Determinants of Non-toxicity in the Gastric Pathogen *Helicobacter pylori*

Received for publication, April 17, 2003

Published, JBC Papers in Press, May 8, 2003, DOI 10.1074/jbc.M304071200

Darren P. Letley‡, Joanne L. Rhead, Rachel J. Twells, Brian Dove, and John C. Atherton$§

From the Division of Gastroenterology and Institute of Infections, Immunity and Inflammation, University of Nottingham, C Floor, West Block, Queen’s Medical Centre, Clifton Boulevard, Nottingham NG7 2UH, United Kingdom

The *Helicobacter pylori* vacuolating cytotoxin gene, vacA, is naturally polymorphic, the two most diverse regions being the signal region (which can be type s1 or s2) and the mid region (m1 or m2). Previous work has shown which features of vacA make peptic ulcer and gastric cancer-associated type s1/m1 and s1/m2 strains toxic. vacA s2/m2 strains are associated with lower peptic ulcer and gastric cancer risk and are non-toxic. We now define the features of vacA that determine the non-toxicity of these strains. To do this, we deleted parts of vacA and constructed isogenic hybrid strains in which regions of vacA were exchanged between toxigenic and non-toxigenic strains. We showed that a naturally occurring 12-amino acid hydrophilic N-terminal extension found on s2 VacA blocks vacuolating activity as its removal (to make the strain s1-like) confers activity. The mid region of s2/m2 vacA does not cause the non-vacuolating phenotype, but if VacA is unblocked, it confers cell line specificity of vacuolation as in natural s1/m2 strains. Chromosomal replacement of vacA in a non-toxigenic strain with vacA from a toxigenic strain confers full vacuolating activity proving that this activity is entirely controlled by elements within vacA. This work defines why *H. pylori* strains with different vacA allelic structures have differing toxicity and provides a rational basis for vacA typing schemes.

Gastric colonization by *Helicobacter pylori* is the main cause of peptic ulceration, gastric carcinoma, and gastric mucoassociated lymphoid tissue lymphomas (1–3). However, although more than half the world’s population is chronically infected with this Gram-negative bacterium, most people remain asymptomatic. Who develops disease depends on strain virulence, host genetic susceptibility, and environmental factors. Several bacterial virulence factors have been linked with virulence, host genetic susceptibility, and environmental factors. Several bacterial virulence factors have been linked with disease: the active form of the vacuolating cytotoxin, VacA (4, 5); presence of a pathogenicity island encoding a type IV secretion system, cag (6–8); and possession of a specific adhesin, BabA (9).

The active form of VacA induces extensive cytoplasmic vacuolation in epithelial cells (10), causes gastro-duodenal damage in a mouse model (11), and increases gastric ulcer risk in *H. pylori*-infected Mongolian gerbils (12). The vacA gene encodes a preprotoxin of ~139 kDa (13–16). This includes an N-terminal signal peptide and a ~50-kDa C-terminal auto transporter domain, both of which are cleaved during toxin secretion through the bacterial membranes (15). The ~90-kDa mature toxin may undergo further processing at an exposed protease-sensitive loop into ~37-kDa N- and ~58-kDa C-terminal fragments (p37 and p58, respectively) (15, 17). The toxin binds to cell surface receptors through the p58 domain (18–20). However, when expressed in epithelial cells, only p37 and an N-terminal fragment of p58 are required for vacuolation (21). Vacuolation is dependent on the insertion of VacA multimers into cell membranes to form anion selective pores (22, 23). The mechanism of subsequent vacuole formation and the cellular origin of the vacuoles from late endosomes have been studied extensively (24–30).

