Phenotypic characterization of a virulence-associated protein, VagH, of *Yersinia pseudotuberculosis* reveals a tight link between VagH and the type III secretion system

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Recently, a number of attenuated mutants of *Yersinia pseudotuberculosis* have been identified using a bioinformatics approach. One of the target genes identified in that study was *vagH*, which the authors now characterized further. VagH shows homology to HemK of *Escherichia coli*, possessing methyltransferase activity similar to that of HemK, and targeting release factors 1 and 2. Microarray studies comparing the wild-type and the *vagH* mutant revealed that the mRNA levels of only a few genes were altered in the mutant. By proteome analysis, expression of the virulence determinant YopD was found to be increased, indicating a possible connection between VagH and the virulence plasmid-encoded type III secretion system (T3SS). Further analysis showed that Yop expression and secretion were repressed in a *vagH* mutant. This phenotype could be suppressed by *trans*-complementation with the wild-type *vagH* gene or by deletion of the negative regulator *yopD*. Also, in a similar manner to a T3SS-negative mutant, the avirulent *vagH* mutant was rapidly cleared from Peyer's patches and could not reach the spleen after oral infection of mice. In a manner analogous to that of T3SS mutants, the *vagH* mutant could not block phagocytosis by macrophages. However, a *vagH* mutant showed no defects in the T3SS-independent ability to proliferate intracellularly and replicated to levels similar to those of the wild-type in macrophages. In conclusion, the *vagH* mutant exhibits a virulence phenotype similar to that of a T3SS-negative mutant, indicating a tight link between VagH and type III secretion in *Y. pseudotuberculosis*.

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INTRODUCTION

Yersinia pseudotuberculosis is a pathogen that causes gastrointestinal disease in humans and animals. Essential for causing disease is a type III secretion system (T3SS) (Gemski *et al.*, 1980) encoded on a 70 kb virulence

plasmid. The T3SS is utilized for translocation of toxins (Yops) into eukaryotic target cells; the toxins promote actin cytoskeleton rearrangements and inhibit phagocytosis (reviewed by Viboud & Bliska, 2005; Cornelis, 2002). The chromosome also contains genes important for virulence; among these is invasins, which by itself can mediate

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Abbreviations: Cy2, 3-(4-carboxymethyl)phenylmethyl-3'-ethyloxycarbocyanine halide *N*-hydroxysuccinimidyl ester; Cy3, 1-(5-carboxypentyl)-1'-propylindocarbocyanine halide *N*-hydroxysuccinimidyl ester; Cy5, 1-(5-carboxypentyl)-1'-methylindodicarbocyanine halide *N*-hydroxysuccinimidyl ester; MTase, *N*⁶-methyltransferase; RF, release factor; SAM, S-adenosyl-L-methionine; T3SS, type III secretion system.

The array data discussed in this publication have been deposited in BμG@Sbase (accession no. E-BUGS-48; <http://bugs.sgul.ac.uk/E-BUGS-48>) and also ArrayExpress (accession no. E-BUGS-48). The array design is available in BμG@Sbase (accession no. A-BUGS-11; <http://bugs.sgul.ac.uk/A-BUGS-11>) and also ArrayExpress (accession no. A-BUGS-11).

bacterial uptake into cultivated cells (Isberg *et al.*, 1987) and is important for invasion in the mouse model of infection. Other virulence loci on the chromosome are type IV pili (Collyn *et al.*, 2002), *ail* and the *psa* locus responsible for the production of pH6 antigen (Yang *et al.*, 1996).

We wanted to explore the possibility of identifying other virulence-associated genes (*vags*) on the chromosome, and to do so in a previous study we took a bioinformatics approach to identify such genes (Garbom *et al.*, 2004). In that study, we aimed at identifying genes with unknown or hypothetical functions that were conserved among pathogenic bacteria, with the goal of evaluating these genes as drug targets for novel antimicrobial compounds. The strategy was to target genes important only for *in vivo* survival and pathogenesis, and not essential for growth outside the host. One of the genes identified in that analysis was *vagH*; the *vagH* gene is homologous to *hemK* in *Escherichia coli*. The *hemK* gene encodes HemK, which has been shown to be an N^5 -methyltransferase (MTase), transferring one methyl group from S-adenosyl-L-methionine (SAM) to the target proteins release factor (RF)1 and RF2 (Heurgue-Hamard *et al.*, 2002; Nakahigashi *et al.*, 2002). Subsequently, several groups have implied that *in vivo* conditions induce the expression of *vagH* homologues in other organisms. John *et al.* (2005) have found the *E. coli* O157:H7 homologue of *vagH* to be induced during infection, and the homologue in *Porphyromonas gingivalis*, one of the causative agents of adult periodontitis, has also been found to be induced in epithelial cell cultures by Park and co-workers (Park *et al.*, 2004).

