

TagA is a secreted protease of *Vibrio cholerae* that specifically cleaves mucin glycoproteins

Rose L. Szabady,¹ Joseph H. Yanta,¹ David K. Halladin,¹
Mark J. Schofield² and Rodney A. Welch¹

Correspondence
Rodney A. Welch
rawelch@wisc.edu

¹Department of Medical Microbiology and Immunology, School of Medicine and Public Health, University of Wisconsin, Madison, WI 53706, USA

²Thermo Fisher, 3747 Meridian Road, Rockford, IL 61101, USA

Vibrio cholerae is a human diarrhoeal pathogen that is a major cause of gastrointestinal disease and death worldwide. Pathogenic *V. cholerae* strains are characterized by the presence of a *Vibrio* pathogenicity island (VPI) that encodes virulence factors, including the toxin co-regulated pilus (TCP). TagA is encoded within the VPI and is positively co-regulated with cholera toxin and TCP. TagA is a sequelogue of the StcE mucinase of *Escherichia coli* O157:H7. We investigated whether this sequence homology reflected a conserved enzymic substrate profile. TagA exhibited metalloprotease activity toward crude purified mucins, salivary mucin and LS174T goblet cell surface mucin. Like StcE, TagA did not cleave general protease substrates, but unlike StcE, TagA did not cleave the mucin-like serpin C1 esterase inhibitor. Both proteins cleaved the immune cell surface mucin CD43, but TagA demonstrated reduced enzymic efficiency relative to StcE. TagA was expressed and secreted by *V. cholerae* under ToxR-dependent conditions. A *tagA*-deficient *V. cholerae* strain showed no defect in a model of *in vitro* attachment to the HEp-2 cell line; however, overexpression of a proteolytically inactive mutant, TagA(E433D), caused a significant increase in attachment. The increased attachment was reduced by pretreatment of epithelial monolayers with active TagA. Our results indicate that TagA is a mucinase and suggest that TagA may directly modify host cell surface molecules during *V. cholerae* infection.

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INTRODUCTION

The Gram-negative bacterium *Vibrio cholerae* causes a secretory diarrhoeal disease in humans that has been the cause of at least seven worldwide pandemics since the 19th century (Sack *et al.*, 2004). Both pathogenic and non-pathogenic *V. cholerae* are found in the environment, and the vast majority of disease is caused by *V. cholerae* of the O1 serotype. Disease-causing strains of *V. cholerae* possess two distinct pathogenic elements, the *Vibrio* pathogenicity island (VPI) and the cholera toxin phage (ctxΦ) (Kaper *et al.*, 1995). The VPI is a 40 kb pathogenicity island that encodes several potential virulence factors and the toxin co-regulated pilus (TCP) (Karaolis *et al.*, 1998, 2001). TCP is a type IV pilus that is required for colonization of the human intestine (Herrington *et al.*, 1988; Taylor *et al.*, 1987). Cholera toxin (CT) is transcriptionally co-regulated with TCP by the ToxR/S/T regulon, which activates

numerous genes of the VPI (Champion *et al.*, 1997; Matson *et al.*, 2007). CT is the major cause of secretory diarrhoea induced by *V. cholerae* and can recapitulate disease when given alone (Levine *et al.*, 1983). However, vaccine strains lacking CT can cause diarrhoea in human volunteers, suggesting that other virulence factors, including some VPI genes, may play a role in disease (Silva *et al.*, 1996; Harrison *et al.*, 2008; Rui *et al.*, 2010).

