Paired cysteine residues are required for high levels of the *Helicobacter pylori* autotransporter VacA

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The *Helicobacter pylori* vacuolating cytotoxin VacA shares homology in its C-terminal domain with many autotransporter proteins, suggesting a similar mechanism of secretion. Like most autotransporters, VacA contains a single pair of cysteine residues located near the C-terminus of the passenger domain. This study aimed to investigate the role of these conserved cysteine residues. This involved changing each cysteine in the VacA passenger domain to serine, quantifying the effect on VacA levels and assessing toxin activity in *H. pylori*. It was shown that both cysteine residues were required for high VacA levels, although mutation of each cysteine reduced toxin amounts to differing extents, implying that their importance was not simply for intramolecular disulphide bond formation. Although less VacA was observed for the cysteine mutants, vacuolating activity was detected, showing that the cysteines were not required for VacA function.

INTRODUCTION

Chronic gastric colonization by *Helicobacter pylori* is the main cause of peptic ulcer disease and a significant risk factor for gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Parsonnet *et al.*, 1994, 1991; Warren & Marshall, 1983). Although more than half the world population is infected with *H. pylori*, most people remain asymptomatic. Who develops disease depends on strain virulence, host genetic susceptibility and environmental factors. One of the major *H. pylori* virulence factors which affects disease progression is the vacuolating cytotoxin VacA (Figura *et al.*, 1989; Tee *et al.*, 1995), which induces extensive cytoplasmic vacuolation in epithelial cells (Leunk *et al.*, 1988), permits cytochrome *c* release from mitochondria leading to apoptosis (Galmiche *et al.*, 2000), causes gastro-duodenal damage in a mouse model (Marchetti *et al.*, 1995) and increases gastric ulcer risk in *H. pylori*-infected Mongolian gerbils (Ogura *et al.*, 2000). The vacuolating activity of VacA has been extensively studied and shown to be dependent on the insertion of VacA multimers into cell membranes to form anion-selective pores (Szabó *et al.*, 1999; Tombola *et al.*, 1999). The mechanism of subsequent vacuole formation and the cellular origin of the vacuoles from late endosomes has been characterized (Cover *et al.*, 1993; Molinari *et al.*, 1997; Papini *et al.*, 1993a, b, 1994, 1996; Ricci *et al.*, 1997).

The *vacA* gene encodes a preprotoxin of ~139 kDa (Cover *et al.*, 1994; Phadnis *et al.*, 1994; Schmitt & Haas, 1994; Telford *et al.*, 1994) which includes an N-terminal signal

peptide and an ~50 kDa C-terminal domain that remains associated with the cell following secretion (Telford *et al.*, 1994). The secreted toxin monomer is 87–95 kDa (Cover & Blaser, 1992; Telford *et al.*, 1994). Such a genetic structure is characteristic of an autotransporter and, indeed, the C-terminal, cell-associated domain shares homology with other autotransporters (Loveless & Saier, 1997). Autotransporters are secreted proteins, generally assumed to direct their own transport across the outer bacterial membrane. However, recently, the autotransporter IgA1 protease of *Neisseria meningitidis* has been shown to have an additional requirement for the general outer membrane assembly protein Omp85 (Voulhoux *et al.*, 2003). Typified by the IgA1 protease of *Neisseria gonorrhoeae*, autotransporter proteins contain a three domain structure: an N-terminal signal sequence which is cleaved upon translocation across the inner membrane via the Sec pathway; a passenger domain which represents the mature secreted protein; and a C-terminal transporter domain which inserts into the outer membrane, forming a channel through which the passenger domain is exported (Pohlner *et al.*, 1987). Transporter domains may either oligomerize to form hexameric pores, as observed for the IgA protease (Veiga *et al.*, 2002), or function as monomers, as suggested for the adhesin-involved-in-diffuse-adherence (AIDA-I) of enteropathogenic *Escherichia coli* (Müller *et al.*, 2005). Following secretion, the passenger domain is cleaved from the autotransporter domain either by its own protease activity or by another protease (Henderson *et al.*, 1998). A fusion of the VacA β -barrel domain and the cholera toxin B subunit, expressed in *H.*

pylori, has been shown to allow export of the toxin B sub-unit, providing evidence of autotransport function (Fischer *et al.*, 2001).

