NJ, USA), affinity-purified polyclonal rabbit anti-OmpA (a gift from Heinz Schwarz, Universität Tübingen, Germany), polyclonal rabbit-anti SipC from our sera collection raised against the SipC peptide ASDEARESSRKS, and polyclonal rabbit anti-ribosomal elongation factor Tu (a gift from M. Vicente, CNB-CSIC, Madrid, Spain). As secondary antibody, goat anti-rabbit IgG conjugated to peroxidase was used for the ECL assay as described by the manufacturer (Amersham-Pharmacia Biotech).

Electron microscopy. The release of membrane vesicles was followed by transmission electron microscopy. Thus, the pellet obtained after high-speed centrifugation of culture supernatants (VES fraction) was fixed in 2% glutaraldehyde/2% paraformaldehyde in PBS buffer pH 7.4 (2 h, room temperature). After three washes (10 min each) in phosphate buffer, the pellets were post-fixed in 1% osmium tetroxide for 1 h at 4 °C. After three additional washes with

PBS buffer pH 7.4, samples were dehydrated with increasing concentrations of ethanol (from 50% to 100%). Samples were then embedded in LR-White resin (London Resin Co., Reading, UK). Ultrathin sections were put onto nickel grids coated with a Formvar film, contrasted with lead citrate/uranyl acetate and visualized at 80 kV in a JEOL1200EX electron microscope. To visualize the integrity of bacterial envelope, the bacteria were fixed in the growth medium by adding a twofold concentrated glutaraldehyde/paraformaldehyde fixer solution. Fixation was maintained for 1 h at room temperature. After spinning down the bacteria, the pellet was washed twice with 0.1 M phosphate buffer pH 7.4 and processed for transmission electron microscopy as described for the pellet containing the VES fraction.

RESULTS

S. enterica mutants deficient in Dam methylase show alterations in envelope stability

The analysis of extracellular proteins present in cultures of *S. enterica* serovar Typhimurium Dam⁻ mutants unveils the presence of a large number of proteins that are absent or scarce in the wild-type (Garcia-del Portillo *et al.*, 1999). Two tentative explanations for this phenotype are as follows: (i) lack of Dam methylase might cause extensive loss of membrane and periplasmic proteins; or (ii) enhanced activity of secretion systems might exacerbate protein release. If the phenotype shown by the Dam⁻ mutant reflects loss of membrane material, a subset of the proteins should be recovered in particulate material from the bacteria-free supernatant. To test this hypothesis, extracellular proteins present in supernatants of cultures from both the wild-type and a Dam⁻ mutant were fractionated by high-speed centrifugation. Two fractions were obtained: particulate material recovered as a pellet (VES), and soluble proteins that remained in the supernatant (VSN). A sample consisting of the entire bulk of extracellular proteins (fraction SP) was also run for comparison. In agreement with a previous report (Garcia-del Portillo *et al.*, 1999), the SP fraction of the Dam⁻ strain contained a higher number of proteins than the isogenic, wild-type strain SL1344 (Fig. 1A). Dam⁻ mutants derived from other strains of the serovar Typhimurium, such as 14028s and LT2, showed a similar complex pattern of extracellular proteins (data not shown).

Analysis of the VES fraction of the Dam⁻ strain showed that over 15 proteins were present in the particulate material (Fig. 1A). Among these proteins, the most abundant were the flagellin subunit FliC and the outer-membrane protein OmpA, as indicated by Western analysis and confirmed by controls performed with isogenic FliC⁻ and OmpA⁻ null mutants (data not shown). Unlike the Dam⁻ mutant, the particulate VES fraction of the wild-type strain contained a reduced number and amount of proteins, of which the most abundant was identified as FliC (Fig. 1A). These data suggested that lack of Dam methylase caused enhanced release of membranous material to the extracellular medium. As expected, analysis of the VSN fractions showed that the wild-type strain secretes flagellin and a