One important step in evaluating the gene product of *vagH* as a potential anti-bacterial drug target is to understand the function that the protein has in our model organism *Y. pseudotuberculosis* and to comprehend the basis of the virulence attenuation of the *vagH* mutant. The target proteins RF1 and RF2 of the MTase activity of HemK are involved in the process of translation. Translation termination occurs when a translating ribosome reaches a stop codon on the mRNA and requires, among other things, class I RFs (Buckingham *et al.*, 1997). Two class I protein RFs are present in all bacteria sequenced so far, RF1 and RF2, whilst eukaryotes only possess one release factor, eRF1. However, in *Saccharomyces cerevisiae* two release factors have been identified, one mitochondrial and one cytoplasmic (Polevoda *et al.*, 2006). In bacteria, RF1 recognizes stop codons UAG and UAA, while RF2 also recognizes UAA and in addition UGA (Scolnick *et al.*, 1968). An invariant motif (GGQ) in release factors from all kingdoms has been identified (Frolova *et al.*, 1999), and it was later found that the glutamine in this motif is post-translationally modified to an N^5 -methylglutamine (Dincbas-Renqvist *et al.*, 2000). HemK has been identified as the enzyme that transfers a methyl group from SAM to RF1 and RF2 (Heurgue-Hamard *et al.*, 2002; Nakahigashi *et al.*, 2002). Deletion of *hemK* in *E. coli* K-12 confers a severe growth defect (Heurgue-Hamard *et al.*, 2002; Nakahigashi *et al.*, 2002). This can be partially restored by an additional mutation in one of the target proteins, RF2, in which the

tyrosine in position 246 is replaced by an alanine or a serine (Nakahigashi *et al.*, 2002). The tyrosine in position 246 renders the RF2 of K-12 more dependent on the methyl transfer for efficient translational termination (Uno *et al.*, 1996). Most other bacteria, including *Y. pseudotuberculosis*, encode an RF2 with an alanine or serine in position 246, probably making these bacteria less dependent on the methyl transfer for efficient termination. This was confirmed in our previous study, in which the growth of the mutant was analysed and found to be similar to that of the wild-type (Garbom *et al.*, 2004). Here, we further analyse the role of the *vagH* gene product in virulence and investigate the MTase activity of VagH. We show that VagH possesses an MTase activity similar to that of HemK, and we perform a phenotypic characterization of the mutant. We establish that the attenuation of the *vagH* mutant is likely due to its effects on the T3SS.

METHODS

Strains, growth media and DNA methods. The *Y. pseudotuberculosis* strain IP32953 used in this study was obtained from E. Carniel, Pasteur Institute, France. This strain was grown on plates containing 10 µg nalidixacin ml⁻¹ in order to select for nalidixacin-resistant clones. One resistant clone (IP32953Nal) was used in all of the experiments and is referred to as wild-type. The *vagH* mutant used in this study had a deletion of amino acids 16–261 in either IP32953Nal (Garbom *et al.*, 2004) or YPIII. The *vagH yopD* double mutant was pIB621 (*yopD*) (Francis & Wolf-Watz, 1998) with an additional deletion of *vagH* created in the same way as described in our previous report for the YPIII strain (Garbom *et al.*, 2004). *E. coli* strains were grown in Luria Broth (LB) or on Luria agar plates. *Yersinia* strains were grown in LB, brain heart infusion (BHI; Oxoid) broth (BHI was supplemented with 5 mM EGTA and 20 mM MgCl₂ to remove calcium) or TMH, a defined rich medium. Antibiotics were used at the following concentrations: nalidixacin, 10 µg ml⁻¹; carbenicillin, 100 µg ml⁻¹; kanamycin, 50 µg ml⁻¹. Preparation of plasmid DNA, restriction enzyme digests, and ligations and transformations into *E. coli*, were performed essentially as described by Sambrook *et al.* (1989). DNA fragments were purified from agarose gels using Ultrafree-DNA (Amicon), according to the manufacturer's instructions.

Enzyme purification. Overnight cultures of *E. coli* DH5α transformed with either pBAD:*vagH::his₆* or pAR:*hemK* (produces His-tagged HemK) were transferred to fresh LB with appropriate antibiotics, and grown at 37 °C to OD₆₀₀ ~0.6. Protein synthesis was induced by adding arabinose (0.2 %), followed by incubation at 37 °C for an additional 4 h. The bacteria were washed twice with sonication buffer (50 mM Na₂PO₄, 300 mM NaCl, 10 mM imidazole). The pellet was resuspended in 10 ml cold sonication buffer supplemented with 0.2 mM PMSF. After sonication, the lysate was centrifuged at 10 000 g for 20 min at 4 °C. The resulting supernatant was passed over a nickel-nitriloacetic acid (Ni-NTA) column (HisTrap, GE Healthcare), the column was washed, and the purified enzymes were eluted with elution buffer (500 mM imidazole in binding buffer). The purified enzymes were then dialysed to the assay buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EDTA, 20 %, v/v, glycerol). The purity of the enzymes was thereafter assessed by SDS-PAGE and Coomassie staining. The protein concentration was determined with Bradford reagent (Sigma).

Preparation of lysates used in MTase assay. Bacteria were cultivated at 26 °C with appropriate antibiotics overnight. The next day the bacteria were diluted 1:20 in fresh LB and grown at 37 °C to OD₆₀₀ 0.8; after this, the bacteria were washed twice in 20 mM Tris,

pH 7.4, and sonicated, and the lysate was thereafter centrifuged to remove cell debris. The protein concentration in the resulting supernatant was determined with Bradford reagent.

Methyltransferase assay. We adopted the methyltransferase assay of Colson *et al.* (1977). Ten micrograms protein substrate lysate, 1 µg VagH or HemK, and 2 µM SAM were mixed to a final volume of 20 µl. At each time point, 3 µl reaction mixture was pipetted onto dry 3MM filter paper (Whatman) soaked in 10% TCA to stop the reaction. The paper was washed twice with 10% TCA for 20 min and twice with 95% ethanol for 10 min. The incorporation of tritium was thereafter quantified by scintillation counting in a liquid scintillation counter (Wallac 1409).

autoradiography. To assess the number of proteins methylated by VagH/HemK, the samples were prepared as described above and 2 × SDS sample buffer was added to stop reactions. The samples were separated by SDS-PAGE and transferred to a nitrocellulose filter by electroblotting. The filter was exposed to a tritium-enhancing screen (GE Healthcare) and thereafter scanned in a Storm 860 scanner (GE Healthcare).

