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Identification of Novel Virulence-Associated Genes via Genome Analysis of Hypothetical Genes

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The sequencing of bacterial genomes has opened new perspectives for identification of targets for treatment of infectious diseases. We have identified a set of novel virulence-associated genes (*vag* genes) by comparing the genome sequences of six human pathogens that are known to cause persistent or chronic infections in humans: *Yersinia pestis*, *Neisseria gonorrhoeae*, *Helicobacter pylori*, *Borrelia burgdorferi*, *Streptococcus pneumoniae*, and *Treponema pallidum*. This comparison was limited to genes annotated as hypothetical in the *T. pallidum* genome project. Seventeen genes with unknown functions were found to be conserved among these pathogens. Insertional inactivation of 14 of these genes generated nine mutants that were attenuated for virulence in a mouse infection model. Out of these nine genes, five were found to be specifically associated with virulence in mice as demonstrated by infection with *Yersinia pseudotuberculosis* in-frame deletion mutants. In addition, these five *vag* genes were essential only in vivo, since all the mutants were able to grow in vitro. These genes are broadly conserved among bacteria. Therefore, we propose that the corresponding *vag* gene products may constitute novel targets for antimicrobial therapy and that some *vag* mutants could serve as carrier strains for live vaccines.

Antibacterial therapy is becoming less effective in the treatment of infections due to the spread of multiple-antibiotic-resistant bacterial strains (59). This has lead to a concerted effort to develop mechanistically novel antimicrobial agents. This search is being facilitated by the wealth of information accumulating in databases via different large-scale genome projects. To date, about 100 bacterial genomes, including several important pathogens have been sequenced, revealing around 25% of the open reading frames (ORFs) to be conserved hypothetical genes without known function (17). Presumably, among this group of ORFs, protein classes that constitute suitable molecular targets for novel therapeutics will be identified. In fact, a number of genome-based studies have already identified homologous gene classes from different bacteria that encode products of unknown or hypothetical function (4, 7, 20, 55). By combining the bioinformatic approaches with mutagenesis, genes essential for in vitro viability have been identified (4, 20, 55). Indeed, these proteins constitute potential targets for the development of novel antibacterial agents that would serve to inhibit bacterial growth.

An alternative approach would be to target genes required only for in vivo growth and not for growth outside the animal host. Finding these nontraditional targets by using a bioinformatic approach is a hitherto-unproven strategy (29). The approach presented here makes use of the fact that several obligatory pathogenic bacteria have genome sizes that are considerably smaller than those of free-living eubacteria. During evolution, these pathogenic bacteria have condensed their genomes by eliminating genes not required for growth in the animal host (52, 60). If a subset of the remaining genes affect growth only in vivo and these genes are common to several different pathogenic bacteria, the corresponding products could then constitute drug targets amenable for attack by mechanistically novel antibacterial agents.

Treponema pallidum contains a circular chromosome of about 1 Mb, indicating that approximately 75% of its previous coding capacity has been lost during evolution (19). Moreover, only 53% of the ORFs have assigned functions, whereas 27% exhibit no database matches. Significantly, 17% show similarity with hypothetical genes from other species. With the aim of identifying novel in vivo survival genes, we analyzed whether this class of hypothetical proteins contained homologues in three other obligatory gram-negative bacterial pathogens (*Yersinia pestis*, *Neisseria gonorrhoeae*, and *Helicobacter pylori*), a gram-negative spirochete (*Borrelia burgdorferi*), and one gram-positive bacterial pathogen (*Streptococcus pneumoniae*). With the exception of *Y. pestis*, all are causative agents of chronic disease in humans (49). This comparison identified 17 different genes with homologues in each of the six different pathogens. The virulence phenotype of each gene knockout was conveniently examined in a murine model of infection with the enteropathogen *Yersinia pseudotuberculosis* serotype I as a tool to study these mutants. Of the 17 identified genes, 5 were found to be specifically required for in vivo survival.

MATERIALS AND METHODS

Bioinformatics. BlastP (ftp.ncbi.nlm.nih.gov) was used for all the comparisons on the protein level (2). The cutoff E-value was routinely set to 10^{-7}, and proteins with lower E-values were considered hits. The conserved hypothetical ORFs and ORFs with unknown function in *T. pallidum* (Nichols) (19) were downloaded at http://www.tigr.org and compared to the proteome of *Y. pestis* (strain CO92) (40) retrieved from http://www.sanger.ac.uk. The protein sequences from *Y. pestis* were then compared to those of *H. pylori* strain 26695 (56), *B. burgdorferi* strain B31 (18), and *S. pneumoniae* strain Tigr4 (54), also down-

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loaded from http://www.tigr.org. A comparison to the unfinished genome sequence of *N. gonorrhoeae* strain FA 1090 was performed at http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table_cgi by using Tblastn. The same cutoff was used in these comparisons. ORFs with homologues in all six genomes were compared to the nonredundant database and to the human proteome downloaded from http://www.ncbi.nlm.nih.gov in order to identify a putative function and to evaluate the level of conservation.