Fig. 1. Cultures of Dam⁻ mutants of *S. enterica* contain a large number of extracellular proteins. (A) Subcellular fractions containing whole-cell-associated protein (TP), total extracellular protein (SP), extracellular protein collected by high-speed centrifugation (VES), and soluble extracellular protein (VSN), were prepared from SL1344 (Dam⁺) and SV1610 (Dam⁻) strains at stationary phase in standing non-shaking conditions. Proteins migrating as invasion factors (SipA, SipB, SipC), flagellin FliC and outer-membrane protein OmpA are indicated. (B) Presence of the invasion protein SipC in extracellular fractions (SP, VES, VSN) of overnight, stationary cultures of SL1344 (Dam⁺) and SV1610 (Dam⁻) strains. (C) Transmission electron microscopy of particulate material (VES fraction) obtained from overnight, stationary cultures of SL1344 (Dam⁺) and SV1610 (Dam⁻) strains. Note the presence of flagella in the sample of the wild-type (wt) strain and numerous membrane vesicles in the case of the Dam⁻ mutant. Bars, 0.2 µm.

small subset of proteins that migrate in gels as the invasion-related determinants SipA, SipB and SipC (Fig. 1A) (Komoriya *et al.*, 1999). Similarly to the VES fraction, the VSN fraction obtained from the Dam⁻ mutant contained a larger number of proteins (Fig. 1A), whose nature and origin are described below.

Table 2. Effect of *dam* mutations on deoxycholate sensitivity

Strain	Relevant genotype	Growth on 1 % deoxycholate	
		Exponential culture	Stationary culture
<i>S. enterica</i>			
SL1344	<i>dam</i> ⁺	++	+++
SV1610	<i>dam</i> -228:: <i>MudJ</i> , isogenic SL1344	—	+
14028s	<i>dam</i> ⁺	+++	+++
SV4392	<i>dam</i> -201:: <i>MudJ</i> , isogenic 14028s	—	++
LT2	<i>dam</i> ⁺	++	+++
SV3000	<i>dam</i> -201:: <i>Tn10dTet</i> , isogenic LT2	—	+
<i>E. coli</i>			
GM28	<i>dam</i> ⁺	++	+++
GM33	<i>dam</i> -3, isogenic GM28	++	+++

It is known that specific invasion proteins, such as the Ipa proteins of *Shigella flexneri*, aggregate in the extracellular medium (Parsot *et al.*, 1995). With this observation in mind, we compared the relative amount of the invasion protein SipC in the extracellular fractions SP, VES and VSN of *Dam*⁺ and *Dam*⁻ strains. Western analysis showed that the amount of SipC secreted by the *Dam*⁻ mutant was lower, in the range of 50 %, than that of the wild-type (compare SP fractions, Fig. 1B). However, most of the SipC protein detected in the SP fraction appeared as non-particulate material (VSN fraction) in both *Dam*⁺ and *Dam*⁻ strains (Fig. 1B). This result suggested that SipC neither forms extensive aggregates nor associates with the membranous particulate material released by *Dam*⁻ mutants (Fig. 1B). To further characterize the nature of the particulate material obtained upon high-speed centrifugation (VES fraction), this material was directly visualized by transmission electron microscopy. The analysis showed that intact flagella constituted the major extracellular particulate material of cultures containing the wild-type strain (Fig. 1C). This observation was consistent with the protein pattern observed in the VES fraction prepared from the wild-type strain (Fig. 1A). In contrast, the VES fraction of the *Dam*⁻ mutant contained numerous membrane vesicles (Fig. 1C). These results indicated that lack of Dam methylation causes exacerbated loss of envelope material in the form of membrane vesicles, a process that is accompanied by the presence of a large number of soluble proteins in the extracellular medium.

