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Overproduction of DNA Adenine Methyltransferase Alters Motility, Invasion, and the Lipopolysaccharide O-Antigen Composition of *Yersinia enterocolitica*▽

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DNA adenine methyltransferase (Dam) not only regulates basic cellular functions but also interferes with the proper expression of virulence factors in various pathogens. We showed previously that for the human pathogen *Yersinia enterocolitica*, overproduction of Dam results in increased invasion of epithelial cells. Since invasion and motility are coordinately regulated in *Y. enterocolitica*, we analyzed the motility of a Dam-overproducing (Dam^{OP}) strain and found it to be highly motile. In Dam^{OP} strains, the operon encoding the master regulator of flagellum biosynthesis, *flhDC*, is upregulated. We show that the increased invasion is not due to enhanced expression of known and putative *Y. enterocolitica* invasion and adhesion factors, such as Inv, YadA, Ail, Myf fibrils, Pil, or Flp pili. However, overproduction of Dam no longer results in increased invasion for an *inv* mutant strain, indicating that Inv is necessary for increased invasion after overproduction of Dam. Since we show that overproduction of Dam results in an increased amount of rough lipopolysaccharide (LPS) molecules lacking O-antigen side chains, this implies that reduced steric hindrance by LPS might contribute to increased invasion by a *Y. enterocolitica* Dam^{OP} strain. Our data add an important new aspect to the various virulence-associated phenotypes influenced by DNA methylation in *Y. enterocolitica* and indicate that Dam targets regulatory processes modulating the composition and function of the bacterial surface.

The DNA adenine methyltransferase (Dam) of gammaproteobacteria catalyzes the methylation of adenine residues at the N^6 position in GATC sequences. Methylation occurs directly after DNA replication with a delay, thereby leaving the newly synthesized daughter strand nonmethylated for a short period of time. Depending on their presence and their affinity, methylation-sensitive regulatory proteins either bind to the hemimethylated DNA during this time and prevent the subsequent methylation of GATC sequences or bind preferentially to fully methylated DNA. Therefore, the methylation status of GATC sequences has an impact on the binding of regulatory proteins and consequently is involved in the regulation of several basic processes of the bacterial cell, such as mismatch repair, chromosome replication, transposition, or transcription of genes (10, 56).

It is therefore not surprising that Dam influences the virulence properties of a variety of bacterial pathogens. Dam-overproducing (Dam^{OP}) and/or *dam* mutant strains of *Salmonella enterica* (*dam*), *Vibrio cholerae* (Dam^{OP}), *Pasteurella multocida* (Dam^{OP}), *Aeromonas hydrophila* (Dam^{OP}), *Haemophilus influenzae* (*dam*), *Klebsiella pneumoniae* (*dam*), *Yersinia pestis* (*dam*), or *Yersinia pseudotuberculosis* (Dam^{OP}, *dam*) show reduced virulence in animal models of infection (11, 20, 23, 25, 26, 30, 34, 42, 46, 48, 55). These in vivo outcomes are most likely the result of the effects of DNA adenine methylation on the regulation of diverse virulence functions (26). For example, it has been demonstrated that Dam influences the proper secretion and translocation of type III effector proteins in *Y. pseudotuberculosis* (Dam^{OP}), *S. enterica* (*dam*), or *A. hydrophila* (Dam^{OP}) (20, 23, 30, 31). Furthermore, host cell invasion and adhesion are affected in strains of *S. enterica* (*dam*) and *H. influenzae* (*dam*) with altered Dam methylation (23, 55). A variety of adhesive pili of *S. enterica* and *Escherichia coli* are phase-variably expressed, with adenine methylation stabilizing the ON or OFF phase (10). A changed methylation status also decreases the swimming motility of *E. coli* (*dam*), *A. hydrophila* (Dam^{OP}), and *S. enterica* (Dam^{OP}, *dam*) (4, 5, 20, 41).

The food-borne human pathogen *Yersinia enterocolitica* is able to cause different gastrointestinal syndromes, ranging from self-limiting enteritis to mesenteric lymphadenitis. In rare cases, *Y. enterocolitica* is able to disseminate to deeper tissues and cause systemic infections (9, 16). We used a Dam^{OP} strain as a tool to identify and analyze methylation-sensitive processes implicated in virulence of this pathogen. By using Dam^{OP} strains as well as *dam* mutant strains, it is possible to alter methylation patterns in regulatory regions of genes, whereby the affinity of transcription factors for these regions can be altered, thereby mimicking a situation that can also be found in vivo (26, 56). In previous studies, we could demonstrate that overproduction of the Dam enzyme in *Y. enterocolitica* leads to a relaxed Ca^{2+} regulation of the Yop/Ysc type III secretion system. This effect depends at least in part on a ClpP-mediated degradation of the regulatory protein LcrG (21). Furthermore, we found that the expression of several in vivo-expressed genes is not changed in Dam^{OP} strains. However, a Dam^{OP} strain cured of the pYV virulence plasmid

