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Identification of the *Salmonella enterica damX* Gene Product, an Inner Membrane Protein Involved in Bile Resistance▽†

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The *damX* gene product of *Salmonella enterica* serovar Typhimurium is a protein located in the inner membrane. DamX migrates as a 70-kDa protein in SDS-PAGE even though the predicted protein size is 46 kDa. Synthesis of DamX protein occurs in both exponential- and stationary-phase cultures. Disruption of *damX* causes severe sensitivity to bile. Lack of the outer membrane protein AsmA suppresses bile sensitivity in *Salmonella damX* mutants.

The *damX* locus, also known as Urf74.3, is an open reading frame (ORF) located in an operon that also contains the *aroB*, *aroK*, *rpe*, *gph*, and *dam* genes (11, 12). The region is highly conserved in *Escherichia coli* and *Salmonella enterica* (2, 13). The known genes contained in the operon are functionally heterogeneous: *aroB* and *aroK* are involved in aromatic amino acid metabolism; *dam* encodes DNA adenine methyl transferase; *trpS* is the gene for tryptophan aminoacyl-tRNA synthetase; and the *rpe* and *gph* genes are involved in carbohydrate metabolism (8, 11). The product of the *E. coli damX* (Urf74.3) gene has previously been described as a 70-kDa protein (8, 11), and two recent studies have shown that DamX accumulates in the *E. coli* septal ring (1, 6). Below, we show that the *damX* gene product of *Salmonella enterica* serovar Typhimurium is an inner membrane protein whose absence causes bile sensitivity. The study has been carried out with strain ATCC 14028. However, the nucleotide sequences of the *damX-dam* chromosomal region are 100% identical in strains LT2 (13) and SL1344 (ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella).

DamX is an inner membrane protein. *In silico* analysis of the DamX secondary structure with the TMpred algorithm (http://www.ch.embnet.org/software/TMPRED_form.html) predicted that DamX has one transmembrane segment. To detect DamX by Western immunoblot analysis, we constructed a DamX protein derivative tagged with a 3× FLAG epitope (24). The primers used for introduction of the 3× FLAG epitope were 5′ GTG GGC AAA ACC GTT GCA TCA GGT TCA GGC CGA TCT GAA AGA CTA CAA AGA CCA TGA CGG 3′ and 5′ GTC AGC GAC ACT CAC AGG CAA TTG CAG GAC ACA GCC TGG ACA TAT GAA TAT CCT CCT TAG 3′. Protein purification, cell fractionation (cytosol, inner membrane, and outer membrane), and Western analysis of the resolved protein extracts were carried out as described elsewhere (17). DamX migrated as a 70-kDa protein under denaturing conditions, as previously described for *E. coli* (11). DamX was found in the *S. enterica* serovar Typhimurium inner membrane (Fig. 1) and was detected in protein preparations from both exponential- and stationary-phase cultures (Fig. 2).

Construction of *damX* deletion mutants. The *S. enterica damX* gene was disrupted by lambda Red recombination (4). The Δ*damX1* deletion was generated with primers 5′ ATG GAT GAA TTC AAA CCA GAA GAC GAG CTG AAA CCC GAT CGT GTA GGC TGG AGC TGC TTC 3′ and 5′ AGC GAC ACT CAC AGG CAA TTG CAG GAC ACA GCC TGG ATT ACA TAT GAA TAT CCT CCT TAG 3′. This deletion eliminated the entire *damX* ORF except 40 bp in the 5′ region. The Δ*damX2* deletion was generated with primers 5′ AGA CGA GCT GAA ACC CGA TCC CAG CGA TCG TCG TAC TGG TAT TCC GGG GAT CCG TCG ACC 3′ and 5′ TCG TGG TGG TCG CTT TCG GCG TCG CGG CTG GCG CTG CCG GGT GTA GGC TGG AGC TGC TTC 3′. The Δ*damX2* deletion eliminated 795 bp, thus leaving about one-third of the *damX* coding sequence (423 bp) at the 3′ end as well as 60 bp at the 5′ end. The external primers for deletion verification by the PCR were 5′ GGA CTA TTT GCC GCA CAT GC 3′ and 5′ AGG TCG CTC TTG ATA TCG GC 3′.