***S. enterica* mutants deficient in Dam methylase are sensitive to deoxycholate**

The level of resistance to deoxycholate shows variations among strains of *S. enterica* serovar Typhimurium: for instance, strain 14028s is resistant to higher deoxycholate concentrations than SL1344 and LT2. However, under the conditions chosen for our experiments (LB

plates containing 1 % sodium deoxycholate), strains SL1344, 14028s and LT2 were all resistant to deoxycholate, while their *Dam*⁻ derivatives were sensitive (Table 2). An additional observation was that, irrespective of their parental origin, *Dam*⁻ mutants were more sensitive to deoxycholate during exponential growth than in the stationary phase (Table 2). These data provide evidence that *Dam*⁻ mutants of *S. enterica* have a defect in envelope stability, mostly during active growth. In contrast to *S. enterica*, a *Dam*⁻ mutant of *E. coli* did not show deoxycholate sensitivity, even during exponential growth (Table 2).

Loss of membrane material in *S. enterica* *Dam*⁻ mutants occurs regardless of the activity of type III secretion systems

Specialized type III secretion systems, such as that involved in flagellar synthesis and the SPI-1-encoded system, secrete most of the proteins that are detected in supernatants of *S. enterica* cultures (Komoriya *et al.*, 1999). To determine whether any of the proteins present in the VSN fraction of *Dam*⁻ mutants were secreted by any of these two type III systems, a *dam* mutation was combined with mutations in the SPI-1 genes *invA* and *invJ* (strains SV4184 and SV4186, respectively), or in the gene encoding the flagellar master regulator *flhD* (strain SV4331). Previous studies have shown that absence of either the inner-membrane protein InvA or the translocator InvJ render *S. enterica* unable to secrete any invasion-related protein effector (Collazo *et al.*, 1995; Collazo & Galán, 1997). In turn, the flagellar master regulator FlhDC is absolutely required for the synthesis of structural or secreted flagellar proteins (Komoriya *et al.*, 1999). Protein analysis of SP, VES and VSN fractions from the single and double mutants indicated that release of particulate membrane material to the extracellular medium occurs whenever a *dam* mutation is present, irrespective of the functional status of the two type III secretion system analysed (SP and VES fractions

Fig. 2. Release of membrane vesicles caused by a *dam* mutation occurs independently of the activity of type III protein secretion systems. (A) Extracts containing total extracellular protein (SP fraction) were prepared from overnight stationary cultures of strains SL1344 (*Dam*⁺), SV1610 (*Dam*⁻), SB136 (*InvA*⁻), SB302 (*InvJ*⁻), SV4184 (*Dam*⁻ *InvA*⁻), SV4186 (*Dam*⁻ *InvJ*⁻), SV4316 (*FlhDC*⁻) and SV4331 (*Dam*⁻ *FlhDC*⁻). The Sips and FliC proteins are indicated. The asterisks mark two proteins only visible in extracts of strains carrying the *dam* mutation. (B) Extracts containing extracellular particulate material (VES fraction) of the same set of strains detailed in (A). The two major proteins

shown in Fig. 2A, B, respectively). Moreover, most of the soluble extracellular proteins that appear in the VSN fraction of the single *Dam*⁻ mutant were still present in VSN fractions from double mutants lacking functional SPI-1 or flagellar type III systems (Fig. 2C). These results raised the question of whether the proteins present in the VSN fraction could be of cytosolic origin, thereby reflecting bacterial lysis. This hypothesis was tested by Western analysis using an antibody against the ribosomal elongation factor Tu, EF-Tu. The results shown in Fig. 2(D) demonstrated that bacterial lysis occurred in cultures of all strains harbouring a *dam* mutation. Lysis affected 2–5 % of the bacterial population, as estimated by comparing the levels of EF-Tu in the extracellular extract versus the levels present in whole-cell-associated extracts (data not shown). Hence, lack of *Dam* methylation causes loss of membrane and cytosolic material associated with the loss of viability in a small proportion of the bacterial population.