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Y. enterocolitica strains		
JB580v	Δ*yenR* ($r^- m^+$) Nal^r, serogroup O:8	32
GHY147	JB580v, pTP166Kan-damΔ, Nal^r Kan^r	22
GHY150	JB580v, pTP166Kan, Nal^r Kan^r	22
GHY174	JB580v, *flhD*::*lacZYA*, Nal^r Cam^r	This study
GHY175	JB580v, *flhD*::*lacZYA*, pTP166Kan-damΔ, Nal^r, Cam^r Kan^r	This study
GHY176	JB580v, *flhD*::*lacZYA*, pTP166Kan, Nal^r Cam^r Kan^r	This study
GHY287	JB580v, *inv*::pEP-inv, Nal^r Cam^r	This study
E. coli strains		
DH5α	Φ80dΔ(*lacZ*)*M15* Δ(*argF-lac*)*U169 endA1 recA1 hsdR17*($r_K^- m_K^+$) *deoR thi-1 supE44 gyrA96 relA1*	Gibco BRL
S17-1λ*pir*	Tp^r Sm^r *recA thi pro hsdR M*$^+$ RP4::2-Tc::Mu::Km Tn*7*λ*pir* lysogen	39
Plasmids		
pFUSE	Cam^r, *mob*$^+$ (RP4), R6K *ori* (suicide vector) *lacZYA*	6
pEP185.2	Cam^r, *mob*$^+$ (RP4), R6K *ori* (suicide vector)	32
pTP166Kan	Kanamycin-resistant derivative (Amp^s) of pTP166	22
pTP166Kan-damΔ	*dam* mutant derivative of pTP166Kan	22
pFUSE-flhD	*flhD* promoter fragment in pFUSE	This study
pEP-inv	Internal fragment of *inv* in pEP185.2	This study

shows an increased ability to invade cultured epithelial cells (22). Invasion processes are critical steps in *Y. enterocolitica* pathogenesis, since infection and colonization of the intestinal lymphoid tissue require transmigration of the bacteria from the intestinal lumen across the epithelial barrier (1, 24).

Therefore, we aimed at identifying the mechanisms leading to the hyperinvasive phenotype after overproduction of Dam in more detail. Furthermore, since the ability to invade epithelial cells is often coregulated with other virulence functions, we wondered if Dam influences these functions as well. In this study, we demonstrate that overproduction of Dam leads to an increased motility of *Y. enterocolitica*. Furthermore, we present data indicating that despite the hyperinvasive phenotype, the expression of a variety of known and putative surface proteins implicated in invasion and adhesion is reduced after overproduction of Dam. In relation to a new role for Dam in the regulation of bacterial pathogenesis, we present data that the lipopolysaccharide (LPS) O-antigen status is also changed, putatively increasing the accessibility of Inv and at least in part being responsible for the increased ability of the bacteria to invade host cells. Altogether, our data indicate that Dam influences diverse virulence-associated surface properties of *Y. enterocolitica*, thereby contributing to the multifaceted functions of DNA methylation in regulating virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, all strains were grown in Luria-Bertani (LB) broth or on agar plates at 26°C for *Y. enterocolitica* or at 37°C for *E. coli*. Antibiotics were used as described previously (22). Overproduction of Dam was achieved by electroporating pTP166Kan or pTP166Kan-damΔ as a negative control into the corresponding *Y. enterocolitica* strain and inducing expression of *dam* from the P_{tac} promoter by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (also for cells used as negative control) 30 min after subculture for each assay. The construction of pTP166Kan and pTP166Kan-damΔ has been described in detail previously (22). Both constructs are derivatives of pTP166, which carries the *E. coli dam* gene under the control of the P_{tac} promoter (33). To allow propagation of the plasmids in *Y. enterocolitica*, the *bla* gene encoding ampicillin resistance was replaced by a kanamycin resistance cassette. In pTP166Kan-damΔ, the P_{tac} promoter and the 5′ part of the *dam* gene have been deleted.

Analysis of motility. Motility was analyzed by inoculating semisolid agar plates containing 0.3% agar with 3 μl of an overnight culture grown at 26°C. After incubation of the plates at 26°C or 37°C for 20 h, the diameters of the halos starting from the points of inoculation were compared. Experiments were performed at least in triplicate.

Quantification of gene expression. The expression of *flhDC* was determined by reporter gene technology. For the construction of a *Y. enterocolitica flhD* promoter-*lacZ* fusion, a 750-bp fragment including the promoter region and the 5′ end of *flhDC* was amplified by PCR using the primer pair GH-flh1/GH-flh2 (Table 2) with genomic DNA of *Y. enterocolitica* JB580v as the template. The

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)[a]
SF-ailRT3	AGCTAGTTCTCTAATAGCCTG
SF-ailRT4	TTTGGAAGTGGGTTGAATTGC
GH-flh1	GCTCTAGAGGTTTACCTCTGCTGCC TTTTA
GH-flh2	CGTACTCATTTTATACATCCC
SF-flp1RT1	TTACGTTACAGCACAAGTTAATGCG
SF-flp1RT2	ATGTTTGTCATGGATGTATCGACG
SF-invRT1	CAGGCTAATATTATCGATCGG
SF-invRT2	CCCGGTCATATCATTGTCATA
SF-myfART1	CACCTGCCTTCCATCTGGTAATG
SF-myfART2	AGAAAGTCGCTTCCACACGCTC
SF-pilLRT1	CAAATGGTGGACATCACTATGCC
SF-pilLRT2	TTCCCGATTCACATCATCAACC
SF-rnaYE1	AATACCGCATAACGTCTTCG
SF-rnaYE2	CTTCTTCTGCGAGTAACGTC
GH3067f	TCCGCTATTACAAGCCGAGT
GH3067r	CTGCGGATGCTTATCGGTAT
GH3071f	TCATAACCCAGGTTGCCATT
GH3071r	CGCGAGATCCATAACATGAA
GH3087f	GTGTGGAACCATGGAATGTG
GH3087r	CACTGACACCATCGCCATAC
GH-inv1	CCATCGATCGCTGAACATAATGAG GCTTT
GH-inv2	GCTCTAGATACGCTTTGACGTGAAT GTCG