VacA contains a single pair of cysteine residues located within the passenger domain with a spacing of 11 residues. The second cysteine in the pair is located 72 residues from the determined C-terminal domain processing site (Nguyen *et al.*, 2001). A striking feature of most autotransporters is a low cysteine content within the passenger domain (see Table 1) (Jose *et al.*, 1995), which is consistent with its translocation through the autotransporter channel in an unfolded state. However, a single cysteine pair is frequently located towards the C-terminal end of the passenger domain, with the most common spacing being 11 residues. Whether and how this cysteine pair contributes to autotransporter function has not been fully assessed. The *Serratia marcescens* autotransporter Ssp-1 is unusual in having two cysteine pairs within the passenger domain. Replacement of either cysteine pair with serine residues results in a small decrease in secretion of the mature protein across the *E. coli* outer membrane, which is only completely abolished when both cysteine pairs are replaced (Miyazaki *et al.*, 1989). However, owing to the atypical number of cysteines within the Ssp-1 passenger domain, it is unclear whether the single cysteine pair found in the passenger domains of other autotransporters has a similar role in secretion. This is of general interest for autotransporters, many of which are important in bacterial pathogenesis, and of particular interest for VacA.

The determinants of production of this major virulence factor are of potential importance in *H. pylori* pathogenesis, and *H. pylori* mutant strains expressing, but not secreting, VacA may be useful in vaccine research. Thus, our aim was to assess the importance of the paired cysteines to VacA autotransport in *H. pylori*.

METHODS

Bacterial strains, plasmids and growth conditions. *H. pylori* strain 60190 (ATCC 49503) was used as the parental strain for the construction of the mutants described in this study. Strain 60190:v1, containing a kanamycin marker insertion in *vacA* (Cover *et al.*, 1994), was used as a VacA-negative control strain. *H. pylori* strains were grown microaerobically on blood agar base 2 supplemented with 7% (v/v) horse blood (Oxoid) at 37 °C in a 5% CO₂ incubator. Broth cultures were grown in brain heart infusion broth (Oxoid) supplemented with 0.2% (w/v) β-cyclodextrin (Sigma-Aldrich) microaerobically (Campypak Plus, BD Biosciences) with shaking. *E. coli* strain XL-1 Blue (Stratagene Europe) was grown using Luria–Bertani broth, supplemented with 1.5% (w/v) Agar No. 1 (Oxoid) where appropriate. Where necessary, media were supplemented with 30 µg kanamycin ml⁻¹, 30 µg chloramphenicol ml⁻¹ or 100 µg ampicillin ml⁻¹ (all from Sigma-Aldrich).

Site-directed mutagenesis of *vacA*. The template used for site-directed mutagenesis of *vacA* was derived from pA148 containing the *vacA* gene from *H. pylori* strain 60190, together with the 3' terminus of the upstream gene *cysS*, and the downstream *fecE* and partial *fecD* sequences cloned as an *EcoRV*–*XhoI* fragment from genomic DNA into *E. coli* pBluescript (a kind gift from Dr T. L. Cover, Nashville, TN, USA). A chloramphenicol resistance marker

Table 1. Cysteine composition of several autotransporter proteins

For this study, cysteine pairs (C-C) have been defined as two cysteine residues in sufficiently close, linear proximity to form a small hairpin loop in the passenger domain, as found in the IgaA1 protease of *N. gonorrhoeae* (Pohlner *et al.*, 1987) and *H. pylori* VacA (Cover *et al.*, 1994). The cysteine pairs described have not been experimentally shown to form disulphide bonds.

Protein	Organism	Function	No. of Cys in passenger domain	C-C pairs (distance)	Accession no.
EspC	<i>E. coli</i>	Endotoxin	2	1 (7)	Q9EZE7
EspP	<i>E. coli</i>	Proteolytic toxin	2	1 (10)	032555
Hap	<i>Haemophilus influenzae</i>	Adhesin	2	1 (11)	P45387
IgA1	<i>H. influenzae</i>	Protease	2	1 (11)	P44969
IgA1	<i>N. gonorrhoeae</i>	Protease	2	1 (11)	P09790
IgA1	<i>N. meningitidis</i>	Protease	2	1 (11)	Q9AIU6
Pet	<i>E. coli</i>	Proteolytic toxin	2	1 (5)	068900
Sat	<i>E. coli</i>	Proteolytic toxin	2	1 (6)	Q9F6T1
SigA	<i>Shigella flexneri</i>	Proteolytic toxin	2	1 (6)	Q9L8L1
VacA	<i>H. pylori</i>	Pore-forming toxin	2	1 (11)	Q48245
Ssp-1	<i>S. marcescens</i>	Protease	5	2 (9/5)	P29805
IcsA	<i>S. flexneri</i>	Intracellular movement	3	1 (4)	Q99Q93
Tsh	<i>E. coli</i>	Haemagglutination	0	0	I27423
IgA	<i>Streptococcus mitis</i>	Protease	0	0	P96466
IgA	<i>Streptococcus oralis</i>	Protease	0	0	033691
SepA	<i>S. flexneri</i>	Protease/inflammation/invasion	0	0	Q99QC6
AIDA-I	<i>E. coli</i>	Adhesin	0	0	Q03155
BrkA	<i>Bordetella pertussis</i>	Adhesin/serum resistance	0	0	Q45340