Research has focused on the most active form of VacA, but most *H. pylori* clinical isolates express less vacuolating or non-vacuolating forms (31). The gene, vacA, is naturally polymorphic and differences are most marked in two areas: the signal region, encoding the signal peptide and the N terminus of the mature protein (which may be type s1 or s2) and the mid region, encoding part of the p58 domain (type m1 or m2) (31). vacA signal and mid regions from all clinical isolates of *H. pylori* can be classified as one of these types, and all combinations of signal and mid region occur naturally, although the s2/m1 structure is rare (31, 32). The vacA allelic type is associated with vacuolating activity in vitro: strains with s1/m1 vacA cause more extensive vacuolation in HeLa cells than those with s1/m2 vacA, and vacA s2/m2 strains are invariably non-vacuolating (31). The existence of stable polymorphisms affecting toxin function is of major biological interest; it may form a paradigm for varying functionality of proteins in other bacteria with high levels of genetic recombination in which mosaic genes are common. However, for *H. pylori*, vacA polymorphism is also potentially of major clinical importance: vacA s2/m2 strains are less frequently associated with both peptic ulceration and gastric carcinoma than vacA s1/m1 or s1/m2 strains (33–38).

In this report, we define the determinants of non-toxicity in *H. pylori* with type s2/m2 vacA. Work by us and others on the most toxic s1/m1 type of vacA has guided our approach. We have previously shown that vacA transcription is higher for some toxic than for some non-toxic strains (39). We and others have also shown that the N terminus of mature s1/m1 VacA in toxic strains is one important determinant of toxicity and that adding an s2-like N-terminal extension blocks activity (21, 40–43). However, one cannot extrapolate from this to imply that the N terminus of natural s2/m2 strains is the cause of their non-toxicity, so we aimed to determine whether this was indeed the case. Naturally occurring vacA type s1/m1 strains cause vacuolation in a wider range of cell lines than s1/m2 strains (19, 44), so we also planned to show whether the m2 mid region was the cause of non-toxicity in natural s2/m2 strains.

* This work was supported in part by a grant from the Medical Research Council (MRC) (United Kingdom). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
§ Supported by an MRC Senior Clinical Fellowship.
Finally, it is unclear whether elements outside vacA in s2/m2 strains contribute to non-toxicity, and we aimed to resolve this issue.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions—**

_H. pylori_ strains (Table I) were grown on blood agar base 2 supplemented with 7% (v/v) horse blood (Oxoid Ltd.) microaerobically at 37 °C (CampyPak Plus, BD Biosciences UK Ltd.). Broth cultures were grown in Iso-sensitest medium (Oxoid Ltd.) with 5% (v/v) FCS (Invitrogen) microaerobically with shaking.

Construction of Isogenic vacA Signal Region Hybrid Strains—The effect of vacA signal region type on toxin production and activity was studied by constructing _H. pylori_ isogenic vacA hybrid strains in which different extents of the signal region had been exchanged between the _s1_ and _s2_ forms on the _H. pylori_ chromosome. Such hybrids were constructed using plasmids pA153::cat and pCTB2::cat, containing the promoter and _5’_ terminus of _vacA_, including the signal region, from strains _Tx30a_ (vacA s2/m2; _Tox−_ and _84-183_ except _vacA_), respectively, with _3’_ terminus of the upstream gene _cysS_ with a chromopatholink resistance marker (chromopatholink acetyltransferase; cat) inserted immediately downstream (see Table I). These pBluescript-derived plasmids act as suicide vectors in _H. pylori_, thus chromosomal recombinants were constructed by natural transformation, allelic exchange, and chloramphenicol resistance as previously described (41) (see Fig. 1). Transformation of pA153::cat and pCTB2::cat into their respective homologous strains resulted in the isolation of control strains _Tx30a/CAT_ and _60190/CAT_, containing the insertion of _cat_ upstream of the _vacA_ promoter. Transformation of _Tx30a_ with pCTB2::cat and _60190_ with pA153::cat gave rise to multiple hybrid strains. DNA extracted from single colonies (45) was typed for _vacA_ signal and mid region by allele-specific PCR, as previously described (31, 46). Only _vacA_ type _s1_ recombinants were identified from the transformation of _Tx30a_ with pCTB2::cat, and one was selected and termed _Tx30a/P1S1_. For the transformation of _60190_ with pA153::cat, type _s1_ and _s2_ recombinants were identified and termed _60190/P2S1_ and _60190/P2S2_, respectively. The precise extent of recombination was determined for these mutants by nucleotide sequencing. pA153::cat and pCTB2::cat were similarly transformed into the toxigenic strain _84-183_ (vacA s1/m1) to give hybrids _84-183/P2S2_ and _84-183/P1S1_, respectively.