[a] GH-flh1 and GH-inv2 contain a restriction site for XbaI (underlined), and GH-inv1 contains a restriction site for ClaI (underlined).

resulting fragment was digested with XbaI and ligated into the XbaI/SmaI-digested vector pFUSE, resulting in plasmid pFUSE-flhD. After conjugation of the plasmid to *Y. enterocolitica* JB580v from *E. coli* S17-1λ*pir*, the merodiploid strain GHY174 (*flhD-lacZYA flhD*$^+$) was generated (Table 1). Homologous integration into the chromosome was confirmed by Southern blot analysis (data not shown).

To determine the expression of *flhDC*, overnight cultures of the corresponding *Y. enterocolitica* strains grown at 26°C were diluted in fresh LB medium and grown for 20 h at 26°C in the presence of 1 mM IPTG to induce overproduction of Dam from pTP166Kan. The bacterial cells were pelleted by centrifugation and washed with 0.85% (wt/vol) NaCl. Subsequently, β-galactosidase activities were quantified as described previously (36) and averaged from three independent experiments, each performed in triplicate.

The transcription of *inv*, *ail*, *flp-1*, *pilL*, *myfA*, *ddhA*, *gne*, and *rosA* was quantified by quantitative reverse transcription-PCR (qRT-PCR) as described recently (21). Briefly, overnight cultures of the Dam^{OP} strain of *Y. enterocolitica* and of the corresponding control strain grown at 26°C were diluted in fresh LB medium and grown at 26°C or 37°C for 4 h in the presence of 1 mM IPTG. After isolation of total RNA, DNase treatment, and randomly primed reverse transcription, relative gene expression was determined using the Light-Cycler system (Roche) and the QuantiTect SYBR Green PCR kit (QIAGEN). Experiments were performed at least in triplicate for each gene with the primer pairs SF-ailRT3 and SF-ailRT4, SF-flp1RT1 and SF-flp1RT2, SF-invRT1 and SF-invRT2, SF-myfART1 and SF-myfART2, SF-pilLRT1 and SF-pilLRT2, GH3067f and GH3067r (*rosA*), GH3071f and GH3071r (*gne*), and GH3087f and GH3087r (*ddhA*) (Table 2). The expression of the 16S rRNA was used as the housekeeping gene control using the primer pair SF-rnaYE1 and SF-rnaYE2 (Table 2).

Construction of an *inv* mutant strain. To construct a *Y. enterocolitica* strain defective in Inv-mediated invasion of host cells, an internal fragment of the *inv* gene was amplified by PCR using the primers GH-inv1 and GH-inv2 (Table 2) and ligated into the suicide vector pEP185.2. The resulting plasmid, pEP-inv, was transferred to *Y. enterocolitica* from *E. coli* S17-1λ*pir* by conjugation and integrated into the chromosome by homologous recombination with selection for chloramphenicol, directly resulting in an *inv* mutant strain. Homologous integration into the chromosome was confirmed by Southern blot analysis and Western blotting using Inv-specific antiserum (data not shown).

Determination of bacterial invasion and analysis of Inv levels. Bacterial invasion in CHO-K1 cells and eukaryotic cell culture was performed as previously described using a standard gentamicin protection assay (22). To determine the amount of Inv after overproduction of Dam, bacteria were grown at 26°C or 37°C for 4 h and overproduction of Dam was induced as described above. Whole-cell lysates corresponding to 3×10^8 bacteria were separated by sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane for immunoblot analysis. The nitrocellulose membrane was blocked for 45 min with 1% skim milk in phosphate-buffered saline (PBS) and incubated for 2 h at room temperature with a rabbit polyclonal antiserum specific for Inv (dilution, 1:2,500 in PBS–0.2% Tween 20), kindly provided by E. Bohn (Tübingen, Germany). After three washes with PBS–0.2% Tween 20, an alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit; dilution, 1:7,500 in PBS; 45 min at room temperature) was used to detect Inv.

Analysis of LPS O-antigen status. LPS was prepared as described previously by Bengoechea et al. (7). Briefly, overnight cultures of bacteria were diluted 1:10 in 5 ml of fresh LB medium and grown at 26°C for 3 h. Afterwards, cells were collected by centrifugation (12,000 × *g*, room temperature, 3 min) and resuspended in lysis buffer (2% deoxycholate, 4% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue in 1 M Tris-HCl buffer, pH 6.8) in a volume adjusted according to the optical density at 600 nm of the cultures. Subsequently, the lysates were boiled for 10 min and subjected to 40 μg proteinase K for 3 h at 55°C. Samples were stored at −20°C until needed. LPS samples were analyzed by sodium deoxycholate-PAGE and silver staining as described previously (35, 53).