(chloramphenicol acetyltransferase, *cat*), derived from pBSC103 (Wang & Taylor, 1990), was inserted upstream of *vacA*, immediately 3' to *cysS* (position 570 in the strain 60190 *vacA* sequence, GenBank/EMBL accession no. U05676) to create pNV1. A kanamycin resistance marker (aminoglycoside phosphotransferase, *aphA*), derived from pILL600 (Labigne-Roussel *et al.*, 1988; Suerbaum *et al.*, 1993), was then inserted 0.5 kb downstream of *vacA* within the *fecE* (*fepC*) gene (position 3693 in GenBank/EMBL accession no. U94318) to form pNV1::km. We have previously shown that inactivation of *fecE* does not affect growth of *H. pylori* on blood agar, or affect VacA levels or activity (Letley *et al.*, 2003). pNV1::km was used as a template for site-directed mutagenesis of *vacA* using the Quikchange Site-Directed Mutagenesis kit (Stratagene Europe), following the manufacturer's protocol and using the primers shown in Table 2. Fortuitously, the codon change TGT to AGT at position 771 created a recognition site for the restriction endonuclease *SpeI* which could be used to screen transformants for the presence of the C771S mutation. Similarly, in addition to the nucleotide substitution at codon 782, a silent substitution at the alanine codon at position 781 (GCA to GCT) was introduced to create a new *NheI* site and disrupt the existing *SphI* site, also allowing mutation screening by restriction analysis. Thus, following transformation of *DpnI*-treated mutagenesis reactions into *E. coli* strain XL-1 Blue, appropriate restriction analysis of plasmid DNA prepared from randomly chosen transformants was performed to identify mutant constructs. Positive transformants were selected and named pC771S and pC782S. Next, these mutations were introduced into the chromosomal *vacA* gene of *H. pylori* strain 60190 by natural transformation with pC771S and pC782S respectively, followed by allelic exchange and kanamycin marker rescue. To determine the presence of the appropriate mutation, a 1248 bp region surrounding codons 771 and 782 was PCR-amplified from genomic DNA of kanamycin-resistant transformants, using primers B3334 (5'-CTTGGAATTATTTTGACGCTAG-3'; position 2622–2643 in the strain 60190 *vacA* sequence) and B1514 (5'-CGTTAGCCCAACATTGGTAG-3'; position 3849–3869) and restriction analysis performed on the product as before. In each case, the appropriate mutation was confirmed by nucleotide sequencing on both strands using an Applied Biosystems 3100 Genetic Analyser (Biopolymer Synthesis and Analysis Unit, University of Nottingham, UK). The resulting *H. pylori vacA* mutant strains were called 60190 C771S and 60190 C782S. In addition to these single cysteine mutations, a third mutant strain was prepared possessing serine substitutions at both cysteine residues. This was constructed using the same methodology: the C771S mutation was first introduced into pC782S to create pDCM; then both mutations were introduced into the chromosomal *vacA* allele of *H. pylori* strain 60190 by natural transformation and allelic exchange with pDCM, forming 60190 DCM. All mutant strains were constructed and characterized in duplicate from separate mutagenesis reactions. Finally, to control for the presence of the kanamycin resistance marker, strain 60190 was transformed with pNV1::km, selecting a kanamycin-resistant transformant which was named 60190km.

Detection of VacA by immunoblotting. *H. pylori* broth cultures were grown for 24 h, resulting in bacterial densities with an OD₅₅₀ of 0.608–0.633. The cultures were centrifuged at 4000 g for 15 min and equal volumes of separated supernatant and cell pellet samples were analysed by SDS-PAGE, followed by transfer to nitrocellulose by electroblotting. Nitrocellulose blots were blocked in PBS/Tween 20 (PBS-T) containing 5% (w/v) milk, then incubated with rabbit antiserum to purified VacA from strain 60190 (Ab123; kindly donated by Dr T. L. Cover, Nashville, TN, USA) diluted 1:10 000. Blots were washed three times in PBS-T, incubated in goat anti-rabbit IgG-horseradish peroxidase conjugate (Sigma-Aldrich), washed six times in PBS-T and visualized by ECL detection (Amersham Biosciences).