Bacterial strains and media. *Y. pseudotuberculosis* strain IP32953 was obtained from E. Carniel at the Pasteur Institute, Paris, France. This strain was grown on plates containing 10 μg of nalidixin ml^{-1} in order to select for spontaneous nalidixin-resistant clones. One resistant clone (IP32953Nal) was used throughout the study and is referred to as the wild type. *Escherichia coli* strain S17-1λpir was used for conjugative transfer of the recombinant plasmids for mutagenesis of *Y. pseudotuberculosis*. *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar plates. *Yersinia* strains were grown in LB broth or brain heart infusion (BHI) (Oxoid) broth. BHI broth was supplemented with 5 mM EGTA and 20 mM $MgCl_2$ (BHI^-) for induction of *Yersinia* outer protein (Yop) synthesis and secretion. For analysis of in vitro growth, Bacto M9 minimal salts supplemented with 0.4% glucose, 10 mM KCl, 10 mM $MgSO_4$, 100 μg of L-aspartate per ml, 1 mM thiamine, and 100 μM pantothenate was used as a minimal defined medium (MDM) at 26°C. Another defined medium, consisting of potassium morpholinopropane sulfonate (MOPS) supplemented with 0.4% glucose, 0.2% Casamino Acids, 2.5 mM $CaCl_2$, and 1 mM Na_2HPO_4 (DMC) (37), was used to assay growth in vitro at 37°C. For solid media, *Yersinia* selective agar base (Difco) was used. Antibiotics were used at the following concentrations: nalidixin, 10 μg ml^{-1}; chloramphenicol, 20 μg ml^{-1}; and carbenicillin and ampicillin, (100 μg ml^{-1}.

Preparation of plasmid DNA, restriction enzyme digests, ligation, and transformation into *E. coli* were performed essentially as described by Sambrook et al. (50). DNA fragments were purified from agarose gels by using Ultrafree-DA bioseparators according to the instructions of the manufacturer (Amicon).

Mutagenesis strategy. (i) Insertion mutants. The insertion mutants were constructed essentially as described previously (45). DNA was amplified with PCR by using primers annealing approximately 10 to 30 nucleotides after the translational start codon of the ORF to be mutated and approximately 350 bp into the ORF. This fragment was cloned into the suicide vector pNQ705 (38). In order to introduce this plasmid into *Y. pseudotuberculosis* (IP32953Nal), conjugal matings were done. Transconjugants were selected on *Yersinia*-specific agar containing chloramphenicol and nalidixin. Correct insertions of the transconjugants were verified by PCR.

(ii) Deletion mutants. The deletion mutants were constructed by allelic exchange as described by Milton et al. (33). In short, DNA regions flanking the gene to be deleted were amplified by PCR, fused in a second PCR, and ligated into the suicide vector pDM4 (33). The flanking DNA regions included 45 nucleotides of the 5′ and 3′ ends of the gene to be deleted. To introduce this plasmid into *Y. pseudotuberculosis* (IP32953Nal), conjugal matings were done with *E. coli* S17-1λpir containing the deletion constructs. Transconjugants were selected on *Yersinia*-specific agar containing chloramphenicol and nalidixin. PCR analysis confirmed that the vector had integrated correctly into the chromosomal DNA. To complete the allelic exchange, the integrated suicide plasmid was forced to recombine out of the chromosome by adding 5% sucrose to the agar plates. The pDM4 vector contains *sacB*, which produce an enzyme that converts sucrose into a product that is toxic to gram-negative bacteria. Transconjugants surviving on plates with 5% sucrose and that in addition were chloramphenicol sensitive were chosen, and correct deletion was confirmed by PCR.

Analysis of Yop synthesis and secretion. Analysis of Yop synthesis and secretion was carried out as described previously by Pettersson et al. (41) with the exception that no Triton X-100 was added. For analysis of secreted Yops, overnight cultures of *Yersinia* strains grown at 26°C were diluted (1:20) in fresh BHI^- medium, grown for 1 h at 26°C, and then shifted to 37°C and grown for an additional 3 h. After the optical density at 600 nm (OD_{600}) was measured, the cells were harvested and the culture supernatant was collected and filtered (0.45-μm-pore-size filter; Sartorius). The proteins from the supernatant were precipitated with 10% trichloroacetic acid. The final pellet from the supernatants was dissolved in sodium dodecyl sulfate (SDS) sample buffer, and the volume of the samples was adjusted in accordance with the OD_{600} of the bacterial cultures. The proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by staining with Coomassie brilliant blue. For analysis of Yop synthesis, the bacterial pellet was dissolved in phosphate-buffered saline and the volume was adjusted according to the OD_{600}. SDS sample buffer was added, and the samples were separated by SDS-polyacrylamide gel electrophoresis. The Yops were visualized by Western blotting with rabbit antisera recognizing the Yops.