**Construction of vacA Mid Region Hybrid Strains—**

The effect of _vacA_ mid region type was studied in a similar manner. Plasmids pNV5 and pNV2, containing _3’_ terminal _3576_ bp and _3587_ bp of _vacA_ with 1 kb and 1.5 kb of downstream sequence from strains _Tx30a_ and _60190_, respectively, with a kanamycin resistance marker (aminoglycoside phosphotransferase; _aphA_) inserted _0.5_ kb _3’_ to _vacA_, within the _fecE_ gene, were introduced into strain _Tx30a_ by natural transformation, allelic exchange, and kanamycin marker rescue. Recombinants were typed for _vacA_ signal and mid region by allele-specific PCR (31, 46). Transformation with pNV5 and pNV2 gave rise to the control strain _Tx30a/KAN_ and the hybrid strain _Tx30a/P1S1M1_.

Site-directed Mutagenesis of vacA—The _vacA_ mutant _Tx30a/P1S1M1_, containing an in-frame deletion of the region encoding the _s2-specific N-terminal extension, was constructed by first deleting this _36-bp region from the cloned _vacA_ fragment in plasmid pA153::cat using inverse PCR with primers _NdeIF_ (5’-GCTTTTTTTCACACCCACGTTTTCATACTTTTT-3’) and _NdeIR_ (5’-AGGCCGCCAGTTCCTTTGAGTCACTTACACCC-3’), which bound to nucleotides _472_ to _501_ and _407_ to _435_ in the _vacA_ sequence of strain _30a_ (31, respectively). Template DNA was removed by _DpnI_ restriction endonuclease digestion (New England BioLabs (UK) Ltd.), and the PCR product was end-polished using _Pfu_ DNA polymerase (Stratagene) and _Pfu_ DNA polymerase (Stratagene, E). Transformation into _Escherichia coli_ strain _DH5α_ Plasmid DNA was extracted, and the presence of the _36-bp deletion screened by PCR using primers _VA1F_ and _VA1R_ (31), and confirmed by nucleotide sequencing. The _vacA_ mutation was introduced into chromosomal _vacA_ of _H. pylori_ strain _Tx30a_ by natural transformation, allelic exchange, and chloramphenicol marker rescue, and the presence of the _vacA_ mutation was confirmed by PCR as before.

**Quantification of VacA—**

VacA production was quantified by antigen detection ELISA as previously described (47). Briefly, duplicate 48-h broth culture supernatant samples of each strain were adsorbed to a microtiter plate overnight at 4 °C, blocked with 3% bovine serum albumin.
Determinants of Non-toxicity in H. pylori

RESULTS

The Type s2 Promoter/Signal Region of vacA Determines the Non-toxic Status of the Tox− Strain Tx30a—Our previous work had shown that vacA s1/m1 strains are usually toxic and vacA s2/m2 strains invariably non-toxic (31). Our first aim was to define whether the promoter/signal region of a candidate non-toxic vacA s2/m2 strain, Tx30a, was directly responsible for its non-vacuolating phenotype. To do this, we used allelic exchange to replace the promoter and signal region of vacA on the Tx30a chromosome with that of strain 60190 (tox++; vacA s1/m1) to make an artificial hybrid s1/m2 vacA construct in a non-toxigenic strain background, which we called Tx30a/P1S1 (see “Materials and Methods” and Fig. 1). Nucleotide sequencing of this hybrid showed the crossover point to be between 588 and 763 in the Tx30a sequence (31) and proved that the promoter region was identical to that in the s1/m1 strain 60190. To ensure that insertion of the chloramphenicol resistance marker (cat), used for the allelic exchange experiment, was not influencing VacA production or activity, we also constructed the control strain Tx30a/CAT with cat inserted between cysS and vacA (see “Materials and Methods”). Insertion of the chloramphenicol cassette in Tx30a did not affect VacA levels in broth culture supernatants, as determined by antigen detection ELISA, and all later comparisons used Tx30a/CAT as control. Replacement of the signal and promoter regions with those of 60190 increased vacA production as determined by ELISA using antisera Ab927 (Tx30a/CAT mean vacA production 0.03 ± 0.01, n = 10, versus Tx30a/P1S1 mean 0.17 ± 0.05, n = 8, p < 0.005, t test). This finding was confirmed by immunoblot using the same antisera (Fig. 2). As expected, water extracts of the non-toxigenic control strain, Tx30a/CAT, did not cause vacuolation of any of the three cell lines tested (Fig. 3A and Table II). However, Tx30a/P1S1 water extracts induced extensive cytoplasmic vacuolation of RK13 cells following overnight incubation (Fig. 3B). Interestingly, the vaculating activity observed for this strain was cell line-specific, because the same water extracts were unable to induce vacuolation of HeLa and AGS cells (Table II). This is consistent with the described phenotype of a naturally occurring vacA s1/m2 strain (19). Thus, naturally occurring differences in vacA signal regions are directly responsible for VacA production and activity.