Quantification of VacA. VacA levels in broth culture supernatant samples of each strain were quantified by antigen detection ELISA, as previously described (Letley *et al.*, 2003), using anti-VacA antiserum, Ab123. ELISA values were expressed as A₄₉₂ units, corrected for bacterial density (OD₅₅₀).

Determination of VacA vacuolating activity. Vacuolating activity was determined for 48 h broth culture supernatants from appropriate *H. pylori* strains (concentrated 20-fold by ultrafiltration) using the epithelial cell lines HeLa and RK13 (a rabbit kidney cell line). Assays were performed by adhering 10⁴ epithelial cells in RPMI1640 medium supplemented with 10% fetal calf serum (both from Life Technologies) to a microtitre plate overnight. The medium was then replaced with fresh medium containing 10 mM ammonium chloride and a fivefold dilution of 0.2 µm-filtered broth culture supernatant. Cells were incubated overnight and then visually assessed for vacuolation by light microscopy.

RESULTS

Substituting serine at VacA positions C771 or C782 reduces secreted VacA levels

We examined the role of the paired cysteine residues in the VacA passenger domain for toxin secretion. To do this, we constructed isogenic *H. pylori vacA* mutants in which serine had been substituted for cysteine at either position 771 (60190 C771S) or 782 (60190 C782S) in the VacA sequence of strain 60190 (GenBank accession no. U05676). Serine is similar in size to cysteine and has similar physicochemical properties, so it would be expected to cause minimal disruption to the structure of VacA, with the exception of abolishing any potential disulphide bond formation between residues 771 and 782. Next, we compared VacA levels for the wild-type strain 60190, a VacA-negative control strain, 60190:v1 (Cover *et al.*, 1994), our control strain, 60190km,

Table 2. Primers used for site-directed mutagenesis of *vacA*

Primer	Sequence (5' to 3')*	Mutation introduced	Restriction site created	Restriction site removed
C771S-F	AACCGCATGGATACT <u>AGT</u> GTGGTGCGAAATAC	C771S	<i>SpeI</i>	—
C771S-R	GTATTTTCGCACCACACT <u>AGT</u> ATCCATGCGGTT			
C782S-F	CTGATGACATTAAAGCT <u>AGC</u> GGTATGGCTATCGG	C782S	<i>NheI</i>	<i>SphI</i>
C782S-R	CCGATAGCCATACCGCT <u>AGC</u> TTTAATGTCATCAG			

*Nucleotide substitutions are shown underlined. Restriction sites used for screening are shown in bold.

and the two isogenic mutant strains, 60190 C771S and 60190 C782S, by immunoblot analysis of 24 h broth cultures of the same cell density (OD_{550} 0.608–0.633). Insertion of the kanamycin marker downstream of *vacA*, within *fecE*, did not alter the amount of VacA present compared with the wild-type strain (Fig. 1, lane 3 versus lane 1), as reported previously (Letley *et al.*, 2003), hence this strain was used as the control for all later comparisons with *vacA* mutant strains. The amount of VacA in 24 h broth culture supernatant was considerably reduced for strain 60190 C771S (Fig. 1a, lane 4) compared with the control strain, 60190*km*. Similarly, the amount of secreted VacA was reduced for strain 60190 C782S (Fig. 1a, lane 5) but, surprisingly, not to the same extent as for strain 60190 C771S. Immunoblot analysis of whole cell samples from the same broth cultures showed mature VacA product for the control strain 60190*km*, and a faint amount for strain 60190 C782S, but no VacA-sized band was observed for strain 60190 C771S (Fig. 1b). This mature-sized VacA protein was most likely secreted from the cell and then adsorbed back onto the surface of the outer membrane, as observed previously (Fitchen *et al.*, 2003, 2005; Pelicic *et al.*, 1999). No accumulation of precursor VacA polypeptide was evident in immunoblots of whole cell preparations (data not shown).