Competitive growth. The competitive growth assay was performed as described by Gutgsell et al. (23). In these experiments IP32953 without nalidixin resistance was used as the wild type. The bacteria were grown individually overnight at 26°C in LB broth containing the proper antibiotics. On the following day the bacteria were inoculated together into the same culture medium without antibiotics and grown at 26 or 37°C, and the growth of the individual strains were monitored by viable counts on plates with or without antibiotics at 26°C.

Cytotoxicity assay. The cultivation and infection of HeLa cells have been described in detail previously (46). Briefly, HeLa cells were grown as monolayers to semiconfluence at 37°C in a humidified atmosphere in 24-well tissue culture plates in modified Eagle medium supplemented with HEPES medium, 10% heat-inactivated fetal calf serum, and penicillin. Before infection, the HeLa cells were washed free of penicillin and fresh medium without penicillin was added. *Yersinia* strains were cultivated in LB broth at 26°C overnight. The next day, the bacteria were diluted (1:300) into fresh cell culture medium. After growth for 30 min at 26°C on a rotary shaker, the bacteria were incubated for 1 h at 37°C. After the addition of bacteria, the infected HeLa cells were centrifuged for 5 min at $400 \times g$ to facilitate contact between the bacteria and the HeLa cells. Thereafter, the tissue culture plates were incubated at 37°C in a humidified atmosphere for 3 h, and the cells were examined every hour to detect changes in morphology.

Oral infections. Bacteria were grown overnight in 100 ml of LB broth cultures at 26°C with aeration. These cultures were collected and resuspended in 50 ml of sterilized tap water. C57BL/6J female mice were deprived of water overnight (18 h) and then given the water containing different bacterial dilutions (5×10^9, 5×10^8 [for the insertion mutants], and 5×10^7 [for the deletion mutants] CFU/ml). Each cage contained three mice, which were allowed to drink the bacterium-containing water ad libitum for 8 h. Each mouse consumed approximately 5 ml of the bacterial suspension. In total, nine mice (three mice for every concentration of bacteria) per bacterial strain were used in each experiment. In order to calculate the 50% lethal dose (LD_{50}) (44), the concentration of bacteria fed to the mice was measured by viable counts. The animal infection study was approved by the Swedish National Board for Laboratory Animals.

RESULTS

Identification of conserved genes with unknown functions among important human pathogens. To identify hitherto-uncharacterized potential virulence-associated genes, *T. pallidum* (19) was chosen for homology comparison with five other human pathogens (Fig. 1). At the time of initiating this study, there were known to be 176 conserved hypothetical genes and 35 genes with an unknown function in *T. pallidum*. These 211 ORFs were first compared to the complete genome of *Y. pestis*, since we aimed to use *Yersinia* to study the virulence phenotypes of the identified genes. The selection criteria identified genes with significant similarity at the protein level (BlastP E-value of $<10^{-7}$). Out of the original 211 selected ORFs in the *T. pallidum* genome, 73 remained after comparison with the *Y. pestis* genome. We then compared these ORFs to the genomes of four other human pathogens preferentially giving chronic infections, i.e., *N. gonorrhoeae*, *H. pylori*, *B. burgdorferi*, and *S. pneumoniae* (49) (Fig. 1). Among these genomes, 17 conserved ORFs were identified and designated virulence-associated genes (*vagA* to *vagQ*) (Table 1). Interestingly, analysis of the National Center for Biotechnology Information nonredundant database indicated that all of the *vag* genes exhibited homologous sequences in at least 35 other microorganisms, including *E. coli* and the two other *Yersinia* human pathogens, *Y. pseudotuberculosis* and *Y. enterocolitica* (data not shown). Of these highly conserved *vag* genes, nine had products that also exhibited similarity (BlastP E-values of $<10^{-7}$) to proteins listed within the first draft of the human genome (31, 58) (Table 1).

To evaluate our selection strategy for identifying potential virulence-associated genes, a collection of 99 genes with in vivo activity demonstrated by either signature-tagged mutagenesis