Lack of *Dam* methylase causes loss of membrane particulate material in stationary-phase bacteria

Shedding of membrane vesicles is known to occur in many Gram-negative bacteria during the active phase of growth (Whitmire & Garon, 1993; Kadurugamuwa & Beveridge, 1995, 1997; Wai *et al.*, 1995; Zhou *et al.*, 1998). Electron microscopy analysis of the VES fraction unveiled the existence of membrane vesicles in the extracellular medium of saturated, stationary cultures of *Dam*⁻ mutants (see Fig. 1C). To determine whether this loss of membrane material due to lack of *Dam* methylase occurred during exponential or stationary growth, the morphology of the bacterial envelope was examined by electron microscopy. These experiments failed to demonstrate visible differences in the envelope along the surface of wild-type and *Dam*⁻ bacteria (Fig. 3A–D). Lack of major alterations in envelope integrity was noticed in both exponential and stationary cultures (Fig. 3A–D). However, the appearance of membranous material and vesicles was consistently reproduced in the samples obtained from stationary-phase cultures of *Dam*⁻ mutants (Fig. 3E). This result indicated that accumulation of membrane particulate material only occurs in stationary cultures of *Dam*⁻ mutants. The possibility that vesicle release starts during exponential growth and increases swiftly cannot be excluded. However, our failure to detect membrane vesiculation in actively growing bacteria is more consistent with the view that loss of membrane material starts when the *Dam*⁻ mutant enters the stationary phase.

present that correspond to FliC and OmpA are indicated. (C) Extracts containing extracellular soluble material (VSN fraction) of the same set of strains detailed in (A). The Sips and FliC proteins are indicated. (D) Immuno-detection of the cytosolic elongation ribosomal factor EF-Tu in the fractions containing soluble proteins (VSN fractions) shown in (C). Note that the strains that release this cytosolic protein are those harbouring the *dam* mutation. The percentage of lysis was estimated as 2–5 % upon comparison of this signal with that obtained from whole-cell protein extracts (not shown). wt, wild-type.

Fig. 3. *S. enterica* Dam⁻ mutants do not display massive envelope alterations. Transmission electron microscopy of bacteria grown to exponential phase (OD₅₅₀ 0.3) (A, B) or to stationary phase (C–E). (A, C) SL1344 (Dam⁺); (B, D, E) SV1610 (Dam⁻). Note the absence of differences in the morphology of the cell envelope in wild-type and mutant bacteria grown to exponential or stationary phase. (E) Shows particulate material and vesicle membranes that were consistently observed in samples prepared from stationary phase cultures of the Dam⁻ mutant. Bars, 0.5 μm.

Dam methylation controls the association of PAL, TolB, Lpp and OmpA proteins with peptidoglycan

The observation that lack of Dam methylase leads to loss of particulate material and cell viability raised the question of whether this phenotype might be triggered by a functional defect in proteins that play a role in the preservation of membrane stability. Among these proteins are the TolQRAB-PAL group and the murein (Braun's) lipoprotein (Lpp). All these proteins bind strongly, even covalently (e.g. Lpp), to the peptidoglycan layer. Lack of any of these proteins triggers extensive membrane leakage (Bernadac *et al.*, 1998; Lazzaroni *et al.*, 1999). To analyse a putative role of Dam methylase in the synthesis or distribution of these proteins in the bacterial cell envelope, a protocol was optimized to

obtain a highly purified peptidoglycan (PG) fraction (see Methods). The relative proportion of TolB, PAL and Lpp proteins that remained firmly associated with purified peptidoglycan was tested by Western analysis. OmpA was also included in this analysis, since it is one of the major proteins present in the outer membrane and it has been reported to associate with peptidoglycan (Koebnik, 1995). PG fractions were prepared from the wild-type and a Dam⁻ mutant in exponential and stationary phase (Fig. 4). Two additional fractions containing the protein present in the membrane, but not firmly associated with peptidoglycan (i.e. solubilized with either 0.4% Triton X-100 or 1% SDS, fractions 'Sol' and 'Insol' respectively), were also included for comparison (Fig. 4). Unlike the results obtained in stationary phase, a substantial decrease in the amount of

Fig. 4. Western blot analysis of the envelope distribution of TolB, PAL, Lpp and OmpA proteins in *Dam*⁻ mutants of *S. enterica*. Envelope extracts were prepared from bacteria grown to exponential phase (EXP) or stationary phase (ST). The different fractions consisted of: Sol, envelope proteins soluble in 0.4% Triton X-100; Ins, envelope proteins insoluble in 0.4% Triton X-100; PG, proteins that remain associated with peptidoglycan after extensive boiling in 4% SDS (see Methods). TolB, PAL, Lpp and OmpA proteins were detected with specific antibodies. The asterisks mark those cases in which a noticeable difference was observed. wt, wild-type.