To confirm our immunoblot data and quantify the differences in VacA levels, we performed an antigen detection ELISA on a minimum of three 24 h broth culture supernatants for each strain using anti-VacA antiserum Ab123, correcting A_{492} units for bacterial density (OD_{550}). Substitution of serine for cysteine at *vacA* codon position 771 caused a 60-fold reduction in the amount of VacA compared with the control strain 60190*km* [60190 C771S mean $\pm$ SE VacA ELISA value 0.017 ± 0.005 corrected A_{492} units, $n=4$, versus 60190*km* mean $\pm$ SE 1.022 ± 0.111 , $n=3$ ($P<0.02$, t test)]. As observed by immunoblot analysis, serine substitution at the second cysteine position (codon 782) caused a more modest decrease in VacA level (strain 60190 C782S mean VacA ELISA value 0.122 ± 0.017 , $n=4$, $P<0.02$),

toxin level being sevenfold higher than that observed for strain 60190 C771S ($P<0.01$). To confirm our VacA ELISA data, we determined VacA levels for the wild-type and mutant strains at a second time point in the growth curve. A minimum of seven broth cultures of each strain were grown for 48 h and VacA levels determined by ELISA as before. VacA levels after 48 h growth were, again, significantly lower for the C771S and C782S mutants compared to the wild-type control [mean VacA ELISA values 0.024 ± 0.006 , $n=8$, and 0.345 ± 0.081 , $n=7$, respectively, versus strain 60190*km* mean 0.943 ± 0.190 , $n=9$ ($P<0.002$ and $P<0.02$, respectively)].

The reduced VacA level observed for mutants with a single cysteine residue is not due to intermolecular disulphide bond formation

One possible hypothesis for reduced VacA levels in culture supernatants of the single cysteine mutants is that the remaining cysteine residue promotes intermolecular disulphide bond formation between VacA precursor molecules, leading to aggregation of the protein in the periplasm, which blocks secretion and leads to subsequent degradation of the protein. The two cysteine residues may differ in their accessibility for intermolecular disulphide bond formation, which could explain the difference in VacA levels between strains 60190 C771S and 60190 C782S. To test this hypothesis, we constructed the mutant strain 60190 DCM, in which both cysteine residues were replaced with serine to prevent intermolecular disulphide bond formation. Our hypothesis predicted that, unlike the single-cysteine substitution mutants, substituting both cysteine residues would not reduce VacA levels compared to those of the wild-type strain. However, VacA levels in 24 h broth culture supernatants were significantly reduced for strain 60190 DCM compared with the wild-type control 60190*km* (mean VacA ELISA values 0.056 ± 0.012 , $n=4$, versus 0.622 ± 0.025 , $n=5$, $P<10^{-6}$). This was confirmed by immunoblot analysis (Fig. 1, lanes 6 and 3). Therefore, we conclude that the reduction in VacA levels observed for the single-cysteine mutant strains was not due to aggregation caused by intermolecular disulphide bond formation involving the remaining cysteine residue.

VacA C782S mutation does not affect toxin activity

We assessed whether VacA secreted by the cysteine mutant strain 60190 C782S was still folded in a biologically active form (the VacA levels for strains 60190 C771S and 60190 DCM were too low for further analysis). We performed vacuolation assays using HeLa and RK13 cell lines with broth culture supernatant from strains 60190*km* and 60190 C782S. Cytoplasmic vacuolation was observed for both HeLa (Fig. 2) and RK13 (data not shown) cell lines, indicating that the serine substitution at position 782 did not adversely affect the biological activity of the toxin.

Fig. 1. VacA levels of wild-type and *vacA* cysteine mutant strains. Twenty-four-hour broth culture supernatants (a) and whole-cell extracts (b) were separated by SDS-PAGE and immunoblotted with rabbit anti-VacA antiserum (Ab123). Broth cultures were of the same cell density (OD_{550} 0.608–0.633) and equal sample volumes were loaded as follows: strains 60190 (lane 1), 60190:v1 (lane 2), 60190*km* (lane 3), 60190 C771S (lane 4), 60190 C782S (lane 5), and 60190 DCM (lane 6).

Fig. 2. Vacuolating activity of strain 60190 C782S. HeLa cells were grown in RPMI 1640 medium supplemented with 10 % fetal calf serum for 24 h in a 96-well microtitre plate (10^4 cells per well) and then incubated overnight with broth culture supernatant from either strain 60190km (a) or 60190 C782S (b) diluted fivefold in medium containing 10 mM ammonium chloride. Vacuolation was assessed by light microscopy. Bar, 30 μ m.