Confirmation That the s2 Promoter/Signal Region of vacA Determines Non-toxic Status Using Tox+ Strain Backgrounds—Using identical methodology, we confirmed our results by performing reciprocal experiments in which we replaced the signal and promoter regions of vacA in strain 60190 (tox++; vacA s1/m1) with those of Tx30a (tox−; vacA s2/m2) to create the hybrid strain 60190/P2S2. Nucleotide sequencing confirmed that the sequence up to nucleotide 1070 had been replaced with that of Tx30a. As for Tx30a, we constructed a control strain of 60190 with the chloramphenicol cassette inserted just after the cysS stop codon; this did not affect VacA production or activity, and 60190/CAT was used as the control in all further experiments. Insertion of the signal and promoter region from Tx30a reduced vacA production as determined by ELISA using Ab123 (60190/CAT mean vacA production 1.01 ± 0.12, n = 8, versus 60190/P2S2 mean 0.19 ± 0.02, n = 4, p < 10−3). As expected, 60190/CAT water extracts caused extensive cytoplasmic vacuolation of all three cell lines studied (Fig. 4A and Table II). In contrast, no vacuolation of HeLa, AGS, or RK13 cells was observed with water extracts of the hybrid strain 60190/P2S2 (Fig. 4C and Table II). Next, we aimed to confirm our results in a third independent strain background. We selected strain 84-183, an easily naturally transformable strain of vacA type s1/m1, which we have previously shown to transcribe VacA less strongly than strain 60190 and to be less strongly vaculating (39). Replacement of the signal and pro-
moter regions of vacA in strain 84-183 with those of strain Tx30a (to make strain 84-183/P2S2) reduced VacA production as determined by ELISA using Ab123 (84-183 mean VacA production 0.20 ± 0.01, n = 6

Table II

| Strain     | vacA type | Vacuolating activitya |
|------------|-----------|-----------------------|
|            |           | HeLa | AGS | RK13 |
| Tx30a      | s2/m2     | -    | -   | -    |
| Tx30a/CAT  | s2/m2     | -    | -   | -    |
| Tx30a/P1S1 | s1/m2     | -    | -   | +    |
| Tx30a/N1   | s2s1/m2   | -    | -   | +    |
| Tx30a/KAN  | s2/m2     | -    | -   | -    |
| Tx30a/M1   | s2/m1     | -    | -   | -    |
| Tx30a/P1S1M1 | s1/m1       | -    | +   | +    |
| 60190      | s1/m1     | +    | -   | -    |
| 60190/CAT  | s1/m1     | +    | -   | +    |
| 60190/P2S1 | s1/m1     | +    | +   | -    |
| 60190/P2S2 | s2/m1     | -    | -   | -    |
| 60190/KAN  | s1/m1     | +    | -   | -    |
| 60190/M2   | s1/m2     | -    | -   | -    |

a Vacuolating activity was assessed by incubating either HeLa, AGS, or RK13 epithelial cells overnight with a water extract of the appropriate strain. Vacuolation was recorded as positive if more than 50% of the cells within a randomly chosen field were vacuolated and negative if the number of vacuolated cells was the same or less than that observed for untreated cells.

b The signal region of this strain is s2 type for the signal peptide including the cleavage site, and s1 type for the mature N terminus.