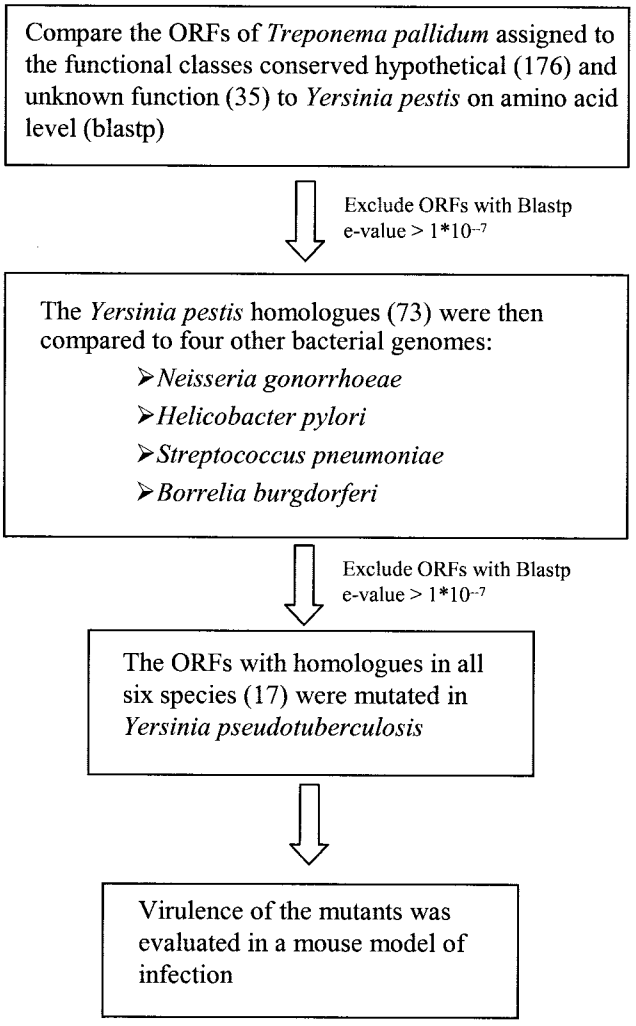

FIG. 1. Outline of the bioinformatic approach to identify novel virulence-associated genes.

(STM) (11, 13, 27, 57) or selected capture of transcribed sequences (22) were compared to the genomes of the six pathogens. Since many of these genes are also known to be virulence associated, they were all designated *vir* genes. We identified five *vir* genes that were conserved among the selected human pathogens (Table 2). These five genes were also highly conserved among many bacteria, including *E. coli*, but no obvious human homologues were detected.

Assessment of virulence phenotypes of novel Vag proteins. To analyze the virulence phenotypes associated with the identified *vag* genes, mutations in all 17 genes were created in the *Y. pseudotuberculosis* serotype I strain IP32953. This enteropathogen is a suitable model organism because infections of mice mimic human infections by the close relative *Y. pestis* (21, 48). In fact, these organisms are so similar that *Y. pestis* is believed to be a clone that evolved from *Y. pseudotuberculosis* serotype I about 1,500 to 20,000 years ago (1). Insertion mutagenesis by a single-crossover event with the suicide plasmid pNQ705 (38) was employed in a primary screening for virulence phenotypes. Fourteen of the 17 *vag* genes could be mutated individually in *Y. pseudotuberculosis*. The remaining three *vag* genes could not be mutated, indicating an essential role in cell viability (Table 1). All but one insertion mutant had an in vitro growth rate comparable to that of the wild type. The one exception, the *vagE* mutant, displayed impaired growth and also exhibited an elongated filamentous morphology that was most pronounced when the strain was incubated at 37°C. Pathogenic *Yersinia* synthesize and secrete virulence factors called Yops. These are translocated into eukaryotic cells by a type III secretion system (12), thereby causing a cytotoxic response in the infected cell (47). To determine whether any of the mutations affected this important virulence mechanism, all 14 insertion mutants were analyzed for their ability to synthesize and secrete Yops as well as for their ability to induce a cytotoxic response in infected HeLa cells (Table 1). While all mutants synthesized Yops, levels were notably reduced in a *vagH* insertion mutant. Moreover, the *vagI* mutant was affected in its ability to secrete Yops into the growth medium (Table 1). However, this reduced ability to secrete Yops did not significantly alter its cytotoxicity towards HeLa cells. In fact, only inactivation of *vagH* resulted in a slightly delayed cytotoxic response in infected HeLa cells, consistent with its reduced synthesis and secretion of Yops (Table 1). One additional mutant, the *vagE* mutant, induced a delayed cytotoxic response in infected HeLa cells. However, this is most likely due to the defect in growth seen with this mutant, since it behaved like the wild-type strain in regard to Yop synthesis and secretion. Thus, 11 of the 14 mutants were indistinguishable from the wild type, whereas the remaining three mutants showed a reduced ability to secrete Yops and/or induce cytotoxicity towards HeLa cells.

Similarly, insertion mutations were generated in the five conserved *vir* genes that were selected from the collection of genes previously shown to be essential for growth in vivo. All of these mutants behaved like the wild-type strain with respect to in vitro growth rate, Yop secretion, and cytotoxicity towards HeLa cells (Table 2).

The insertion mutants were all screened for virulence in the mouse infection model. The oral route of infection was chosen, since this peripheral route of infection is likely to engage several levels of the host defense. Oral infection of the 14 *vag* mutants showed that 9 (64%) were attenuated for virulence, whereas the remaining 5 mutants were as virulent as the wild-type strain (Table 1). Insertional mutagenesis of the five *vir* genes resulted in three attenuated mutants (60%), whereas the remaining two mutants were as virulent as the wild-type strain (Table 2). This confirms that our approach to identify novel virulence-associated genes among conserved hypothetical genes was as efficient as the biased approach based on genes previously associated with virulence in one of the selected organisms.