To achieve separation and clear spots in two dimensions, the supernatant from the sonicated bacteria was passed over an ion-exchange column (GE Healthcare) to remove impurities. The eluted fractions were tested together with VagH and ³H-SAM to assess in which fractions the incorporation was present. These fractions were pooled and the methyltransferase assay was performed as above. After this, the sample was dialysed overnight against a buffer of 20 mM Tris, pH 7.4, 8 M urea and thereafter 1% CHAPS was added. First-dimension IEF was performed on immobilized pH gradient strips (11 cm, pH 4.0–7.0, linear) in an Ettan IPGphor system (GE Healthcare). Second-dimension separation was performed on 12% SDS-PAGE gels. The gel was thereafter treated in the same way as the 1D gel described above. For identification of proteins by MS, a preparative gel with unmethylated protein lysate was run. This gel was Coomassie-stained for visualization of proteins.

Mouse infection. In all mouse-infection studies, C57BL/6J female mice of similar age and weight were used (5–7 weeks, weighing ~15 g). Bacteria were grown overnight in 100 ml LB broth cultures at 26 °C with aeration. These cultures were collected and resuspended in 50 ml sterilized tap water. Mice were deprived of water overnight (18 h) and then given contaminated water containing different bacterial dilutions (5×10^9 , 5×10^8 and 5×10^7 c.f.u. ml⁻¹). Each cage contained three mice which were allowed to drink *ad libitum* the bacteria-containing water for 8 h. Each mouse consumed approximately 5 ml bacterial suspension. In total, nine mice (three mice for each concentration of bacteria) per bacterial strain were used in each experiment. The concentration of the bacteria fed to the mice was measured by viable counting. The animal infection study was approved by the Swedish National Board for Laboratory animals (CFN).

For analysis of the colonization of different organs with *Yersinia*, mice were deprived of water overnight (18 h) and then given the contaminated water containing 5×10^9 c.f.u. ml⁻¹ of the different strains (wild-type, *vagH* mutant and virulence-plasmid cured), as described above. At each time point, two mice per strain were killed, the spleen and Peyer's patches were dissected, and bacterial numbers in each organ were determined by growth on plates containing nalidixic acid. The animal infection study was approved by the CFN.

For determination of ID₅₀ by the intraperitoneal route of infection, overnight cultures of *Yersinia* grown at 26 °C were diluted in PBS in 10-fold serial steps. Mice were injected intraperitoneally with bacterial concentrations of 10^3 , 10^5 and 10^7 c.f.u. ID₅₀ was calculated according to Reed & Muench (1938).

Microarray analysis. The preparation of RNA and microarray analysis were performed in essence as described by Robinson *et al.*

(2005) and Taylor *et al.* (2005). *Y. pseudotuberculosis* IP32953 or *vagH* mutant were cultured in LB broth to OD₆₀₀ 0.5 (LKB Ultrospec plus). An aliquot of 2 ml was placed into RNA Protect (Qiagen) and RNA was extracted using the RNeasy kit (Qiagen).

Y. pseudotuberculosis IP32953 RNA was used as the control and labelled with 1-(5-carboxypentyl)-1'-propylindocarbocyanine halide *N*-hydroxysuccinimidyl ester (Cy3)-dCTP. The *vagH* mutant strain was treated as the test sample and was labelled with 1-(5-carboxypentyl)-1'-methylindocarbocyanine halide *N*-hydroxysuccinimidyl ester (Cy5)-dCTP (Amersham Biosciences). Labelling of 2–3 µg of denatured RNA was performed using SuperScript II (Invitrogen) in the presence of 5 mM dATP, 5 mM dGTP, 5 mM dTTP, 2 mM dCTP and 750 pM Cy5-dCTP or Cy3-dCTP. After incubation at 25 °C in the dark for 10 min, followed by incubation at 42 °C for 90 min, control- and test-strain labelled RNA were mixed and purified together using a single Qiagen mini-elute column. Microarray slides were prehybridized in 3.5 × saline sodium citrate (SSC) buffer, 0.1%, w/v, SDS and 10 mg BSA ml⁻¹ at 65 °C. All the *Y. pestis* CO92 predicted coding sequences were represented on the DNA microarray. The microarray slides were spotted PCR products produced by the bacterial microarray group, St George's Hospital Medical School, London, UK; details of the construction of the microarray are given by Stabler *et al.* (2003). Gene numbers refer to the published CO92 genome sequence (Parkhill *et al.*, 2001). The denatured DNA was applied to the microarray slide in hybridization solution (4 × SSC, 0.3%, w/v, SDS) and hybridized for 18 h at 65 °C. The slides were then washed once in 1 × SSC, 0.05%, w/v, SDS at 65 °C, and twice in 0.06 × SSC for 2 min. All hybridizations were performed in duplicate.

Microarray slides were scanned using an Affymetrix 428 scanner (MWG Biotech), the scanned images were loaded into Imagene 5.0 (Biodiscovery), and empty spots were flagged. Quantified data were loaded into Genespring 6.0 (SiliconGenetics). The data were normalized using LOWESS normalization with 40% of the data used for smoothing and a cut-off value of 0.01. Data were interpreted using the 'log of ratio' with the cross gene error model switched off. A 1.5-fold difference in expression level between the wild-type and the mutant was set as the cut-off. Genes that demonstrated at least a 1.5-fold difference in expression level were tested by Student's *t* test to determine that the difference was statistically significant. The Benjamini and Hochberg false discovery rate was used to correct for multiple testing. The results are a compilation of the gene-expression profiles of three biological replicates grown on different days and two arrays of each replicate also performed on different days, resulting in a total of six arrays.