Consequences of *damX* disruption. Both the Δ*damX1* and the Δ*damX2* alleles conferred sensitivity to sodium deoxycholate (DOC) (Table 1) but not to sodium dodecyl sulfate, crystal violet, or malachite green (data not shown). Because *dam* mutants are extremely sensitive to bile (21) and the *damX* gene lies immediately upstream of *dam* (11), these experiments did not ascertain whether the Δ*damX1* and Δ*damX2* deletions caused bile sensitivity on their own or by a polar effect on *dam* gene expression. To clarify this point, two kinds of experiments were carried out: (i) tests for SOS induction using the *cea*::*lac* fusion of plasmid pGE108 (23) as a reporter (18, 19) and (ii) analysis of expression of a translational *std*::*lac* fusion, which is extremely sensitive to Dam methylation (7). The main conclusion from these experiments, whose results are summarized in Table 2, was that the Δ*damX1* deletion caused a certain degree of polarity on *dam* gene expression, reflected in its ability to

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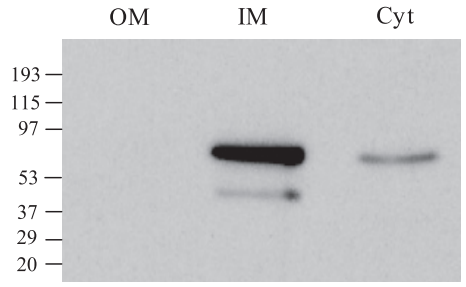

FIG. 1. Distribution of DamX protein tagged with a 3× FLAG epitope in subcellular fractions of *S. enterica* serovar Typhimurium. Anti-FLAG western hybridization is shown for three fractions: the outer membrane (OM), the inner membrane (IM), and the cytosol (Cyt). The volumes loaded for all fractions were normalized to the same number of bacteria (5×10^7 CFU). Prestaining molecular mass standards in kDa are indicated.

cause moderate induction of *std*::*lac* expression. The polarity was mild, however, as indicated by the fact that neither Δ*damX1* nor Δ*damX2* caused significant SOS induction (Table 2).

Examination of the nucleotide sequence in the *S. enterica damX-dam* region provided an explanation for the partial polarity of the Δ*damX1* deletion. The *E. coli dam* gene is transcribed from five promoters, and one of them (p_4) is located within the *damX* coding region (10). *In silico* analysis of the *Salmonella damX* ORF unambiguously predicts the existence of nearly canonical −10 and −35 promoter modules in a region which has been eliminated by the Δ*damX1* deletion but not by Δ*damX2* (see Fig. S1 in the supplemental material). A polar effect on *dam* expression may likewise explain SOS induction by transposon insertions in *damX* (15) as well as the invasion defect of *Salmonella enterica* serovar Typhi *damX* mutants (9).

A nonpolar *damX* deletion causes bile sensitivity on its own. Lack of polarity of the Δ*damX2* deletion on the downstream *dam* gene was confirmed by digestion of genomic DNA preparations with restriction endonucleases DpnI, Sau3AI, and NdeII. All these enzymes recognize the sequence 5′ GATC 3′. NdeII activity is blocked by Dam methylation, while DpnI cuts methylated DNA only, and Sau3AI cuts both methylated and unmethylated DNA. The DNA restriction pattern in the Δ*damX2* mutant was similar to that of the wild type (*dam*$^+$) strain, thus ruling out polarity (Fig. 3).

Evidence that lack of DamX causes bile sensitivity on its own (and not by polarity on *dam*) was also obtained by complementation analysis using a chromosomal duplication (3). A *damX*$^+$/Δ*damX2* merodiploid was 10-fold more resistant to DOC than the Δ*damX2* mutant and 20-fold more resistant than a Δ*damX2*/Δ*damX2* merodiploid (Table 1).

Lack of AsmA suppresses bile sensitivity in *damX* mutants. Previous studies have shown that bile sensitivity of *S. enterica dam* mutants is suppressed by two classes of recessive mutations: (i) in the mismatch repair genes *mutH*, *mutL*, and *mutS* (18) and (ii) in the *asmA* gene (17). The MIC analyses represented in Table 1 indicated that lack of AsmA suppresses DOC sensitivity in a *damX* background, while inactivation of the MutHLS system does not. This is a relevant difference between *dam* and *damX* mutants despite the fact that both mutant types are bile sensitive. Lack of AsmA has previously been shown to activate the Mar regulon, which may in turn activate efflux pumps and other protective devices (17). As a consequence, *asmA* mutations suppress bile sensitivity in genetic backgrounds as diverse as *dam*, *phoP*, and *wec* (17). This study adds *damX* to the list.

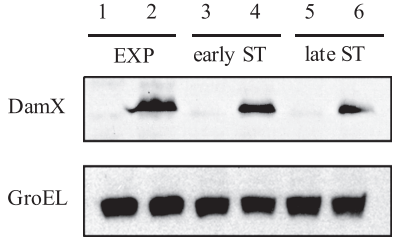

FIG. 2. Levels of DamX protein in *S. enterica* cultures grown in LB medium at 37°C with shaking. Protein preparations were made from cultures in exponential phase (EXP; optical density at 660 nm [OD_{660}] = 0.2), early stationary phase (early ST; OD_{660} = 2.5), and late stationary phase (late ST; OD_{660} = 3.4). All samples were prepared from 4×10^8 cells. Levels of the heat shock protein GroEL were monitored as internal loading controls. The strains were ATCC 14028 (*damX*$^+$; lanes 1, 3, and 5) and SV5448 (*damX*$^+$::3×FLAG; lanes 2, 4, and 6).