TolB, PAL, Lpp and OmpA that associate with peptidoglycan was observed in the actively growing *Dam*⁻ mutant (Fig. 4). These differences were more marked for the TolB and PAL proteins (Fig. 4). To confirm that these differences were not related to loss of membrane material by the *Dam*⁻ mutant, the relative amount of a cytosolic protein, the ribosomal factor EF-Tu, was monitored in cytosolic fractions prepared from the same number of bacteria used to prepare the envelope fractions. In this case, an equivalent amount of EF-Tu was observed for both the wild-type and the *Dam*⁻ mutant (data not shown). Altogether, these results suggest that Dam methylation plays a relevant role in controlling the distribution and relative amount of

Fig. 5. *E. coli* *Dam*⁻ mutants do not exhibit a phenotype of membrane instability. Subcellular fractions TP, SP, VES and VSN (see legend to Fig. 1) were prepared from isogenic *E. coli* strains GM28 (*Dam*⁺) and GM33 (*Dam*⁻) grown to stationary phase. Unlike *S. enterica*, no accumulation of proteins in the extracellular medium was observed. wt, wild-type.

peptidoglycan-associated proteins when bacteria undergo active growth. This regulation is apparently exerted to a higher degree in the case of the envelope-stabilizing proteins TolB and PAL.

Dam methylation is not required for maintenance of envelope integrity in *E. coli*

S. enterica and *E. coli* are closely related genera. Most of the 'membrane-leakage' phenotypes associated with loss of function in Tol-PAL or Lpp proteins have been demonstrated in *E. coli* (Bernadac *et al.*, 1998). Therefore, we were interested to test whether Dam methylase also played a role in the control of membrane integrity in *E. coli*. A fraction of total extracellular protein present in the culture supernatant (fraction SP) was prepared from isogenic *E. coli* wild-type and *Dam*⁻ strains. Unlike *S. enterica*, lack of Dam methylase in *E. coli* did not produce massive accumulation of proteins into the extracellular medium (Fig. 5). Furthermore, when the extracellular proteins were separated by high-speed centrifugation in the corresponding particulate and soluble fractions, the VES fraction proved to have a low protein content (Fig. 5). In the VSN fraction (soluble extracellular fraction), a major protein migrating as flagellin was observed (Fig. 5). In contrast to *S. enterica*, no major differences were observed in the amount or distribution of TolB, PAL, OmpA and Lpp proteins between wild-type and *Dam*⁻ *E. coli* strains during exponential or stationary growth phases (data not shown). A concomitant observation is that *dam* mutations do not cause deoxycholate sensitivity in *E. coli* (Table 2). These observations indicate that Dam methylation is not required for envelope stability in *E. coli*.

DISCUSSION

In a previous study we showed that Dam methylase mutants of *S. enterica* secrete high amounts of proteins into the culture medium (Garcia-del Portillo *et al.*, 1999). Complementation with a plasmid-borne *dam* gene restored the wild-type pattern of protein secretion, indicating that protein hypersecretion was solely caused by the *dam* mutation (Garcia-del Portillo *et al.*, 1999). This phenotype, first described for Dam⁻ mutants of SL1344, has been now reproduced in Dam⁻ derivatives of other strains of *S. enterica* serovar Typhimurium, such as the laboratory strain LT2 and the mouse-virulent strain 14028s.