Proteomics. *Y. pseudotuberculosis* IP32953 or *vagH* mutant were cultured in TMH supplemented with 2.5 mM CaCl₂ (a rich defined medium inhibiting Yop expression) at 37 °C to OD₆₀₀ 0.5. The bacteria were thereafter collected by centrifugation. The bacterial pellet was solubilized in lysis buffer (20 mM Tris, pH 7.4, 8 M urea, 1% CHAPS) and sonicated to disrupt cells. The disrupted cells were centrifuged and the protein concentration was determined with Bradford reagent. Ten micrograms protein lysate was labelled either with Cy3 or with Cy5. A mixture of the two lysates (total 10 µg) was labelled with 3-(4-carboxymethyl)phenylmethyl-3'-ethyloxycarbocyanine halide *N*-hydroxysuccinimidyl ester (Cy2). First-dimension IEF was performed on immobilized pH gradient strips (24 cm, pH 3.0–10.0, linear) in an Ettan IPGphor system (GE Healthcare), each sample containing wild-type, *vagH* mutant and a combination of the two lysates differentially labelled. Second-dimension separation was performed on 12% SDS-PAGE gels (20 × 24 cm). Images were acquired on a Typhoon 9400 scanner (GE Healthcare), and relative quantification of matched gel features was performed by DeCyder DIA and BVA software (GE Healthcare). For analysis of proteins, a preparative gel was run with unlabelled wild-type protein lysates and the gel was stained with Coomassie brilliant blue, the proteins of

interest were excised, and MS and protein identification were carried out by the Wallenberg Consortium North (WCN) Expression Proteomics Facility, IMBIM, Uppsala University, Sweden.

Yop secretion and YopB expression. Yop synthesis and secretion were carried out as described previously by Pettersson *et al.* (1999), with the exception that no Triton X-100 was added. Overnight cultures of *Yersinia* strains grown at 26 °C were diluted 1:20 in fresh BHI–Ca²⁺ medium, grown for 1 h at 26 °C, then shifted to 37 °C and grown for an additional 3 h. After measuring OD₆₀₀, the cells were harvested, and the culture supernatant was collected and filtered (0.45 µm pore size, Sartorius). The proteins in the supernatant were precipitated with TCA, and thereafter separated by SDS-PAGE and visualized by staining with Coomassie brilliant blue. For analysis of YopB expression, bacteria were grown in TMH medium supplemented with 5 mM EGTA for depletion of calcium for 2.5 h at 37 °C. Total samples (pellet and supernatant mixed) were mixed with 2 × SDS sample buffer, loaded according to OD₆₀₀ and separated by SDS-PAGE, blotted onto nitrocellulose filters and thereafter analysed with YopB-specific mAbs followed by a secondary antibody conjugated to horseradish peroxidase (HRP). The light signal was detected using ECL (GE Healthcare) and light-sensitive film (GE Healthcare).

Intracellular survival in macrophages and HeLa cells. The mouse-macrophage cell line J774 was grown in modified Eagle's medium (MEM)α (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS). The macrophages were grown at 37 °C and 5% CO₂ in a humidified cell incubator. Cells (5 × 10⁵ ml⁻¹) were plated in 24-well tissue-culture plates and grown until confluent. *Yersinia* strains for infection were cultivated in LB at 26 °C overnight. After growth overnight the bacteria were washed three times with PBS and the bacterial concentration was adjusted to 5 × 10⁶ bacteria ml⁻¹. Before infection, the macrophages were washed three times with PBS and fresh medium was added. To each well, 5 × 10⁶ bacteria were added. After uptake of bacteria for 30 min, the bacteria were washed away and MEMα containing 20 µg gentamicin ml⁻¹ was added. After another 30 min, the cells were washed once again and MEMα supplemented with 4 µg gentamicin ml⁻¹ was added to the cells. After 0, 5 and 24 h, the cells were washed with PBS and lysed with 0.1% Triton X-100 for 5 min. Bacterial numbers were determined by viable counting on selective plates.

Survival in the presence of macrophages or HeLa cells. The assay was performed as described elsewhere (Bartra *et al.*, 2001). Briefly, the mouse-macrophage cell line J774 was kept under the same conditions as those described above for the assay for intracellular survival. Macrophages (2 × 10⁵ ml⁻¹) were seeded in a 24-well tissue-culture plate and grown until confluent. Bacteria grown overnight in BHI–Ca²⁺ medium were diluted with tissue-culture medium, and 400 µl of the solution was added to the cell-coated wells which had been washed free of antibiotics. Thirty minutes after the addition of the bacteria, the overlying medium was removed and replaced with 250 µl fresh medium (*t*=0 h). Cells were harvested by first removing the overlying medium, followed by lysis with lysis buffer (0.1% Triton X-100, 15 mM NaCl, 1 mM Tris, pH 8). The lysate was then analysed by plating on selective agar plates. The same procedure, with the exception that HeLa cells were grown in MEM supplemented with 10% FCS, was used to investigate survival in the presence of HeLa cells.