RESULTS

Overproduction of Dam alters motility of *Y. enterocolitica* by increasing expression of *flhDC*. In a previous study, we showed that overproduction of the Dam enzyme in *Y. enterocolitica* leads to an increased ability to invade cultured epithelial cells independently of the pYV-encoded YadA protein (22). Invasion phenotypes are often related to functions of the flagellum and to motility, for example, in *S. enterica* or *Listeria monocytogenes* (17, 40). Interestingly, motility and the ability to invade eukaryotic cells are affected by Dam methylation in *S. enterica* (4, 5). Several studies with *Y. enterocolitica* indicate that expression of motility and the *inv* gene encoding Inv, the primary invasion factor in cell culture models, are coordinately regulated. Strains could be isolated which show a significant decrease in *inv* expression but are hypermotile when grown at 26°C. Furthermore, a *clpB* mutant of *Y. enterocolitica* is deficient in Inv production and in motility (2, 3).

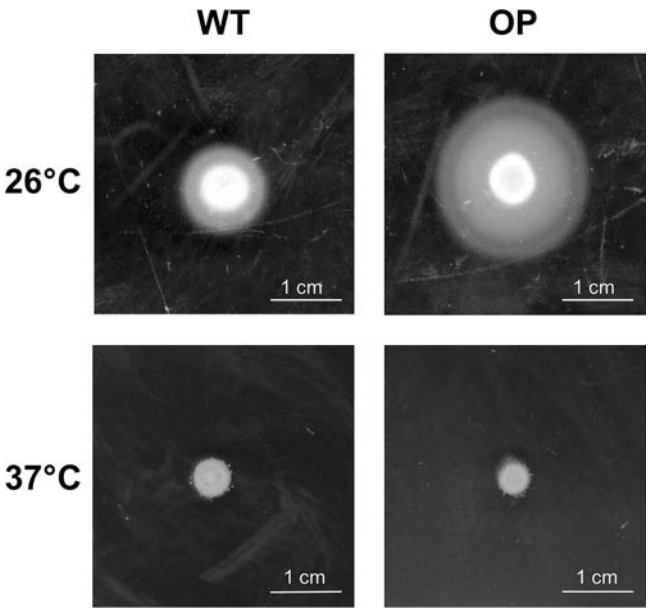

FIG. 1. Motility is increased after overproduction of Dam at 26°C. A *Y. enterocolitica* Dam^{OP} strain (OP; GHY150) and a control strain (WT; GHY147) were spotted on motility agar plates and incubated at 26°C or 37°C for 20 h. The agar plates shown are representative of at least three independent experiments.

Due to these observations, we analyzed whether overproduction of Dam might also influence the motility of *Y. enterocolitica.* Therefore, we spotted a Dam^{OP} strain and a control strain on soft agar plates and incubated the plates at 26°C or 37°C. As shown in Fig. 1, the Dam^{OP} strain is indeed hypermotile at 26°C compared to the control strain (diameter of the halo, 2.3 ± 0.17 cm, compared to 1.4 ± 0.06 cm). Motility is temperature regulated in *Y. enterocolitica*, with bacteria being motile at 26°C but not at 37°C. Both the Dam^{OP} strain and the control strain are nonmotile at 37°C, indicating that overproduction of Dam does not induce the expression of motility under nonpermissive conditions but alters motility under permissive conditions.

Motility is regulated by a complex cascade, which is well characterized for *Enterobacteriaceae*, including *Yersinia* species (12, 47, 58). Interestingly, the promoter region of the *flhDC* operon, encoding the master regulator of flagellum biosynthesis, contains a nonmethylated GATC site in *E. coli* (54), indicating a putative role for Dam in the regulation of *flhDC* expression. Therefore, we speculated on whether overproduction of Dam might induce hypermotility in *Y. enterocolitica* via an upregulation of *flhDC* transcription. To analyze this, a *lacZ* transcriptional fusion to *flhDC* was constructed and the resulting β-galactosidase activity was monitored. After incubation for 20 h at 26°C, a mean *flhDC* transcription of 3,925 ± 430

TABLE 3. Relative gene expression after overproduction of Dam

Gene, temp (°C)	Rel. expression[a]	*P* value[b]	Fold change[c]
inv, 26	0.181 ± 0.0194	0.0008*	↓ 5.52
inv, 37	0.573 ± 0.2061	0.023*	↓ 1.75
ail, 26	0.729 ± 0.0653	0.002*	↓ 1.37
ail, 37	0.875 ± 0.3599	0.51	↓ 1.14
pilL, 26	0.363 ± 0.2505	0.014*	↓ 2.75
pilL, 37	0.902 ± 0.2339	0.519	↓ 1.11
myfA, 26	0.902 ± 0.1761	0.393	↓ 1.11
myfA, 37	0.922 ± 0.5869	0.83	↓ 1.08
flp-1, 26	0.184 ± 0.1070	0.0001*	↓ 5.43
flp-1, 37	1.080 ± 0.8303	0.811	↑ 1.08
ddhA, 26	0.680 ± 0.5963	0.32	↓ 1.47
ddhA, 37	1.325 ± 0.2514	0.088	↑ 1.33
gne, 26	0.599 ± 0.5557	0.2	↓ 1.67
gne, 37	1.205 ± 0.3425	0.36	↑ 1.21
rosA, 26	0.567 ± 0.5211	0.15	↓ 1.76
rosA, 37	1.120 ± 0.2364	0.43	↑ 1.12

[a] Relative expression. Relative mRNA amounts were determined by qRT-PCR and related to mRNA levels in control cells without overproduction of Dam, set as 1. Values are means ± standard deviations.