for both, p < 10^-5). As expected, strain 84-183 caused vacuolation in HeLa cells, whereas strain 84-183/P2S2 did not (data not shown). In complementary experiments, we replaced the signal and promoter regions of strain 84-183 with those of strain 60190 (to make strain 60190/P2S2), and this increased VacA production (to mean 0.31 ± 0.02, n = 8, p < 0.005). Both strains vacuolated HeLa cells (data not shown). These experiments show that replacing the type s1 promoter and signal region in a tox+/strain with a type s2 region from a tox- strain reduced VacA production and abolished activity. Interestingly, strains 60190/P2S2 and 84-183/P2S2, which both have a hybrid s2/m1 vacA structure, grew similarly to their respective controls on blood agar and in broth. Thus the reason that strains of vacA type s2/m1 are uncommon in nature is not that they have obvious self-toxicity or a growth disadvantage, at least in vitro.

The vacA Promoter Region Determines Differences in VacA Production, but the Signal Region Determines Differences in Vacuolating Activity—Having shown directly that the vacA signal and promoter regions in vacA s2/m2 strain Tx30a were together an important determinant of its non-toxigenic status, we now aimed to define the role of each region individually.
From the previous experiments it was unclear whether the low level of VacA production in strains Tx30a and 60190/P2S2 was the reason these strains were non-toxic or whether there were other determinants of non-toxicity. We aimed to address this by screening for transformants from our previous experiments where the recombination point was between the promoter region and signal sequence coding region. Screening of mutants generated by transforming strain Tx30a with the promoter and signal regions of vacA from 60190 yielded no such transformants. However, the transformation of 60190 with pA153::cat yielded a transformant, which typed as s1 by allele-specific PCR, and we called this 60190/P2S1. Sequence analysis showed that this hybrid contained a promoter region and ribosomal binding site identical to that of the type s2 strain Tx30a, but the signal sequence remained 60190 vacA. The exact 3’ crossover point was between nucleotides 795 and 835 in the 60190 vacA sequence (13). Studying this hybrid allowed us to determine the relative effects of the s2 promoter and signal regions in the 60190 strain background. Replacing the promoter region only, significantly reduced VacA ELISA levels using Ab123 (60190/P2S1 mean 0.34 ± 0.09, n = 4, p < 0.005, versus 60190/CAT, not different from 60190/P2S2). However, in contrast to 60190/P2S2, which did not express vaculating activity, 60190/P2S1 caused vacuolation in all three cell lines (Fig. 4B and Table II). Thus the lack of vaculating activity observed for 60190/P2S2 was not simply due to reduced VacA production as 60190/P2S1 produced similar levels of VacA but still retained vaculating activity. This shows that, as expected, the promoter region, not the signal region, determines VacA production. However, the signal region is responsible for determining VacA activity.

The N-terminal Hydrophilic Extension on Mature Type s2 VacA Determines Its Non-vaculating Phenotype—A striking difference in the signal region between vacA type s1 and s2 strains is the presence of a 12-amino acid extension to the non-toxicogenic, s2 form of VacA (31). The mature N terminus of s1 type VacA is markedly hydrophobic, whereas the s2-specific, N-terminal amino acid extension is strongly hydrophilic (Fig. 5). We hypothesized that the presence of this hydrophilic extension blocks the vaculating activity of VacA produced by s2 strains such as Tx30a. To test this, we made an in-frame deletion in Tx30a vacA of the 36 nucleotides encoding this extension, thus removing it from the mature VacA protein (see “Materials and Methods”). We named the resulting isogenic mutant strain Tx30a/N1. Removal of the N-terminal amino acid extension reversed the non-vaculating phenotype of Tx30a, such that water extracts of Tx30a/N1 caused extensive vacuolation of RK13 cells (Fig. 3C). Tx30a/N1 did not vacuolate AGS or HeLa cells (Table II). Note that Tx30/N1 has a type m2 mid region, so this result is consistent with the described phenotype of a naturally occurring vacA s1/m2 strain (19). To ensure that the vaculating activity of Tx30a/N1 was not due to a greater VacA production compared with Tx30a/CAT, we determined the amount of VacA in water extracts by ELISA using Ab123. VacA levels were no greater for Tx30a/N1 than Tx30a/CAT (means 0.11 ± 0.02 for both, n = 10 and 4, respectively, p = ns). Thus, the non-toxicogenic phenotype of the vacA s2/m2 strain Tx30a is due to the hydrophilic extension on the N terminus of mature VacA.