RESULTS AND DISCUSSION

Methyltransferase activity of VagH

Since VagH shows high homology to the *E. coli* methyltransferase HemK (63% identity), we wanted to establish if VagH shared this enzymic activity. To assess this, we

adopted the methyltransferase assay developed by Colson (1977). Total protein lysates prepared from different strains of *Y. pseudotuberculosis* were incubated with [³H]SAM and purified VagH. When the protein lysate was prepared from wild-type bacteria, the incorporation of [³H]CH₃ was low in both the presence and the absence of purified VagH (Fig. 1). In contrast, when VagH was added to the protein lysate prepared from *vagH* mutant bacteria, a sixfold increase in incorporation of [³H]CH₃ was observed. These results showed that VagH is a methyltransferase capable of using SAM as a methyl donor, and also indicated that the targets of the enzymic activity of VagH are saturated in protein lysates of wild-type bacteria. In addition, we evaluated the *E. coli* homologous enzyme HemK and its ability to methylate proteins in *Yersinia*. We found that HemK exhibited similar activity, and methylated proteins to the same extent as VagH (Fig. 1). Thus, VagH is a methyltransferase that exhibits the same activity as the functional orthologue HemK.

Targets of VagH MTase activity

Next, we wanted to analyse the target proteins of the VagH-dependent MTase activity. We repeated the above experiments, but instead of precipitating the proteins after the reaction we added SDS sample buffer to stop the reaction, and the samples were thereafter separated by SDS-PAGE. The proteins were transferred to a nitrocellulose

Fig. 1. VagH is a SAM-dependent methyltransferase. Total protein lysates prepared from either *vagH* mutant or wild-type bacteria were incubated with [³H]SAM for measurement of incorporation of methyl groups into the lysate (L). The incorporation without addition of enzyme (E) (VagH or HemK) was low, as was also observed when VagH was added to protein lysates prepared from wild-type bacteria. In contrast, when VagH/HemK was added to a lysate prepared from *vagH* mutant bacteria, an increase in incorporation of [³H]CH₃ was observed, showing that VagH possesses a methyltransferase activity similar to that of HemK. No incorporation of [³H]CH₃ was found when the wild-type strain was used, indicating that this strain is fully methylated. □, *vagH* (L) + HemK (E); ○, *vagH* (L) + VagH (E); ◆, wild-type (L) + *vagH* (E); ■, wild-type (L); ▲, wild-type (L) + VagH (E); ●, *vagH* (L).

membrane and the signal was quantified by autoradiography. Two bands appeared at molecular masses of ~45 and 53 kDa (Fig. 2a). The theoretical molecular masses of the *Y. pseudotuberculosis* homologues of the known targets in *E. coli*, RF1 and RF2, are 40.55 and 41.29 kDa, respectively. Due to the differences between predicted and observed molecular masses, the methylated proteins were characterized further. First, we separated the methylated proteins in two dimensions (Fig. 2b). The analysis revealed that the upper band migrated at pI 4.75 and the lower band at pI ~5. These pI values correspond to the theoretical pI values of RF2 and RF1, respectively (4.74 and 5.07). Second, we analysed the proteins by MS. The spot migrating at pI 4.75 was identified as RF2, but the second protein could not be identified due to contamination of the fraction by other proteins. The pI and the molecular mass of this protein, however, strongly suggested that it was RF1. To confirm this, we therefore overexpressed His-tagged RF1 in the *vagH* mutant and subjected the lysate to the analysis described above. A methylated protein band close to the protein migrating at 45 kDa appeared in elevated amounts when RF1 was overexpressed, demonstrating that RF1 was also targeted by VagH (Fig. 3) and that the lower band/spot likely corresponds to RF1. However, we were puzzled that RF2 showed a mobility corresponding to that of a 53 kDa protein, and we therefore also overexpressed His-tagged RF2 from *Y. pseudotuberculosis* (RF2y) and compared it to overexpressed His-tagged RF2 cloned from *E. coli* K-12 (RF2e). Surprisingly, the recombinant RF2y migrated close to 53 kDa, whereas RF2e migrated closer to 45 kDa (Fig. 3). The difference in migration between these very similar proteins is not easily explained based on the sequences of the two proteins and, in addition, the methylation status does not affect the migration of the protein, since an unmethylated RF2y shows the same apparent

Fig. 2. Protein substrates of the VagH MTase activity. Protein lysates from *vagH* mutant bacteria were incubated with or without VagH and [3 H]SAM and separated by SDS-PAGE (a) and blotted to nitrocellulose filters. The filters were scanned in a Storm scanner. Two radioactively labelled bands appeared in the lanes loaded with lysate incubated with enzyme, indicating that at least two proteins are targeted by VagH. To further separate the proteins, the reactions were subjected to 2D gel analysis (b). The upper spot was identified as RF2. The pI (5) and size (45 kDa) of the lower spot correspond to theoretical values for RF1.

Fig. 3. The protein substrates of VagH are RF1 and RF2. To demonstrate RF1 and RF2 of *Y. pseudotuberculosis* (RF2y) as targets of VagH, His-tagged variants of the proteins were expressed in the *vagH* mutant. RF2 (RF2e) from *E. coli* K-12 was also expressed in the *vagH* mutant. The methyltransferase assay was performed as described in the legend to Fig. 2. Lane 1, *vagH* mutant; lane 2, RF1 overexpressed in the *vagH* mutant; lane 3, RF2y overexpressed in the *vagH* mutant; lane 4, RF2e overexpressed in the *vagH* mutant. An increase in the intensity of one band was observed, indicating that VagH targets RF1 and RF2; this activity was identical to that of HemK. The bands showing increased intensity corresponded to the sizes of overexpressed RF1, RF2y and RF2e, as determined by immunoblotting with anti-His-tag antibody (data not shown).

molecular mass after separation by SDS-PAGE. We also used HemK as the MTase in this assay, and this gave rise to similar results as for VagH (Fig. 3). We concluded that VagH specifically methylates RF1 and RF2 in the same way as HemK, further supporting the close functional relationship between the two enzymes.