[b] Significance of difference ($P < 0.05$) from level in control cells without overproduction of Dam was calculated by Student's *t* test and is indicated by an asterisk.

[c] *n*-fold change (↓, downregulation; ↑, upregulation) compared to relative expression in control cells without overproduction of Dam.

Miller units was obtained for the Dam^{OP} strain, showing a 1.5-fold upregulation of *flhDC* transcription in comparison to results for the wild-type control strain (2,685 ± 55 Miller units; $P = 0.0013$). We and others have described similar modest effects on gene expression after overproduction of Dam for most Dam-regulated genes (21, 41). Furthermore, modest differences in *flhDC* expression can have a strong effect due to the multiplying effects of the downstream regulatory cascade. These results might indicate that the hypermotility after overproduction of Dam in *Y. enterocolitica* is mediated via an upregulation of the *flhDC* operon. We identified five GATC sequences in the 5′ untranslated region of the *flhD* gene, at 63 nucleotides (nt), 190 nt, 448 nt, 476 nt, and 693 nt (numbering is with respect to the *flhD* start codon), respectively, as potential targets of the Dam enzyme. However, we were not able to detect any differences in the methylation pattern of the *flhDC* upstream regulatory region by an adapted Southern blot analysis described previously (21). Our data indicate that all GATC sites present within this region are at least hemimethylated and that there is no switch from nonmethylated to fully methylated DNA after overproduction of Dam (data not shown). Nevertheless, a switch between hemimethylated and fully methylated DNA cannot be detected with the method used and remains a possibility; a similar mechanism was proposed for the Dam-dependent regulation of the flagellar gene *fliC* in *S. enterica* (5).

Overproduction of Dam reduces *inv* transcription and the steady-state level of Inv. In a previous study, Young et al. showed that overexpression of *flhDC* from a plasmid in *Y. enterocolitica* resulted in an approximately twofold increase in *inv* transcription but in decreased amounts of Inv, suggesting transcriptional as well as posttranscriptional levels of regulation that are influenced directly or indirectly by FlhDC (57). Therefore, we assumed that the altered *flhDC* expression after overproduction of Dam might be responsible for the previously described increased invasion of epithelial cells by the Dam^{OP} strain by altering Inv levels (22). To address this, we incubated the Dam^{OP} strain and the control strain for 4 h at 26°C or 37°C and subsequently used qRT-PCR analysis to monitor *inv* transcription. Surprisingly, as shown in Table 3, we found that *inv* transcription is not upregulated but is downregulated 1.7-fold at 37°C and even 5.5-fold at 26°C after overproduction of Dam ($P = 0.023$ and 0.0008, respectively). To determine if the Dam-induced downregulation of *inv* transcription leads to a reduced protein level or if posttranscriptional levels of regulation, as proposed by Young et al. (57), might be involved in mediating the observed invasion phenotype, we analyzed whole-cell lysates of cells grown at 26°C or 37°C by immunoblotting with antibodies directed against the Inv protein. In accordance with the distinct downregulation of *inv* transcription at 26°C, the steady-state level of Inv was strongly reduced after overproduction of Dam at this temperature. After incubation at 37°C, the reduction of Inv amounts after overproduction of Dam was less obvious; however, the Inv level did not increase (Fig. 2). These data indicate that overproduction of Dam negatively affected expression of *inv*, further confirming the coordinated expression of motility and invasion properties in *Y. enterocolitica*. Nevertheless, despite the lowered expression of Inv, the Dam^{OP} bacteria had an altered ability to invade epithelial cells, suggesting that overproduction of Dam influences other functions associated with invasion in *Y. enterocolitica*, thereby compensating for reduced *inv* expression.

Overproduction of Dam decreases the transcription of known and putative invasion factors. The reduced steady-state levels of Inv after overproduction of Dam raised the question of which factor might be responsible for the observed hyperinvasive phenotype. Since invasion was analyzed with *Y. enterocolitica* strains cured of the pYV virulence plasmid, we could exclude a role for the plasmid-encoded invasion factor YadA (15, 22). A third *Y. enterocolitica* invasion factor is encoded by the *ail* gene (38). Although the ability of Ail to promote invasion is relatively modest, we could not exclude that Ail might play a more important role for the invasion phenotype under Dam^{OP} conditions. To address this question, we analyzed the putative influence of overproduction of Dam on *ail* transcrip-

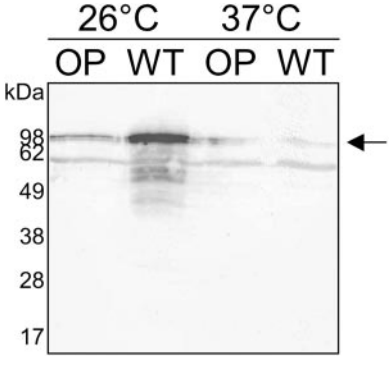

FIG. 2. Steady-state levels of Inv are decreased at 26°C after overproduction of Dam. Whole-cell lysates of a *Y. enterocolitica* Dam^{OP} strain (OP; GHY150) and a control strain (WT; GHY147) grown at 26°C or 37°C for 4 h were transferred to nitrocellulose and subjected to Western blot analysis using a polyclonal anti-Inv antiserum. An arrow indicates the expected size of full-length Inv.