It could not be excluded that the *vag* genes were members of polycistronic transcription units. Therefore, in-frame deletions of the *vag* genes were constructed to verify that the virulence phenotype was due to the mutated *vag* per se and not due to polar effects on downstream genes. The deletion mutants were constructed by allelic exchange by using the suicide plasmid pDM4 (33). Deletion mutants could be constructed for eight of the nine virulence-associated genes. A deletion in *vagD* could not be constructed even though the mutagenesis construct could efficiently recombine with homologous sequences both

TABLE 1. Phenotypic characterization of mutants with insertion mutations in 17 conserved ORFs with an unknown or hypothetical function

Gene	*Y. pestis* Sanger[a] CDS	*E. coli* homologue	Predicted function[b]	Growth in rich medium at 26/37°C[c]	Yop secretion[e]	Cytotoxicity[f]	Virulence[g] (oral infection)	Human homologue[h]
vagA	YPO2010	*ychF*	GTPase	+++/+++	+++	+++	Attenuated	Yes
vagB	YPO3269	*yfiF*	RNA methyltransferase	+++/+++	+++	+++	Attenuated	Yes
vagC	YPO3277	*rluD*	RNA pseudouridine synthase	+++/+++	+++	+++	Attenuated	Yes
vagD	YPO1062	*mesJ*	Cell division	+++/+++	+++	+++	Attenuated	No
vagE	YPO0547	*yabc/mraW*	Methyltransferase	+++/+	+++	+	Attenuated	Yes
vagF	YPO2213	*yciL*	RNA pseudouridine synthase	+++/++	+++	+++	Attenuated	No
vagG	YPO0243	*smf*	DNA uptake	+++/+++	+++	+++	Attenuated	No
vagH	YPO2018	*hemK*	Methyltransferase	++/+++	+	+	Attenuated	Yes
vagI	YPO2055	*yebC*	Unknown	+++/+++	+	++	Attenuated	Yes
vagJ	YPO1604	*yceG*	Catabolism/anabolism of chorismate	+++/+++	+++	+++	Virulent	No
vagK	YPO2607	*nadD*	Nucleotidyltransferase	+++/+++	+++	+++	Virulent	No
vagL	YPO3547	*yraL*	Methyltransferase	+++/+++	+++	+++	Virulent	No
vagM	YPO1607	*ycfH*	Metal-dependent hydrolase	+++/+++	+++	+++	Virulent	Yes
vagN	YPO1106	*yfjB*	GTPase	+++/+++	+++	+++	Virulent	Yes
vagO	YPO3816	*yhhF*	Methyltransferase	ND[d]	ND	ND	ND	No
vagP	YPO1049	*uppS*	Undecaprenyl pyrophosphate synthetase	ND	ND	ND	ND	Yes
vagQ	YPO2875	*engA*	GTPase	ND	ND	ND	ND	No

[a] http://www.sanger.ac.uk.
[b] Putative function assigned to the product of the *E. coli* homologue.
[c] +++, wild-type growth; ++, intermediate growth; +, slow growth.
[d] ND, not determined. Repeated attempts to isolate these mutants failed.
[e] +++, wild-type level; +, low level.
[f] Most mutant strains generated a cytotoxic response within 45 min, similar to the case for the wild-type strain (+++). However, some mutants generated a cytotoxic response that was delayed for 0.5 to 1 h (++) or for 1 to 2 h (+).
[g] Two different bacterial concentrations (10^9 and 10^7 CFU/ml) were used for infection. For the wild-type strain a dose of 10^7 CFU/ml was lethal, and at least 50% of the mice died within 5 to 8 days. A virulent strain cured of the virulence plasmid, which is essential for virulence, did not kill the mice within 12 days even at the highest dose used. An attenuated strain was not lethal at a dose of 10^7 CFU/ml but could have a delayed lethal effect at a dose of 10^9 CFU/ml.
[h] Homologues with BlastP E-values of $<10^{-7}$.

upstream and downstream of the gene. This suggests that retention of *vagD* confers a selective advantage for *Yersinia*.

All of the deletion mutants were comparable to the wild type with respect to growth in rich medium, notwithstanding the fact that *vagC* shows similarity to the gene for ribosomal large-subunit pseudouridine synthase D (*rluD*), an essential requirement for growth in *E. coli* (43). However, while deletion of some other *E. coli* pseudouridine synthase genes has no effect on exponential growth in rich medium (14), some can confer a growth disadvantage during competition with the wild-type strain (23). This was not the case for a *vagC* deletion in *Y. pseudotuberculosis*, however, because even under competitive conditions, growth was still equal to that of the wild-type strain (data not shown). The deletion mutants were also grown in MDM. Four of the deletion mutants (the *vagA*, *vagC*, *vagG*, and *vagI* mutants) demonstrated a growth rate comparable to that of the wild-type strain in MDM at 26°C, whereas the growth rate of the *vagH* deletion mutant in MDM at 26°C was slightly reduced compared to that of the wild-type strain. For analysis of the growth rate at 37°C, a defined medium supplemented with Casamino Acids (DMC) was used, as *Y. pseudotuberculosis* did not grow in MDM at 37°C. It had been observed earlier that enteropathogenic *Yersinia* strains require additional nutrients at 37°C compared to 26°C (6). Similar to what was seen with growth in MDM, the *vagA*, *vagC*, *vagG*, and *vagI* deletion mutants grew as well as the wild-type strain in DMC at 37°C, whereas the *vagH* deletion mutant showed a more severe defect in growth rate than the wild-type strain