Replacing the m2 Mid Region of a Non-toxicogenic Strain with an m1 Mid Region Is Not Sufficient to Render It Toxogenic—Having shown that non-toxic vacA s2/m2 strains could be rendered toxic by replacing the signal region with an s1 region or by removing the N-terminal amino acid extension on the mature VacA protein, we now aimed to assess whether these strains could also be rendered toxic by changing the mid region to an m1 type. To do this we replaced the vacA mid region of strain Tx30a with that of strain 60190 (tox+; vacA s1/m1) to make a vacA s2/m1 construct in a non-toxicogenic strain background, which we termed Tx30a/M1 (see “Materials and Methods”). Sequence analysis confirmed that the vacA mid region sequence had been exchanged for the 60190 sequence downstream of nucleotide 828. This strain also gained the 3’ region of vacA from 60190, but this region, which encodes the C-terminal bacterial outer membrane transporter, which is cleaved from mature VacA after export, is very similar between all vacA alleles (48). As the kanamycin resistance marker used for allelic exchange was located 3’ to vacA within fecE, we also constructed the control strain Tx30a/KAN containing just the marker insertion.

Insertion of the kanamycin cassette in fecE did not affect growth on blood agar or VacA activity (Table II), and all later comparisons used Tx30a/KAN as the control. Inactivation of fecE, encoding the ATP-binding protein component of an Fe3⁺–dicitrate ABC transporter, has previously been shown not to affect Fe3⁺ or Fe3⁺ transport, or growth on brain heart infusion–FCS medium (49). Replacing the vacA m2 mid region with an m1 type did not render the strain toxigenic; neither Tx30a/KAN nor Tx30a/M1 caused vacuolation in any of the three cell lines tested (Table II and Fig. 3D). VacA levels in water extracts of Tx30a/M1 were not significantly different to those of Tx30a/KAN as determined by ELISA using an m2-specific antibody (Ab927) (means 0.32 ± 0.01, n = 4 and 0.35 ± 0.004, n = 2). However, using an m1-specific antibody (Ab929) VacA levels appeared 2-fold higher for Tx30a/M1 (means 0.45 ± 0.01, n = 4 and 0.22 ± 0.01, n = 2, p < 10⁻³). Although it is not possible to directly compare VacA production for these strains by ELISA, owing to antigenic differences in the mid region, it is worth noting that, even if VacA levels were higher in Tx30a/M1 water extracts, no vacuolating activity was observed. This shows that merely replacing the m2 mid region with an m1 mid region is insufficient to render a non-toxicogenic vacA s2/m2 strain toxigenic. Presumably, the type s2 N-terminal extension is sufficient to block toxic activity. Interestingly, strain Tx30a/M1, which has a hybrid s2/m1 vacA structure, grew similarly to Tx30a and Tx30a/CAT on blood agar and in broth. This con-
firms, in a Tx30a background, our findings in the 60190 and 84-183 backgrounds, that possession of vacA with the naturally uncommon s2/m1 structure is not obviously disadvantageous in vitro.

vacA from a Toxigenic Strain Is Sufficient to Render a Non-toxigenic Strain Fully Toxigenic—The failure of an m1 vacA mid region to render tox- strain Tx30a toxigenic could have been because the m1 mid region was non-functional in this Tx30a/s1M1 hybrid. To refute this, we next replaced the s2 signal and promoter regions with s1 regions derived from strain 60190, to make a vacA s1/m1 construct in a non-toxigenic Tx30a background. Our second and more important aim in making this hybrid was to replace type s2/m2 vacA in Tx30a with type s1/m1 vacA from 60190 to assess to what extent single copy chromosomal complementation with toxigenic vacA conferred activity in a non-toxigenic strain background.