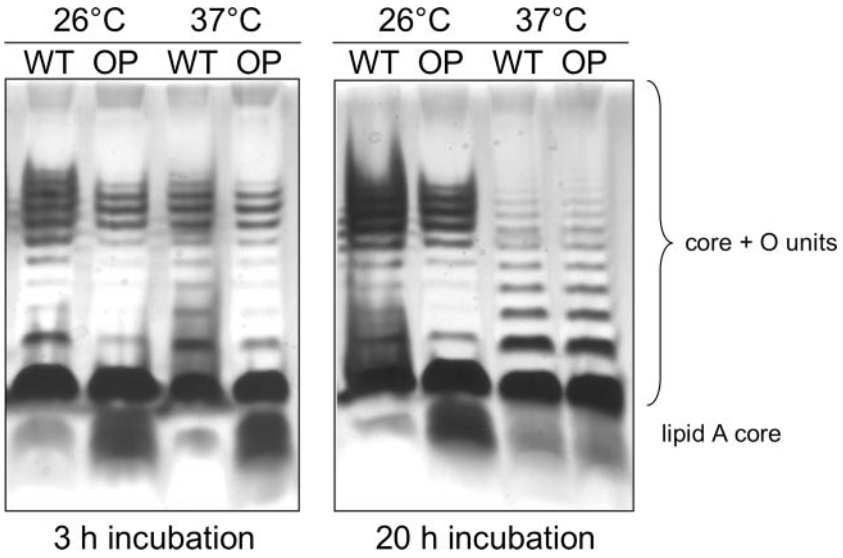

FIG. 3. The amount of lipid A core without conjugated O-antigen units is increased after overproduction of Dam. A Dam^{OP} strain (OP; GHY150) and a control strain (WT; GHY147) of *Y. enterocolitica* were grown at 26°C or 37°C for 3 h or 20 h. LPS was isolated by proteinase K treatment and analyzed by sodium deoxycholate-PAGE and silver staining.

tion. To this end, we incubated the Dam^{OP} strain and a control strain at 26°C or 37°C and subsequently monitored *ail* transcription by qRT-PCR. As shown in Table 3, *ail* transcription is slightly downregulated at 26°C (1.37-fold; $P = 0.002$) but not at 37°C. Although this effect is moderate, the data indicate that the hyperinvasive phenotype caused by overproduction of Dam is at least not due to increased *ail* expression.

The downregulation of *ail* and *inv* transcription after overproduction of Dam suggested that further factors involved in invasion might be present in *Y. enterocolitica*. Pili and fimbriae often mediate binding to eukaryotic cells. Therefore, we analyzed if genes coding for Myf fibrils, a surface appendage homologous to the pH 6 antigen of *Y. pseudotuberculosis* (28, 29), are differentially transcribed after overproduction of Dam. Our qRT-PCR analyses show that overproduction of Dam does not influence *myfA* transcription, indicating that the Myf fibril is not involved in the hyperinvasive phenotype of Dam^{OP} *Y. enterocolitica* strains (Table 3).

Other operons encoding putative type IV pili have been identified in the *Y. enterocolitica* genome, which are encoded by the $YAPI_{YE}$ and YGI-1 pathogenicity islands (13, 14, 49, 50). To investigate the putative role of the $YAPI_{YE}$ (*pil*)-encoded and YGI-1 (*flp*/*tad*)-encoded pili in invasion/adherence of *Y. enterocolitica* after overproduction of Dam, we monitored the transcription of the *pil* and *tad* operons. To this end, we incubated the Dam^{OP} strain and the control strain of *Y. enterocolitica* as described above and performed qRT-PCR analyses with primers directed against the first genes of the operons, *pilL* and *flp-1*, respectively. Our results indicate that at 26°C the transcription of *pilL* and that of *flp-1* are markedly downregulated after overproduction of Dam (*pilL*, 2.75-fold; $P = 0.014$; *flp-1*, 5.43-fold; $P < 0.0001$) (Table 3). At 37°C, *pilL* transcription is not significantly influenced by overproduction of Dam ($P = 0.519$). This also holds true for the mean transcription of *flp-1* at 37°C ($P = 0.811$), although we obtained extremely variable results when comparing single experiments, which is mirrored in the considerable standard deviation (Table 3). Nevertheless, since the hyperinvasive phenotype of Dam^{OP} strains is highly reproducible and since we were not able to visualize pilus structures under these conditions on the bacterial surface by electron microscopy (data not shown), putative pili do not seem to be involved in the increased ability to invade tissue culture cells after overproduction of Dam.

Taken together, by transcriptional analyses we were not able to identify an adherence/invasion factor that is responsible for the hyperinvasive phenotype of Dam^{OP} *Y. enterocolitica* strains. In contrast, overproduction of Dam in *Y. enterocolitica* results in downregulation of a relatively broad spectrum of genes encoding known and putative surface proteins conferring adhesion to and/or invasion of host cells.