TABLE 2. Phenotypic characterization of mutants with insertion mutations in a control group of virulence-associated genes conserved among selected bacteria

Gene	*Y. pestis* Sanger[a] CDS	*E. coli* gene	Function	Growth in rich medium at 26/37°C[b]	Yop secretion[c]	Cytotoxicity[d]	Virulence[e] (oral infection)
virA	YPO2567	*pta*	Phosphate acetyltransferase	+++/++	+++	+++	Attenuated
virB	YPO0324	*uvrA*	Exonuclease ABC subunit A	+++/+++	+++	+++	Attenuated
virC	YPO0372	*miaA*	tRNA transferase	+++/++	+++	+++	Attenuated
virD	YPO3097	*manB*	Phosphomannomutase	+++/++	+++	+++	Virulent
virE	YPO3393	*pbpF*	Penicillin-binding protein 1B	+++/+++	+++	+++	Virulent

[a] http://www.sanger.ac.uk.
[b] See Table 1, footnote *c*.
[c] See Table 1, footnote *e*.
[d] See Table 1, footnote *f*.
[e] See Table 1, footnote *g*.

TABLE 3. Phenotypic characterization of mutants with in-frame deletion mutations in novel virulence-associated genes and *vir* genes

Gene	Growth in rich medium at 26/37°C[a]	Yop secretion[c]	Cytotoxicity[d]	LD_{50}[e] (CFU/ml)	Relative oral attenuation (LD_{50} for mutant/LD_{50} for wild type)
vagA	+++/+++	+++	+++	5.2×10^9	104
vagB	+++/+++	+++	+++	$<7.4 \times 10^7$	1
vagC	+++/+++	+++	+++	1.2×10^9	24
vagD	ND[b]	ND	ND	ND	ND
vagE	+++/+++	+++	+++	1.8×10^8	4
vagF	+++/+++	+++	+++	2.2×10^8	4
vagG	+++/+++	+++	+++	1.6×10^9	32
vagH	+++/+++	+	++	$>5 \times 10^9$	>100
vagI	+++/+++	+++	+++	3.2×10^9	64
virA	+++/+++	+++	+++	1.6×10^9	32
virB	+++/+++	+++	+++	$>2 \times 10^9$	>100
virC	+++/+++	+++	+++	1.4×10^9	28

[a] See Table 1, footnote *c*.
[b] ND, not determined. Repeated attempts to isolate this mutant failed.
[c] See Table 1, footnote *e*.
[d] See Table 1, footnote *f*.
[e] The LD_{50} was calculated as described by Reed and Muench (44). The LD_{50} for the wild-type strain was 5×10^7 CFU/ml.

under these growth conditions compared to when grown in MDM at 26°C. We also confirmed that Yop secretion and cytotoxicity towards HeLa cells were similar to those of the wild-type strain for all but one mutant (Table 3). The notable exception was the *vagH* mutant, which was severely affected in Yop synthesis and secretion although still capable of mounting a cytotoxic response, although slightly delayed, towards HeLa cells. Deletion mutations generated in the three conserved *vir* genes (*virA*, *virB*, and *virC*) did not alter patterns of Yop synthesis and secretion, nor did they perturb cytotoxicity towards HeLa cells.

To investigate the virulence potential of these mutant strains we again used the oral route of infection. Five of the eight *vag* deletion mutants (the *vagA*, *vagC*, *vagG*, *vagH*, and *vagI* mutants) were significantly attenuated in comparison to the wild-type strain. The *vagB*, *vagE*, and *vagF* deletion mutants maintained wild-type virulence (Table 3). All of the *vir* deletion mutants were attenuated in this mouse model (Table 3). These results show that *vagA*, *vagC*, *vagG*, *vagH*, and *vagI* encode products that are essential for virulence. Furthermore, *vagB*, *vagE*, and *vagF* are not essential per se but are contained within operons involved in virulence. Interestingly, the product of *vagH* somehow modulates the production of virulence effectors.

DISCUSSION

Identification of novel virulence genes that are essential only during in vivo growth. Most bacterial genome-based searches have focused on identification of genes that are essential for bacterial viability as potential targets for new types of antimicrobial compounds (4, 20, 55). In this study, we have evaluated a new concept in the identification of drug targets. On the basis of a bioinformatic approach, we sought to identify novel genes that are strictly required for in vivo growth. Such genes may constitute novel targets for therapeutics, since they are required for bacterial survival in the host.