Virulence

Analysis of the sequence of the region containing *vagH* revealed that *vagH* is the last gene in a potential operon consisting of three genes. The genes, and also the gene order, of this operon are conserved among *E. coli*, *Salmonella enterica* and *Y. pseudotuberculosis*. The gene upstream of *vagH* is *prfA*, encoding RF1, one of the targets of the VagH enzyme. The two genes downstream of *vagH*, *sirB1* and *sirB2*, show homology to two regulators of the virulence locus *Salmonella* pathogenicity island (SPI)-1 in *S. enterica* (Johnston *et al.*, 1996; Rakeman *et al.*, 1999). The *sirB1/B2* genes are necessary for full expression of *sirC*, which positively regulates the SPI-I-mediated uptake of *Salmonella* into eukaryotic cells (Rakeman *et al.*, 1999), indicating that *sirB1/B2* are involved in the virulence function of *Salmonella*. To ensure that the effect on virulence of the *vagH* mutation was not due to downstream effects on the expression of *sirB1/B2* in *Y. pseudotuberculosis*, these genes were deleted and the virulence of this double mutant was evaluated. No effect of the mutation on virulence was observed (data not shown). We also evaluated the virulence of a *vagH* mutant by the intraperitoneal route of infection. We found that the mutant was 500-fold less virulent than wild-type (ID₅₀: wild-type, 3.2×10^3 c.f.u.; *vagH*, 1.6×10^6 c.f.u.), further strengthening the role of *vagH* in the

virulence of *Y. pseudotuberculosis*. The mice were also infected orally with lethal concentrations of bacteria (10^9 c.f.u. ml^{-1}) and the colonization of bacteria in different organs such as Peyer's patches and spleen was monitored over time (Fig. 4). The mutant could not colonize the mice, either in Peyer's patches or spleen, and it behaved much like an avirulent virulence-plasmid-cured strain.

Microarray and proteomic analysis of the *vagH* and wild-type strains

To further characterize the *vagH* mutant, we performed microarray analysis on the mutant and compared this to the wild-type strain. The bacteria were cultivated in LB at 37 °C, mRNA was collected from bacteria in exponential phase, and the corresponding cDNA was hybridized to a DNA chip containing the genes that encode all predicted ORFs of *Y. pestis*. In this analysis, few genes (22) were up- or down-regulated more than 1.5-fold (see Table 1). Six ribosomal genes were found to be down-regulated in the *vagH* mutant, in accordance with the results obtained by Nakahigashi *et al.* (2002) when the *hemK* mutant of *E. coli* was analysed. In *E. coli*, as many as 260 genes are differentially expressed in the *hemK* mutant (Nakahigashi *et al.*,

2002). *E. coli* is much more affected in growth by a deletion of *hemK* than the corresponding mutant in *Y. pseudotuberculosis* (Garbom *et al.*, 2004; Nakayashiki *et al.*, 1995), and this could explain the difference in expression patterns between the two strains. Of the remaining 16 genes affected in the *vagH* mutant, half showed the same regulatory pattern as the *hemK* mutant, while six showed a different pattern and two genes were not found in the *E. coli* study. No gene that was obviously involved specifically in virulence was found in the microarray analysis of the *vagH* mutant. However, the effect on the ribosomal genes seemed to be consistent, and therefore effects in the *vagH* mutant on translation rather than transcription would be expected. To analyse this, we conducted a proteomics study.

Again, the bacteria were cultivated in rich medium, and the protein lysates prepared from exponentially growing bacteria were separated by 2D gel electrophoresis. We used the fluorescence difference gel electrophoresis (DIGE) system, which has the advantage of reducing gel-to-gel variation by running several samples on the same gel, employing differential labelling of the lysates with the dyes Cy2, Cy3 and Cy5. We could identify 400 protein spots (triplicates), and among these surprisingly few, 10 proteins, showed significant differences in expression levels when the two strains were compared ($P < 0.05$). Five of these could be identified using MS; a list of these proteins is presented in Table 2. No overlap was found with the microarray analysis, indicating that transcription and translation are affected differently in the *vagH* mutant. This difference between transcription and translation could be expected, since RF1, RF2 and other ribosomal components are affected in the *vagH* mutant compared to the wild-type. One interesting protein emerging from this analysis, from the point of view of virulence, was a hypothetical protein (YP03050) with similarities to iron-binding proteins in other pathogens. Acquisition of iron within the animal is an important virulence trait (Buchrieser *et al.*, 1998), and to assess if the virulence attenuation of the *vagH* mutant was due to the down-regulation of this iron-binding protein we deleted the gene in *Y. pseudotuberculosis*. The virulence of this mutant was evaluated by oral infection of mice; no difference was found upon comparison with the wild-type (data not shown).

Interestingly, YopD, a known virulence determinant in *Y. pseudotuberculosis* (Rosqvist *et al.*, 1991), was found in twofold increased amounts in the *vagH* mutant compared to the wild-type. YopD is encoded on the virulence plasmid of *Y. pseudotuberculosis* and is part of a T3SS that is required for virulence (Gemske *et al.*, 1980). This finding was surprising, as we have found previously that the T3SS is repressed in the *vagH* mutant (Garbom *et al.*, 2004). The bacteria were, in contrast to the earlier study, grown under non-inducing conditions (+Ca). Since YopD is a negative regulator of the T3SS (Francis & Wolf-Watz, 1998; Williams & Straley, 1998), the upregulation of YopD is in accordance with the earlier observation of a down-regulation of the T3SS in the *vagH* mutant.