Overproduction of Dam modulates LPS O-antigen status. The observation that the transcription of invasion/adhesion factors is decreased or not changed and also that the amount of Inv protein is decreased in a Dam^{OP} strain but that at the same time the strain shows a hyperinvasive phenotype suggested that there might be a change in the surface structure of the Dam^{OP} strain increasing the accessibility of an invasion factor. Pierson showed that a mutation affecting the LPS profile of *Y. enterocolitica* increased the Ail-mediated entry into mammalian cells 20-fold, although the expression of *ail* was not changed (45). Furthermore, defects within the LPS O-antigen status lead to an increased motility and reduced transcription of the *inv* gene (7). Due to these observations, we wondered if the invasion and motility phenotype of a Dam^{OP} strain of *Y. enterocolitica* is caused or at least influenced by a modulation of the LPS structure. To analyze this in more detail, we incubated the Dam^{OP} strain and the control strain of *Y. enterocolitica* at 26°C or 37°C for 3 h and subsequently analyzed the LPS. As demonstrated in Fig. 3, overproduction of Dam indeed modulates the LPS O-antigen status. At 26°C and at 37°C, we detected increased amounts of lipid A core without O-antigen units after overproduction of Dam, whereas the

proportion of core units with small O-antigen units was decreased. Interestingly, the amount of molecules with the preferred O-antigen chain length of about 7 to 10 repeats was not considerably changed after overproduction of Dam, putatively indicating that Dam methylation affects the stability of shorter LPS species or influences the addition of the first O-antigen units to the growing chain. However, in accordance with the study by Pierson (45), the increased occurrence of shorter LPS species after overproduction of Dam is a plausible explanation for an altered accessibility of an invasion factor on the bacterial surface; this might contribute to the hyperinvasive phenotype caused by overproduction of Dam despite the reduced expression of invasion and adhesion factors analyzed here.

Expression of LPS is temperature regulated in *Y. enterocolitica*; at 26°C, transcription of the O-antigen gene cluster is induced, while it is repressed at 37°C (8). Therefore, we repeated LPS isolation after 20 h of growth at 26°C and 37°C, since the effect of the growth temperature on the expression of LPS becomes most obvious in stationary-phase cultures. As can be seen in Fig. 3, the change in LPS structure after overproduction of Dam is still visible in overnight cultures grown at 26°C, but not at 37°C, when expression of the O-antigen cluster is repressed, indicating that overproduction of Dam does not interfere with the temperature regulation of LPS expression but modulates expression at the permissive temperature.

Overproduction of Dam does not influence transcription of LPS O-antigen genes. Since overproduction of Dam modulates the LPS O-antigen status, we wondered if transcription of the corresponding genes might be directly or indirectly affected by DNA methylation. The O-antigen gene cluster consists of two transcriptional units controlled by the promoters P_{wb1} and P_{wb2}, respectively, with transcription of the latter being indirectly modulated by the RosAB system (8). We performed qRT-PCR to analyze the transcription of *ddhA*, *gne*, and *rosA*, representing the first genes in the respective operons, in a strain overproducing Dam for 3 h and a control strain at 26°C and 37°C. As shown in Table 3, there was no significant change in the transcription of these genes in response to overproduction of Dam, indicating that the modulation of the LPS structure of Dam^{OP} *Y. enterocolitica* is mediated posttranscriptionally.

Inv is necessary for increased invasion after overproduction of Dam. The qRT-PCR data revealed effects of Dam on the transcription of genes associated with adhesion and invasion, but the factor responsible for the observed hyperinvasive phenotype after overproduction of Dam remained elusive. We anticipated that if we mutated the factor necessary for increased invasion, the effect of overproduction of Dam would no longer be detectable. Since Inv is the major invasion factor of *Y. enterocolitica*, we constructed a mutant strain missing a functional Inv protein. Subsequently, CHO-K1 cells were infected with strain GHY287 (*inv*::pEP-inv) overproducing Dam from pTP166Kan or carrying the control plasmid pTP166KandamΔ. The Dam^{OP} strain and the control strain did not differ in their abilities to invade eukaryotic cells [GHY287 (pTP166Kan), 3.8% ± 1.99% invasion; GHY287 (pTP166KandamΔ), 4.01% ± 1.93% invasion]. These data show that an intact Inv protein is necessary for increased invasion after overproduction of Dam, indicating that Inv and not another adhesion/invasion factor is involved in the observed increased invasion phenotype after overproduction of Dam. These data further indicate that indeed the steric accessibility of Inv due to a changed LPS structure is involved in the increased invasion of a Dam^{OP} strain.

DISCUSSION

In previous studies, we were able to demonstrate that overproduction of the Dam enzyme in *Y. enterocolitica* affects the regulation of several virulence properties, such as type III secretion or invasion (21, 22). Since invasion processes are often coordinately regulated with other virulence functions, this study was conducted to analyze Dam's influence on further virulence-associated genes and to identify molecular mechanisms underlying the hyperinvasive phenotype caused by overproduction of Dam. Indeed, we could demonstrate that motility is increased after overproduction of Dam. This effect is mediated by an upregulation of *flhDC* encoding the master regulator of the flagellar biosynthesis cascade (58). However, we were not able to detect a direct switch from nonmethylated to methylated GATC sequences within the upstream regulatory region of *flhDC* after overproduction of Dam in *Y. enterocolitica*. In *E. coli*, a nonmethylated GATC sequence is present in the noncoding region of the *flhDC* operon (54). However, in a global screen for Dam-regulated genes of *E. coli*, *flhDC* could not be identified (41). Interestingly, in *S. enterica* the expression of *fliC* encoding a flagellar structural protein is influenced by DNA hemimethylation (5). Regulation by hemimethylation remains a possibility for the increased *flhDC* transcription after overproduction of Dam in *Y. enterocolitica* as well, since hemimethylation and methylation of both strands cannot be distinguished by the assay used. The hypothesis of direct regulation by Dam is further supported by the fact that the transcription of *flhDC* is regulated by H-NS and DNA structure, which is strongly affected by the methylation status (47, 56). In this context, it is interesting to note that Pérez-Gutiérrez and colleagues describe a relationship between the absence of LPS in rough mutants and H-NS expression, which underlies *flhDC* upregulation and altered secretion by the pYV-encoded Ysc type III secretion system (44). Since we previously described an effect of overproduction of Dam on type III secretion in *Y. enterocolitica* (21), the expression of LPS, motility, invasion, and type III secretion is obviously under the coordinated control of a complex regulatory network which can be modulated by Dam. This network might also include posttranscriptional effects via other factors. The ATP-dependent protease ClpP might be involved in this process, since the turnover of FlhDC is influenced by ClpXP in *S. enterica* and since *clpP* transcription is increased after overproduction of Dam in *Y. enterocolitica*, at least during incubation at 37°C (21, 51, 52). The detailed mechanism underlying the Dam-dependent increased motility in *Y. enterocolitica* remains to be elucidated.