In an attempt to characterize the basis for this phenotype, we separated the extracellular proteins by high-speed centrifugation into two fractions: (i) particulate material; and (ii) soluble material. In the Dam⁻ mutant, over 15 proteins were visible in the particulate material. Some of these proteins were identified as flagellin (FliC), the outer-membrane proteins OmpA, OmpC, OmpX, and the murein lipoprotein Lpp by Western blotting and mass spectrometry analysis (data not shown). This result provided us with the first evidence for an involvement of Dam methylation in the maintenance of envelope integrity. In addition, electron microscopy analysis of fractions containing particulate material showed that stationary cultures of Dam⁻ mutants contained large amount of membrane vesicles in the extracellular medium, whereas only flagella were visible in extracts from the wild-type strain. Interestingly, direct examination of whole bacteria in either exponential or stationary growth phases failed to show massive envelope defects in the Dam⁻ mutant. Nevertheless, and in agreement with a recent study (Heithoff *et al.*, 2001), our results show that Dam⁻ mutants are highly sensitive to deoxycholate during active growth. The apparent contradiction between this sensitivity and the inability to observe major defects in actively growing Dam⁻ mutants might indicate the existence of minor alterations in envelope stability sufficient to promote sensitivity to bile acids. These envelope alterations might be cumulative and, upon entrance into the stationary phase, culminate in envelope breakage in a subpopulation of bacteria.

The existence of an envelope defect in exponential cultures of Dam⁻ mutants was confirmed by subcellular fractionation analysis: a set of proteins required for the maintenance of envelope stability were found to associate at lowered levels with the peptidoglycan of Dam⁻ mutants. Among these proteins, a marked alteration of the association of TolB and PAL to peptidoglycan was observed in mutants lacking Dam methylase. Previous studies have shown that deficiency of any of these proteins in *E. coli* triggers release and leakage of membrane and periplasmic material into the growth medium (Bernadac *et al.*, 1998; Lazzaroni *et al.*, 1999). Our study provides the first evidence for a similar envelope-stabilizing role of these proteins in *S. enterica* and, in addition, raises the hypothesis that such a role is controlled by Dam methylation. The fact that the

alterations in protein-peptidoglycan association were noticeable only in actively growing bacteria indicates that the envelope defect caused by lack of Dam methylation might reflect up-regulation of a natural process that occurs under conditions of high growth rate and cell wall turnover. This assumption agrees with previous studies that demonstrated the shedding of membrane vesicles as a process occurring predominantly in actively growing bacteria (Kadurugamuwa & Beveridge, 1995, 1997; Zhou *et al.*, 1998). Interestingly, and unlike Lpp and OmpA, lack of Dam methylation seems to affect the total amount of TolB and PAL proteins present in the envelope of actively growing bacteria. This observation contrasts with the similar levels of mRNA transcripts of the *tolQRAB* and *pal* operons detected in Dam⁺ and Dam⁻ strains (unpublished observations), which suggests the existence of post-transcriptional regulatory mechanisms acting on TolB and PAL, and controlled by Dam methylation. These post-transcriptional regulatory mechanisms could be sustained by the stimulation of a normal physiological process (i.e. cell wall turnover). Alternatively, Dam methylation might control the synthesis of an as yet unknown cell function required for proper maintenance or positioning of the Tol-PAL protein complexes. The fact that the association to peptidoglycan of all the envelope proteins analysed was altered at some degree by the lack of Dam methylase suggests that a basic process of envelope physiology may be altered.

In addition to the set of proteins present in membranous material, about 25–30 proteins were visible by Coomassie staining in the soluble, extracellular fraction (VSN) from Dam⁻ mutants. Although we first considered that hypersecretion of VSN proteins could reflect some imbalance in protein secretion systems, two observations revealed that this was not the case. First, with the exception of the flagellin subunit FliC, the pattern of proteins present in the VSN fraction was unaltered when either the flagellar or invasion-specific type III systems were non-functional. Because most proteins secreted by *S. enterica* are either flagellar proteins or virulence-related proteins (Komoriya *et al.*, 1999), we reasoned that many proteins present in the VSN fraction could derive from lysed bacteria. Furthermore, the detection of a ribosomal factor in the VSN fraction confirmed that the Dam⁻ mutant undergoes lysis upon entering stationary phase. Some 2–5 % of the Dam⁻ bacterial population was estimated to lyse in stationary phase. Despite this small percentage, the concentration of extracellular proteins by acid precipitation hampered the visual analysis of 'secreted' proteins. An example was the SipC protein, which is secreted at a 50 % rate in a Dam⁻ mutant (Fig. 1B) (Garcia-del Portillo *et al.*, 1999), but was virtually masked by the bulk of proteins present in the extracellular fraction (SP) of the Dam⁻ mutant.