Fig. 4. A *vagH* mutant is avirulent. C57BL/6J female mice were infected by the oral route with the different strains. At days 2, 3, 4, 5 and 8, two mice in each group were analysed, the Peyer's patches (PP) and spleen were homogenized, and bacterial numbers were determined by viable counting. The mutant could not colonize PP (a) or spleen (b) and behaved in a similar manner to a strain cured of the 70 kb T3SS-expressing virulence plasmid. *All mice in this group were dead at day 8.

Table 1. Differential regulation of ribosomal genes in a *vagH* mutant

mRNA was prepared from exponentially growing bacteria, and corresponding cDNA was hybridized to a DNA chip containing genes from *Y. pestis*. Differentially transcribed genes obtained from wild-type and *vagH* mutant were identified (see Methods). Six ribosomal genes were found to be down-regulated in the *vagH* mutant.

Name	Fold change*	Function
<i>rpsJ</i>	-2.5	30S ribosomal protein S10
<i>rpsC</i>	-2.3	30S ribosomal protein S3
<i>rplC</i>	-2.2	50S ribosomal protein L3
<i>rpmC</i>	-2.1	50S ribosomal protein L29
<i>rplB</i>	-2.5	50S ribosomal protein L2
<i>rplW</i>	-1.9	50S ribosomal protein L23
<i>nuoJ</i>	-1.8	NADH dehydrogenase I chain J
<i>sucD</i>	-2.1	Succinyl-CoA synthase alpha chain
<i>sucB</i>	-1.9	Dihydrolipoamide succinyltransferase component
YPO2375	3.9	Putative aldo/keto reductase
<i>mglA</i>	2.1	Galactoside transport ATP-binding protein
<i>mglB</i>	2.1	Galactose-binding protein
YPO3139	1.9	Putative 6- <i>O</i> -methylguanine DNA methyltransferase
<i>ureC</i>	1.7	Urease alpha subunit
<i>mgtB</i>	2.3	Mg ²⁺ transport ATPase protein B
YPO2674	1.8	Putative exported protein
YPO0819	2.0	Putative carbonic anhydrase
<i>dps</i>	1.9	Putative DNA binding protein
YPO3279	3.5	Putative sigma 54 modulation protein
<i>ribB</i>	1.9	3,4-Dihydroxy-2-butanone 4-phosphate synthase
YPO1649	2.0	Conserved hypothetical protein
<i>cysH</i>	2.0	Phosphoadenosine phosphosulfate reductase

*A negative value signifies that the gene has a lower expression in the *vagH* mutant compared with wild-type.

A link between the T3SS and the methyltransferase activity of VagH

These results prompted us to analyse the Yop secretion in the *vagH* mutant, the wild-type and a *trans*-complemented *vagH* mutant to investigate if the methyltransferase activity of VagH could be linked to the earlier-observed lowered Yop secretion in a *vagH* mutant. It was found that the *vagH* mutant (Fig. 5) was severely affected in the expression and secretion of the Yops. This defect could be

trans-complemented with the wild-type gene and with *E. coli hemK*⁺ (data not shown), but not with a gene encoding an enzymically inactive VagH. This demonstrates that the enzymic activity of VagH is essential for Yop secretion. The results from the proteomic analysis showed that YopD was present in twofold higher amounts in a *vagH* mutant compared to wild-type. To analyse if YopD is indeed involved in the repression of Yop expression in a *vagH* mutant, we created a *vagH yopD* double mutant and analysed the expression of YopB (Fig. 6). The analysis revealed

Table 2. Proteome analysis of the *vagH* mutant compared with that of the wild-type

Of 400 different proteins, 10 showed a different expression pattern when the *vagH* mutant was compared to the wild-type. Proteins successfully identified by MS are listed.

Protein name	Fold change*	Function
Mae2	1.8	NAD-dependent malic enzyme
KBL	1.5	2-Amino-3-ketobutyrate CoA ligase
YopD	1.8	T3SS regulator/translocator
LpdA	1.6	Dihydrolipoamide dehydrogenase
YPO3050	-2.8	Putative exported protein, iron binding domain

*A negative value signifies that the protein has a lower expression in the *vagH* mutant compared with wild-type.

Fig. 5. The secretion of Yops is repressed in a *vagH* mutant. The bacteria were grown under Yop-inducing conditions, BHI- Ca^{2+} at 37 °C, and the supernatant was precipitated and separated by SDS-PAGE, followed by staining with Coomassie brilliant blue. The following strains were used: *vagH* mutant (*vagH*), *vagH* mutant trans-complemented with an active-site mutant of VagH [*vagH/pVagH(PP-AA)*], *vagH* trans-complemented with the wild-type gene (*vagH/pVagH*), wild-type (WT). Secreted Yops are indicated in the Figure.

that a deletion of *yopD* restores expression of YopB to wild-type levels in the *vagH* mutant, showing that the lowered Yop expression in the *vagH* mutant can be suppressed by deletion of the negative-regulator YopD gene.