Since it has been reported that motility is required to initiate host cell invasion in *Y. enterocolitica*, it might be concluded that motility is the critical factor inducing the hyperinvasive phenotype after overproduction of Dam (57). Since this is true only for experiments in which contact between the bacteria and the host cells is not induced by centrifugation and since we determined the invasion frequencies of the Dam^{OP} strain and the control strain after inducing contact, increased motility

after overproduction of Dam is not exclusively responsible for the hyperinvasive phenotype. Moreover, in our study we present evidence that increased *flhDC* transcription after overproduction of Dam goes along with a decreased *inv* transcription as well as a reduced (or at 37°C at least an unchanged) steady-state level of Inv, although it was reported that overexpression of *flhDC* from a plasmid results in increased *inv* transcription (57). A possible explanation for this discrepancy might be the fact that Dam has pleiotropic effects within a cell, representing a situation clearly different from overproduction of just one specific protein. Interestingly, a rough mutant of *Y. enterocolitica* is hypermotile and downregulates *inv* expression, further supporting a close regulatory connection of LPS expression, motility, and invasion (7). Interestingly, however, in contrast to the case with the Dam^{OP} strain, invasion is reduced in the rough mutant. This discrepancy might be explained by the difference between rough mutants without LPS on the one hand and a modified LPS structure after overproduction of Dam on the other. Alternatively, further effects of Dam on surface structures influencing invasion efficiency cannot be excluded. The invasion assay using an *inv* mutant strain overproducing Dam clearly indicates that Inv is necessary for the Dam-mediated hyperinvasive phenotype. This is surprising, since transcription of *inv* and also steady-state levels of Inv are decreased after overproduction of Dam. The data suggest an altered steric accessibility of the remaining Inv protein on the bacterial surface. This hypothesis is supported by the observation that mutations affecting LPS increased the Ail-mediated entry of *Y. enterocolitica* into host cells, although *ail* expression was not affected (45). Therefore, we postulated that a decreased amount of proteins promoting internalization might be accompanied by a changed LPS O-antigen status. Indeed, we could demonstrate that overproduction of Dam leads to an increase in the amount of rough LPS molecules lacking O-antigen side chains. Although the amount of core units with 7 to 10 repeats is not considerably changed, our data show that the population of LPS molecules is shifted to shorter species after overproduction of Dam. This indicates that overproduction of Dam results in an altered accessibility of Inv, with this being at least in part responsible for the Dam-induced hyperinvasive phenotype.

The amount of lipid A is strongly increased in Dam^{OP} *Y. enterocolitica*. Since lipid A is an important signal for the induction of the innate immune system in the host via Toll-like receptor 4, this might have implications for the course of an infection (37). However, since overproduction of Dam results in increased invasion of the wild-type strain but not the *inv* mutant strain, we can exclude the possibility that lipid A induces increased endocytosis in the invasion assay.

During infection of the host, *Y. enterocolitica* is facing various biotic surfaces composed of different cell types. For the various stages of infection, different factors might be predominant for promoting adhesion or internalization. This is supported by the fact that YadA, Inv, Ail, and MyfA are maximally expressed under conditions distinctly differing from each other in temperature, pH, or growth phase (18, 19, 28, 43). Furthermore, depending on the surface of the host cell, a competition between YadA and Inv for activation of different phagocytic responses was proposed for *Y. pseudotuberculosis* (27). Due to these observations, it can be speculated that overproduction of Dam does not directly influence the expression of an individual invasin/adhesin but influences a global program regulating the expression of diverse surface proteins with the outcome of adaptation to a specific environment during infection. Since it was reported that the LPS O-antigen status is involved in the regulation of the expression of virulence factors such as *inv* and *flhDC* (7), the LPS structure might well be a critical factor influenced by Dam and causing downstream effects, such as increased motility or reduced transcription of genes. Which additional factors might be involved in this putative global regulation network remains to be elucidated. An interesting player in this context might be ClpP, since ClpP modulates the expression of *ail* and type III secretion and since the transcription of *clpP* increases after overproduction of Dam (21, 43).

In conclusion, overproduction of Dam clearly leads to a new organization of the bacterial surface concerning proteins and appendages and of the LPS structure. DNA methylation by the Dam enzyme seems to contribute to an adaptation of the bacterial surface to specific stages during an infection, an idea that is also underlined by various reports of different pathogens with defects in virulence due to defects in DNA methylation (26).

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