Among a selection of six human pathogens, including one obligate intracellular bacterium, 17 conserved genes with unknown functions were identified. Among these 17 conserved *vag* genes, five (*vagA*, *vagC*, *vagG*, *vagH*, and *vagI*) were directly associated with virulence, since nonpolar mutants were still avirulent. In addition, four *vag* genes were contained within operons associated with virulence. Thus, a high proportion of the *vag* genes were associated with virulence, demonstrating that the in silico screening strategy identified novel virulence-associated genes and gene clusters. Interestingly, these genes are conserved among a large number of both gram-positive and gram-negative bacteria that were not included in the screen.

The common theme among the identified *vag* genes is their relationship with fundamental cellular processes, such as DNA or RNA processing. However, despite their predicted functions, the genes were not essential for growth in vitro. Importantly, these genes are generally conserved among a large number of different bacteria that are not necessarily pathogenic. One interesting example is *vagA*, which shows similarity to *E. coli ychF*. The corresponding protein contains the four structural motifs characteristic of GTP-binding proteins. YchF belongs to a group of 11 universally conserved bacterial GTPases (8), and in addition, *ychF* homologues have been found in all three domains of life (34). Therefore, it was an unexpected finding that both *Yersinia vagA* insertion and nonpolar deletion mutants were not affected in growth yet were strongly attenuated for virulence compared to the wild-type strain. This correlates to signature-tagged mutagenesis data showing that the *vagA/ychF* homologue in *Neisseria meningitidis* was required for virulence in an infant rat model (53). Thus, it seems likely that *ychF* homologues are virulence associated in several pathogens.

The *vagC* homologue in *E. coli*, *rluD*, encodes one of the 10 known putative pseudouridine synthases in this bacterium. Pseudouridine is the most abundant modified nucleoside and was the first to be discovered in RNA. Pseudouridine synthases catalyze the isomerization of specific uridines in cellular RNA to pseudouridines. This modification has been proposed to play a structural role in polyribonucleotides, which may explain

why some genetic mutants exhibit difficulties in translation and display a growth disadvantage compared to the wild-type strain (reviewed by Charette and Gray [10]). An RluD-deficient strain of *E. coli* is blocked in 23S rRNA pseudouridine formation and is growth restricted (43). Recent data also indicate that RluD may have two different functions, since a point mutant unable to form specific pseudouridines in *E. coli* 23S rRNA restores normal growth in an RluD null mutant (24). Surprisingly, neither the insertion nor the in-frame deletion *vagC* mutant of *Y. pseudotuberculosis* was impaired for growth in vitro, even during competition with the wild-type strain. Our *vagC* null mutants were not defective in Yop secretion or cytotoxicity yet were clearly attenuated in mice.

The importance of RNA-modifying proteins in virulence was further supported by studies of *miaA*, a member of our control group (Table 2), which encodes a product that cooperates with other proteins to modify ms^2i^6A nucleoside in tRNA (5). Consistent with a role in *Yersinia* virulence, MiaA is also involved in *Shigella* virulence, involving a mechanism affecting the expression of the transcriptional activator VirF (16). Even though VirF is a regulatory protein that positively regulates the type III secretion system of *Shigella*, we could not find a link between MiaA and type III secretion in *Yersinia*.

Interestingly, *vagG* is located within the gene region *fms-aroE*, which harbor genes associated with virulence in *Salmonella*. The *fms-aroE* intergenic region was shown to be associated with the virulence phenotype of host cell endosome aggregation that is necessary for intracellular replication of *Salmonella* within host cell endosomes, a process that also involves the type III secretion system within SPI-2 (25). The *fms-aroE* intergenic region is conserved in *Yersinia*, but not all of the genes are conserved in the other genomes compared in this study. However, *smf* was shown not to be involved in the endosome aggregation phenotype induced by *Salmonella* (25), but the role of *smf* in *Salmonella* virulence has not been further elucidated. The *vagG* mutant of *Yersinia* was attenuated in mice yet grew normally in vitro, efficiently synthesized Yops, and could induce a rapid cytotoxic response towards infected HeLa cells. The *smf* homologues in several other bacteria, such as *S. pneumoniae*, *Haemophilus influenzae*, *H. pylori*, and *Bacillus subtilis*, are implicated in the uptake of DNA in these naturally competent bacteria (3, 9, 30, 39). However, no link between virulence and *smf* has been established in these pathogens, although a role for the *smf* homologue *dprA* in *N. meningitidis* virulence has been reported (53).