Survival and replication within macrophages and growth in the presence of eukaryotic cells

The main role of the T3SS in *Y. pseudotuberculosis* is to inhibit phagocytosis by macrophages (Rosqvist *et al.*, 1988). In addition, it has been shown that survival and replication inside macrophages may be important virulence traits for *Y. pseudotuberculosis* (Grabenstein *et al.*, 2004). This is not dependent on the T3SS encoded on either the virulence plasmid or the chromosome (Pujol & Bliska, 2003). Since we could not exclude that factors other than T3SS contributed to the attenuated phenotype of the *vagH* mutant, we

Fig. 6. The repression of YopB expression in a *vagH* mutant is suppressed by deletion of *yopD*. The bacteria were grown in TMH- Ca^{2+} at 37 °C, and total samples were separated by SDS-PAGE; the amount of YopB was analysed by Western blotting with mAbs recognizing YopB (α -YopB). A *vagH* mutant produced less YopB than wild-type (WT) bacteria; this could be suppressed by an additional deletion of *yopD*.

also wanted to analyse the intracellular survival of the mutant in macrophages. To avoid the effects of the T3SS on the initial interaction with the macrophages, the bacteria were grown under conditions repressing the T3SS (2.5 mM Ca^{2+} and 26 °C) before addition to the macrophages. The bacteria were incubated with J774.1 mouse macrophages to allow the bacteria to enter the cells. Thirty minutes after infection, non-adherent bacteria were washed away and gentamicin was added to kill extracellular bacteria. After this, the number of bacteria was evaluated at different time points (0, 5 and 24 h) by plating and viable counting. The mutant and wild-type showed a similar pattern of replication (Fig. 7a). Both strains increased in number, indicating that both could replicate inside the macrophages.

Fig. 7. Mφ intracellular replication is not affected in the *vagH* mutant. To assess the ability of the *vagH* mutant to replicate inside macrophages, the mouse-macrophage cell line J774.1 was infected with wild-type (light-grey bars) or *vagH* mutant bacteria (dark-grey bars). (a) Extracellular bacteria were killed using 20 μg gentamicin ml^{-1} and the replication of the intracellular bacteria was followed by viable counting. The y axis values in (a) are a percentage of the c.f.u. per well at $t=0$. There was no significant difference in intracellular survival between the two strains. (b) Macrophages were infected with wild-type or *vagH* mutant strains, and the total number of viable bacteria was estimated after 0 and 8 h. (c) The same assay as in (b) was employed using HeLa cells.

It is also known that when T3SS mutants of *Y. pseudotuberculosis*, in contrast to the wild-type, are added to a macrophage culture, the mutants are cleared from the culture (Bartra *et al.*, 2001). We tested the *vagH* mutant and the wild-type in this assay, inducing the T3SS by growth at 37 °C in calcium-depleted medium prior to addition to the macrophages. Eight hours after infection, the number of viable *vagH* mutant bacteria was half that of the wild-type, indicating that the macrophages were killing the *vagH* mutant more efficiently than the wild-type (Fig. 7b). However, no difference in viability was noticed when the two strains were instead cultivated in the presence of an epithelial cell line (HeLa cells) (Fig. 7c). In the study of Bartra *et al.* (2001), a difference between a T3SS⁺ and a T3SS⁻ strain in proliferation in presence of HeLa cells was observed. The reason for the difference compared with our study is probably that the *vagH* mutant, in contrast to a T3SS mutant, still possesses a functional T3SS, although impaired. For instance, the *vagH* mutant is capable of inducing cytotoxicity in HeLa cells (Garbom *et al.*, 2004), indicative of a functional T3SS and translocation of the effector YopE (Rosqvist *et al.*, 1990). In the interaction with macrophages, the attenuated mutant cannot inhibit phagocytosis and therefore does not proliferate to the same extent as the wild-type, but in the interaction with HeLa cells, which are not professional phagocytes, the amount of translocated effectors might be enough for inhibition of phagocytosis, allowing the mutant to proliferate. These results suggest that the difference between the two strains in the interaction with macrophages is associated with the reduced expression of the T3SS in the mutant, and also indicate that the T3SS is not involved in intracellular macrophage survival, in accordance with the results of Pupol & Bliska (2003).

Conclusions

VagH is homologous to HemK of *E. coli*, and here we show that the two enzymes also exhibit identical function. VagH, like HemK, possesses a methyltransferase activity, and the targets of this activity are RF1 and RF2. We also demonstrate that a deletion of *vagH* has little effect on the transcriptome and proteome of *Y. pseudotuberculosis*, but that the negative regulator of T3SS YopD is present in twofold higher amounts in the *vagH* mutant than in the wild-type strain under non-inducing conditions. This is in agreement with the previously reported down-regulation of the T3SS observed for a *vagH* mutant which in this study we show is dependent on the methyltransferase activity of VagH. However, under inducing conditions, the amount of YopD is lower in the *vagH* mutant (data not shown). This reflects the fact that YopD, in addition to being a negative regulator, is a protein essential for translocation of the effector molecules delivered by the T3SS, and is therefore upregulated when the bacteria are grown under inducing conditions. To assess the relevance of the upregulation of YopD, we created a *vagH yopD* double mutant and analysed the expression of YopB under T3SS-inducing

conditions. In a *vagH* mutant, the levels were very low, but in a *vagH yopD* double mutant, the amount of YopB was similar to that observed for the wild-type strain. Thus, repression of the T3SS is likely the cause of the avirulent phenotype of the mutant, and importantly the mutant behaves like a *Y. pseudotuberculosis* strain cured of the virulence plasmid (encoding the T3SS) in a mouse model of infection, i.e. the mutant is cleared in Peyer's patches following oral dosing, which is generally the case for mutants defective in the T3SS. Finally, we also show that the virulence attenuation is most likely not due to defects in intracellular survival, since the mutant replicated as well as the wild-type in the cytosol of macrophages. Further studies are required to find the molecular mechanisms that link VagH to the T3SS; such studies are in progress in our laboratory.

Having established that VagH is a methyltransferase, we now have the tools to further investigate the potential of VagH as a drug target. A compound inhibiting VagH could at least be effective against pathogens encoding a T3SS; we are currently investigating the possibility of identifying such compounds.

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