DISCUSSION

The genetic structure of *vacA* is typical of an autotransporter (Schmitt & Haas, 1994): the toxin is encoded as a protoxin, including an N-terminal signal sequence and a C-terminal domain which is cleaved upon secretion of the mature polypeptide and remains associated with the cell envelope (Telford *et al.*, 1994). Comparison of the VacA C-terminal domain with 17 functionally characterized autotransporters identified weak homology within this region (e.g. 12 % identity between VacA and EspC), including the presence of 14 putative amphipathic β -strands typical of outer-membrane-spanning domains (Loveless & Saier, 1997). Furthermore, it has been shown elsewhere that the VacA C-terminal domain, when fused to the cholera toxin B subunit, is able to secrete the latter to the *H. pylori* cell surface (Fischer *et al.*, 2001). Although many autotransporters contain a pair of cysteine residues located close to the C-terminus of the passenger domain, their role in autotransporter function has not been addressed. VacA contains such a pair of cysteine residues which are conserved between the *vacA* alleles of different *H. pylori* strains and located 84 and 73 residues from the proposed C-terminal cleavage site in strain 60190. In this study, we have shown that both of these residues are required for high levels of mature, secreted VacA in culture supernatants and propose that they are involved in either the translocation of mature VacA through the autotransporter domain or the folding of the VacA protein into a stable structure before, during or after its export. Either of these propositions would be consistent with the lack of accumulation of a VacA precursor protein in the cysteine mutants owing to proteolysis by periplasmic or outer membrane proteases. In a previous study, *H. pylori* isogenic transposon mutants, in which the autotransporter domain of VacA was disrupted, were unable to secrete the toxin into the supernatant and no accumulation of precursor was observed

within the cell, leading the authors to suggest that it was rapidly degraded in the periplasm (Schmitt & Haas, 1994).

One model of autotransporter secretion proposes that a transient hairpin structure is required to direct translocation of the C-terminal end of the passenger domain through the β -barrel translocator domain (Jose *et al.*, 1995). A disulphide bond between the pair of cysteine residues located at the C-terminal end of the VacA passenger domain could stabilize such a hairpin, which would be disrupted by substitution of serine for either cysteine. However, our results are not fully consistent with this hypothesis. While the reduction in VacA level observed for the single and double VacA cysteine mutants is consistent with reduced secretion efficiency, the difference in the amount of VacA observed for strains 60190 C771S and 60190 C782S suggests that the role of the cysteine residues is not merely disulphide bond formation, as we would have expected both mutations to have similar effects on VacA level.

Besides directing their own translocation across the outer membrane, there is evidence that autotransporters also contain intramolecular chaperone sequences which are required for the stable folding of the passenger domain into a protease-resistant form, either during or following translocation (Achtman *et al.*, 1999; Maurer *et al.*, 1999; Ohnishi *et al.*, 1994; Oliver *et al.*, 2003a, b; Suzuki *et al.*, 1995). Such regions have often been identified within the linker or junction region immediately following the C-terminal end of the passenger domain and N-terminal to the β -barrel domain (Ohnishi *et al.*, 1994; Suzuki *et al.*, 1995). However, the suggested intramolecular chaperone domain of BrkA is located within the C-terminus of the passenger domain, 40–131 aa from the processing site (Oliver *et al.*, 2003b). A similarly located region in VacA would include the paired cysteine residues, which are located 84 and 73 residues from

the proposed processing site (Nguyen *et al.*, 2001). The location of a potential intramolecular chaperone sequence at the C-terminus of VacA might be expected, given that this end of the passenger domain is most likely translocated through the β -barrel pore first (Henderson *et al.*, 1998; Jose *et al.*, 1995; Pohlner *et al.*, 1987); translocation of the chaperone region first would allow folding of the passenger domain as it exits the pore. Thus, we favour a model in which the cysteine residues are involved in an intramolecular chaperone function, guiding the folding of the passenger domain into the correct conformation. Substitution of the cysteine residues may alter the conformation of this folding region, disrupting the correct folding of mature VacA into a stable form, explaining the lower amount of mature VacA and absence of accumulated unprocessed toxin in the cysteine mutants. The lower amount of mature VacA in mutants 60190 C771S and 60190 DCM compared with 60190 C782S suggests a more pivotal role for C771 in such a putative chaperone sequence.

Autotransporters are an interesting group of proteins whose passenger domains have diverse functions. Many are important in bacterial virulence and some, such as VacA, are central to pathogenicity. Understanding the mechanism of bacterial protein autotransport will provide essential information to elucidate the basis for this role and may allow novel methods of producing high quantities of specific proteins in 'clean' forms secreted efficiently into bacterial culture supernatants.

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