vagH, which has a predicted function in protein translation, was also found to be associated with virulence in *Yersinia*. The gene product VagH shows similarity to *E. coli* HemK, an *N*(5)-glutamine methyltransferase that modifies peptide release factors (28, 36). The *hemK* gene is ubiquitous in both gram-negative and gram-positive bacteria, pointing to an essential role in cell viability, yet loss of *vagH* did not affect the growth of this *Yersinia* mutant in rich medium. However, a *vagH* mutant did grow more slowly in a less-nutrient-rich defined medium than did the wild-type strain. Further, both mutant variants synthesized and secreted reduced amounts of Yops. Nevertheless, a *vagH* mutant still induced a cytotoxic response, although slightly delayed, towards HeLa cells. The *hemK* genes of *E. coli* and *Yersinia* are located just downstream of *prfA* (the gene encoding release factor 1 [RF1]). HemK methylates both RF1 and RF2 in vitro. In the case of *E. coli* K-12, in vitro N^5-methylation of the glutamine residue is important for the activity of RF2 (28) but contributes little to the activity of RF1 (15). In contrast to the case for a *vagH* mutant of *Y. pseudotuberculosis*, deletion of *hemK* in *E. coli* K-12 resulted in a severe growth defect in rich medium, which could be reversed either by transcomplementation of *hemK* or by a suppressor mutation in RF2 (28, 36). This may in part be due to an unusual variant of RF2 in *E. coli* K-12 which is more dependent on a functional HemK than RF2 in other *E. coli* strains. Thus, the physiological role of HemK in other bacteria remains to be clarified. Interestingly, SirB, which is encoded by the gene located just downstream of *hemK*, was recently shown to be an indirect regulator of the expression of one of the type III secretion systems in *Salmonella* encoded within SPI-1 (42). However, preliminary data (S. Garbom and H. Wolf-Watz, unpublished data) indicate that *sirB* in *Yersinia* is not involved in virulence in mice and does not affect the expression of Yops. Thus, the role for VagH in Yop synthesis and virulence warrants further investigation.

The function of the *vagI* gene, which shows similarity to the *E. coli* conserved hypothetical gene *yebC*, is unknown. In *Y. pseudotuberculosis vagI* is the third gene in what might be an operon consisting of six genes. The gene order of these six genes is also conserved in *E. coli*. However, the region is not particularly well conserved among the other bacteria included in this study. The nonpolar *vagI* mutant secreted normal amounts of Yops and showed cytotoxicity towards HeLa cells, but it was strongly attenuated, demonstrating that *vagI* is essential for survival of the pathogen in vivo.

Numerous studies using other techniques, such as STM, for in vivo selection of virulence-associated genes have identified many genes involved in bacterial survival in vivo (reviewed in references 32 and 51). A study using STM to identify essential genes in vivo in *N. meningitidis* (53) identified 65 virulence-associated genes, including homologues of *vagA* and *vagG*. However, a polar effect on downstream genes was not excluded in that study. Nevertheless, the study suggests that the *vag* genes associated with virulence in *Yersinia* also may be general virulence factors not earlier described. Although many previous studies using diverse elegant but often laborious methods, such as STM and in vivo expression technology (26), for identifying in vivo-expressed genes have identified a substantial number of such genes (32, 51), it is obvious that there are yet more genes to be discovered. This highlights the importance of developing new complementary methods for identifying virulence-associated genes.

Impact on antibacterial research. It is apparent that treatment of infections with broad-spectrum antibiotics stimulates the rapid emergence of multiply resistant bacterial strains, making restricted usage of these antibiotics an urgent requirement. Therefore, this demands the development of new antibacterial agents by engaging strategies that specifically counteract the development of resistance. Antibacterial therapy, which involves chemical attenuation of the pathogen during infection without targeting growth, requires that the host's immune defense clears the infectious agent. To date, this is an unproven, novel strategy to cure infections. Targeting of gene products directly associated with virulence will likely have this characteristic, since mutations in the targeted genes will select

not for resistance but rather for attenuation of the pathogen. In this study, we identified five novel *vag* genes that are involved in virulence of *Y. pseudotuberculosis*, since strains harboring nonpolar in-frame mutations of these genes were attenuated in vivo. Presumably, this was not due to a growth defect in vitro, because these mutants, with one exception, grew normally in laboratory culture. Rather, these Vag proteins must be strictly involved in processes of pathogen survival under in vivo conditions. These Vag proteins constitute possible molecular targets for novel bacterial therapeutic discoveries, particularly if their homologues in other bacteria are also required for survival of the pathogens in vivo.

In summary, we have presented a successful strategy to identify novel virulence-associated factors. Analysis of the widespread involvement of these factors in virulence of other pathogens and whether these proteins can be targeted by different antibacterial drugs is the theme for future investigation. Understanding Vag function may also unravel novel virulence traits, which is particularly important in cases where homologues exist in severe pathogens that are intrinsically difficult to study. These studies may even facilitate the understanding of functions in their human counterparts. It is interesting that six of the nine *vag* genes found to be associated with virulence in *Yersinia* also have human homologues. This may not exclude them as potential targets for development of antibacterial agents, since a number of antibiotics used today target bacterial genes which have human homologues, showing a similarity or homology of as much as 28% (35).

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