TABLE 1. MICs of sodium deoxycholate in *S. enterica* strains carrying *dam* and *damX* deletion alleles

Strain	Genotype	MIC of DOC (%)[a]
ATCC 14028	wt	6.0
SV4536	Δ*dam-230*	0.2
SV5116	Δ*damX1*	0.4
SV5307	Δ*damX2*	0.4
SV5441	DUP [Δ*damX2*/Δ*damX2*]	0.2
SV5443	DUP [Δ*damX2*/*dam*$^+$]	4.0
SV5538	Δ*damX2* Δ*asmA*::Km^r	6.0
SV5539	Δ*damX2* Δ*mutH*::Km^r	0.4

[a] MICs are medians of results from >5 independent determinations. Overnight cultures in LB were diluted 1:50 in fresh LB and incubated at 37°C with shaking until late exponential phase (OD_{660} = 0.6 to 0.8). Cultures were then diluted to obtain a concentration of 3,000 cells/ml. Aliquots were transferred to microtiter plates whose wells had previously been filled with aqueous solutions of DOC (0.2%, 0.4%, 0.6%, 0.8%, 1%, 2%, 3%, 4%, 5%, 6%, and 7%). After overnight incubation at 37°C, bacterial growth was visually monitored.

TABLE 2. Effects of *dam* and *damX* mutations on the expression of *cea*::*lac* and *stdA*::*lac* fusions

Genotype[a]	β-Galactosidase activity[b]	
	cea::*lacZ*	*sdtA*::*lacZ*
Wild type	54.75 ± 2	<1
Δ*damX1*	57.49 ± 2	15 ± 1
Δ*damX2*	55.36 ± 3	<1
Δ*dam-230*	727.38 ± 63	1273 ± 88

[a] The strains were SV4933 (ATCC 14028/pGE108), SV5119 (Δ*damX1*/pGE108), SV5488 (Δ*damX2*/pGE108), SV4930 (Δ*dam-230* /pGE108), SV5206 (*stdA*::*lacZ*), SV5287 (*stdA*::*lacZ* Δ*damX1*), SV5486 (*stdA*::*lacZ* Δ*damX2*), and SV5208 (*stdA*::*lacZ* Δ*dam-230*). Strains SV4930 and SV4933 were described in reference 18. Strains SV5206 and SV5208 were described in reference 7.

[b] Strains were grown in LB at 37°C with shaking. Aliquots for β-galactosidase analysis were extracted at an OD_{660} of 0.8. β-Galactosidase activities were assayed using the $CHCl_3$-sodium dodecyl sulfate permeabilization procedure (14). Data are averages and standard deviations from 3 experiments.

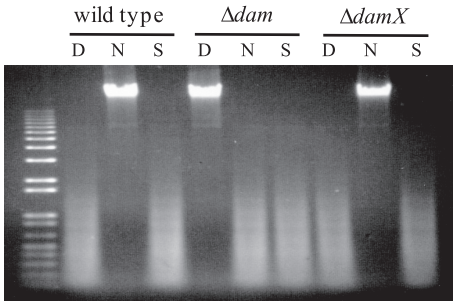

FIG. 3. Digestion of genomic DNAs isolated from the wild type, a *dam* mutant (SV4536), and a mutant carrying the Δ*damX2* allele (SV5307) by restriction enzymes DpnI (D), NdeII (N), and Sau3AI (S).

Relevance of envelope integrity for bile resistance. The importance of the cell envelope as a barrier for bile salts is illustrated by the variety of envelope alterations that cause bile sensitivity. The current list includes mutations that alter the outer membrane (17, 20), the lipopolysaccharide (5, 16), and the enterobacterial common antigen (22). It is thus interesting that DamX, an inner membrane protein, is likewise required for bile resistance. The high sensitivity of *damX* mutants to DOC suggests that lack of DamX may cause a major change in envelope structure or function. Unlike *S. enterica dam* mutants (21), however, *S. enterica damX* mutants do not show envelope instability (see Fig. S2 in the supplemental material). The relevance of DamX as an envelope component is supported by two recent studies indicating that *E. coli* DamX may participate in cell division (1, 6). However, the observation that DamX is produced by both dividing and nondividing *Salmonella* cells (Fig. 2) suggests that DamX may be a constant component of the *S. enterica* inner membrane.

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