To replace the s2 signal and promoter regions in Tx30a/s1 with s1 regions from 60190, we transformed Tx30a/M1 with pCTB2:cat to produce the hybrid strain Tx30a/P1S1M1. Sequence analysis of vacA from this hybrid confirmed that the gene had been replaced with that of the toxigenic strain 60190 with the exception of a 190-bp region from nucleotides 639 to 828 encoding part of the p37 domain of the VacA protein, which was still derived from Tx30a. Water extracts of Tx30a/P1S1M1 induced extensive vacuolation of all three cell types studied, similar to 60190 and controls 60190/CAT and 60190/KAN (Table II and Fig. 3E). VacA levels in the Tx30a/P1S1M1 and 60190/KAN water extracts were also similar as determined by ELISA using Ab929 (means 0.50 ± 0.10 and 0.43 ± 0.01, respectively, n = 4 for both, p = ns). This confirms that the m1 mid region is functional in a Tx30a background and that vacA itself from a toxigenic strain is sufficient to confer full toxigenic activity. Thus differences between tox+ and tox− strains elsewhere on the chromosome do not contribute to differences in VacA toxicity between strains.

The m2 Mid Region from a Non-Toxigenic vacA s2/m2 Strain Confers Cell-line Specificity of Vakuolating Activity to a Toxigenic s1/m1 Strain—The type m2 mid region of a naturally occurring vacA s1/m2 strain has previously been shown to confer specificity of vacuolization such that the strain vaculates RK13 cells but not HeLa or AGS cells (19). Our previous experiment described above in which we created Tx30a/P1S1 with its s1/m2 structure, suggests that the m2 mid region from an originally non-toxigenic vacA s2/m2 strain also confers cell specificity. To prove that the m2 vacA mid region is directly responsible for this effect, we replaced the type m1 mid region in strain 60190 with the m2 mid region from Tx30a to create the vacA s1/m2 hybrid strain 60190/M2. Sequence analysis confirmed that the mid region had been replaced with the m2 form downstream of nucleotide 1696. As before we also constructed the control strain 60190/KAN containing the kanamycin marker insertion in fecE. Water extracts of 60190/M2 and 60190/KAN vaculated RK13 cells equally (Fig. 4D). However, in contrast to the control, which also caused extensive vacuolation of HeLa and AGS cells, 60190/M2 did not cause vacuolation of either of these cell types (Table II). The amount of VacA in water extracts of 60190/M2 appeared nearly 2-fold lower than those of the control 60190/KAN, as determined by ELISA using the m1-specific antibody, Ab929 (means 0.23 ± 0.01 and 0.43 ± 0.01, n = 4 for both, p < 0.01−), but over 3-fold higher using the m2-specific antibody, Ab927 (means 0.83 ± 0.04 and 0.25 ± 0.005, n = 4 for both, p < 0.05−). Immunoblotting confirmed these findings (data not shown). Given that the ELISA values obtained with each antibody would have been underestimated for VacA alleles of the opposite mid region type, it is likely that the actual VacA amounts for the hybrid and control strains were similar. This experiment shows that the m2 mid region from a non-toxigenic vacA s2/m2 strain is functional in determining cell line specificity. Notably also, the vaculating phenotype of 60190/M2 was the same as that obtained for Tx30a/P1S1, showing that the activity of the s1/m2 form of vacA is independent of strain background.

**DISCUSSION**

Compared with toxigenic strains of *H. pylori*, non-toxigenic vacA s2/m2 strains are associated with a much lower risk of peptic ulceration and gastric adenocarcinoma (31, 36, 37, 50). In this study we have shown why vacA s2/m2 strains are non-toxigenic, the primary determinant being a 12-amino acid N-terminal extension on the VacA protein, which blocks toxin activity regardless of production level or p58 binding region type. Removal of this extension confers toxicity. The m2 vacA mid region in non-toxigenic s2/m2 strains is not the cause of non-toxicity, but is functional in conferring cell specificity. Chromosomal complementation with single copy vacA from a toxigenic vacA s1/m1 strain shows for the first time that elements outside vacA are not needed for full vacuolating activity.