Release of outer-membrane vesicles is a physiological process that occurs naturally in many bacterial pathogens. For instance, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Actinobacillus pleuropneumoniae*, entero-

haemorrhagic *E. coli* (EHEC) and *Helicobacter pylori* are all known to shed membrane vesicles into the culture medium (Kadurugamuwa & Beveridge, 1995, 1997, 1999; Wai *et al.*, 1995; Keenan *et al.*, 2000). In some cases, these vesicles have been related to the transport of pathogenic factors. Examples include the VacA toxin of *H. pylori* (Keenan *et al.*, 2000), Shiga toxin and lipopolysaccharide by STEC (Shiga-toxin producing *E. coli*) and EHEC (Wai *et al.*, 1995; Meno *et al.*, 2000; Yokoyama *et al.*, 2000). In other cases outer-membrane vesicles have been shown to contain muramidases and other periplasmic enzymes (Kadurugamuwa & Beveridge, 1995, 1999). When these membrane vesicles fuse with the membrane of a different bacterial species, their content can trigger loss of viability of the recipient strain, or a physiological 'incorporation' of this heterologous material into its envelope structure (Li *et al.*, 1998; Kadurugamuwa & Beveridge, 1999). If outer-membrane shedding is a normal physiological process in *S. enterica*, it seems reasonable that it must be subjected to tight control. Our study suggests that Dam methylation may participate in such a control.

Unlike *S. enterica*, lack of Dam methylation did not cause any noticeable effect on envelope integrity or stability in *E. coli*. Biochemical analyses of protein content in extracellular fractions, deoxycholate sensitivity experiments, and studies of protein-peptidoglycan interactions were all unable to show any difference between Dam⁺ and Dam⁻ strains of *E. coli*. The basis of this unexpected difference between *Salmonella* and *E. coli* is at present unknown. One may, however, recall that the *S. enterica* genome contains genes with a putative role in peptidoglycan remodelling which are absent in *E. coli* (Hilbert *et al.*, 1999).

Envelope instability might contribute to virulence attenuation of *S. enterica* Dam⁻ mutants in the mouse typhoid model. Enhanced release of membrane vesicles through infected cells and tissues might cause overstimulation of the host immune response, as shown for *Neisseria meningitidis* (Mirlashari *et al.*, 2001). Release of outer-membrane proteins such as OmpA, PAL and Lpp to the extracellular medium can occur in the presence of serum (Hellman *et al.*, 2000a, b), and this process has been proposed as a major phenomenon mediating elicitation of immune host defence. Therefore, overstimulation of host defences during infection might render Dam⁻ mutants unable to cause disease. These effects on the host would also sustain the vaccine properties of *S. enterica* Dam⁻ mutants (García-del Portillo *et al.*, 1999; Heithoff *et al.*, 1999, 2001).

ACKNOWLEDGEMENTS

We are grateful to Drs J.-C. Lazzaroni, H. Schwarz, M. Inouye and M. Vicente for providing antibodies, and to Drs M. G. Marinus, J. E. Galán and E. A. Groisman for sending bacterial strains. Strain construction was carried out by Dr David Cano. This work was supported by grants from the European Union (QLK2-1999-00310) and the Comunidad de Madrid (08.2/0045.1/2000). M.G.P. is supported by a postdoctoral

fellowship from the Comunidad de Madrid. A.I.P. is the recipient of a predoctoral fellowship from the Fundación Ramón Areces.

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Received 31 October 2001; revised 12 December 2001; accepted
19 December 2001.