Mature VacA from toxigenic vacA type s1 strains has a hydrophobic N-terminal region, which can insert into lipid bilayers (51). In non-toxigenic vacA type s2 strains this region is preceded by a 12-amino acid hydrophilic N-terminal extension, which we have shown blocks vacuolating activity. Partially deleting the hydrophobic N terminus of type s1 VacA (for example, amino acids 6–27) also blocks vacuolating activity, and, although VacA still forms pores in artificial membranes, they form more slowly and are less anion selective (42). We have previously shown that adding the s2-specific hydrophilic extension to the N terminus of type s1 VacA abolishes vacuolating activity (41). However, although this slows pore formation in artificial membranes, it does not abolish it nor change the anion selectivity of these pores (43). Thus, it remains unclear whether the hydrophilic N-terminal extension of s2 VacA blocks membrane insertion but that this is not necessary for pore formation in artificial lipid membranes, or whether it acts through another mechanism, for example, through changing the conformation of the active p37 subunit of VacA.

Our key finding is that type s2/m2 VacA is functionally vacuolating once the hydrophobic N-terminal extension is removed. Why *H. pylori* should possess a functional “blocked” form of VacA is a fascinating enigma. One possibility is that the toxin becomes activated in vivo through cleavage of this extension. However, if this occurs, it is insufficient to render such strains pathogenic. A second possibility is that VacA possesses an important biological function other than inducing vacuolation, which is not blocked by the s2 extension. For example, it may perform other functions ascribed to VacA such as increasing epithelial permeability, stimulating epithelial cell apoptosis (52), inhibiting antigen presentation (53), or binding to cytoskeletal proteins (54).

Wild-type *H. pylori* strains of vacA type s2/m1 have only rarely been isolated (32, 55, 56), and we have identified only one that expresses VacA. Population genetic analyses show that vacA structure is characterized by frequent recombination events between vacA from different strains (57–60), so vacA s2/m1 structures would be expected to arise in vivo as frequently as s1/m2 structures. In this study, we constructed the vacA s2/m1 structure in both originally toxigenic and originally non-toxigenic strain backgrounds, and these strains grew indistinguishably under laboratory conditions from other strains. Thus, although we speculate that s2/m1 vacA offers a selective disadvantage or fails to offer a selective advantage in vivo, the nature of this remains unclear.
An important finding in our study is that single copy chromosomal replacement of s2/m2 vacA in a non-toxic strain with s1/m1 vacA from a toxigenic strain confers full vacuolating activity similar to that of the parent toxigenic strain. This shows that production and vacuolating activity of VacA are not dependent on chromosomal elements outside vacA. Furthermore, as full vacuolating activity was conferred without amino acids 639–828 (which are fairly well conserved between vacA alleles) being replaced, we can infer that these residues do not contribute to reduced VacA activity.

Pathogenic strains with the s1/m1 type of VacA have been extensively characterized, including the determination of the complete genome sequence for two such strains (61, 55, 56). We have shown that strains with the type s2 signal sequence (which are fairly well conserved between vacA alleles) being replaced, we can infer that these residues do not contribute to reduced VacA activity.

Further studies are needed to determine the full significance of the mid region of both s2/m2 strains and s1/m2 strains and this region (31, 46), and our study confirms that this test has a rational biological basis. We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56).

We have now concentrated on the s2/m2 type of VacA found in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56).

We have now concentrated on the s2/m2 type of VacA found in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56).

We have now concentrated on the s2/m2 type of VacA found in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56). We have shown that strains with the type s2 signal sequence are present in non-pathogenic, non-toxigenic strains and the common hybrid s1/m2 type and uncommon hybrid s2/m1 type (32, 55, 56).
Determinants of Non-toxicity in H. pylori

Gibson, R., Merberg, D., Mills, S. D., Jiang, Q., Taylor, D. E., Vovis, G. F., and Trost, T. J. (1999) Nature 397, 176–180

63. Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty, B. A., Nelson, K., Quackenbush, J., Zhou, L. X., Kirkness, E. F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H. G., Glodek, A., McKenney, K., Fitzgerald, L. M., Lee, N., Adams, M. D., Hickey, E. K., Berg, D. E., Gocayne, J. D., Utterback, T. R., Peterson, J. D., Kelley, J. M., Cotton, M. D., Weldman, J. M., Fujii, C., Bowman, C., Watthey, L., Wallin, E., Hayes, W. S., Wendman, J. M., Borodovsky, M., Karp, P. D., Smith, H. O., Fraser, C. M., and Venter, J. C. (1997) Nature 388, 539–547

64. Pérez-Pérez, G. I., and Blaser, M. J. (1987) Infect. Immun. 55, 1256–1263