One potential VPI-encoded virulence factor is TagA (ToxR-activated gene A), a homologue of the StcE mucinase of enterohaemorrhagic *E. coli* (EHEC). The small intestinal niche of *V. cholerae* is coated with a protective layer of thick mucus to which the bacteria preferentially adhere (Yamamoto *et al.*, 1988). Mucinase activity of *V. cholerae* was first described over 50 years ago as a complex with both neuraminidase and protease activities that were eventually attributed to the 32 kDa haemagglutinin/protease (HA/P) (Finkelstein *et al.*, 1983; Stewart-Tull *et al.*, 1986; Burnet, 1949; Schneider & Parker, 1982). HA/P is an elastase-like general metalloprotease that cleaves a wide variety of substrates, including ovomucin, BSA, casein, elastin and CT (Finkelstein *et al.*, 1983; Häse & Finkelstein, 1990; Booth *et al.*, 1983, 1984). Although HA/P promotes mucin gel penetration (Silva *et al.*, 2003), it is not

Abbreviations: BSM, bovine submaxillary mucin; C1-INH, C1 esterase inhibitor; CT, cholera toxin; EHEC, enterohaemorrhagic *E. coli*; HA/P, *V. cholerae* haemagglutinin/protease; MUC7, mucin 7; PGM, porcine gastric mucin; SLiMe, StcE-like metalloprotease; StcE, secreted protease of C1 esterase inhibitor; TagA, ToxR-activated gene A; TCP, toxin co-regulated pilus; VPI, *Vibrio* pathogenicity island; WGA, wheatgerm agglutinin.

required for virulence (Silva *et al.*, 2006; Finkelstein *et al.*, 1992; Häse & Finkelstein, 1991). Rather, it provides detachase activity that may allow bacteria to disengage from the intestinal epithelium and disseminate (Finkelstein *et al.*, 1992). Expression profiling suggests that HA/P is active late in infection and regulated oppositely to virulence genes (Zhu *et al.*, 2002). Penetration of the mucus layer is presumably important during initial colonization, and a role for a specific mucinase early in infection remains to be described.

TagA is encoded by the VPI and is part of the ToxR/S/T regulon (Withey & Dirita, 2005). Microarray analysis of stool samples from patients with cholera detected *tagA* transcript, suggesting that it is expressed during infection (Bina *et al.*, 2003). TagA is a putative 115 kDa secreted lipoprotein that is homologous to StcE (Secreted protease of C1 esterase inhibitor), a metalloprotease secreted by *E. coli* O157:H7. StcE is the prototypic member of the SLiMe (StcE-like metalloprotease) family, a group of large proteins of Gram-negative bacteria that share a consensus protease domain with a conserved zinc metalloprotease active site. StcE cleaves specific mucin-type O-glycosylated proteins, including C1 esterase inhibitor (C1-INH), CD43 and mucin 7 (MUC7), and increases the ability of *E. coli* O157:H7 to intimately adhere to epithelial cells (Grys *et al.*, 2005; Lathem *et al.*, 2002; Szabady *et al.*, 2009). The StcE homologue of *Aeromonas hydrophila* contributes to virulence in a mouse peritonitis model (Pillai *et al.*, 2006), and evidence suggests that StcE may be involved in colonization of the rabbit intestine by EHEC (Ho *et al.*, 2008). Here we investigate the hypothesis that TagA, similar to StcE, is a secreted mucinase and contributes to colonization of the intestinal epithelium.

METHODS

Bacterial strains and plasmids. Bacterial strains were maintained in LB medium with 50 % glycerol at -80°C . Bacteria were grown under ToxR-inducing conditions at 30°C in LB medium pH 6.5 (Miller & Mekalanos, 1988). Ampicillin was used at $100\ \mu\text{g}\ \text{ml}^{-1}$. *V. cholerae* O1 El tor strain N16961 was obtained from ATCC (Manassas, VA, USA). Classical biotype *V. cholerae* O395 Sm^R and JJM43 (*V. cholerae* O395 Sm^R *toxR43*) were kind gifts from R. K. Taylor (Dartmouth College, Hanover, NH, USA). MC4100 λ pir containing pKAS32 was a kind gift from V. J. DiRita (University of Michigan, Ann Arbor, MI, USA).

A *tagA* deletion in *V. cholerae* O395 Sm^R was constructed using allelic exchange as described by Skorupski & Taylor (1996). Briefly, DNA flanking *tagA* was PCR amplified from the VPI (GenBank accession no. AF325733) using the oligos A (5'-CGGGTACCCGGAAGCAGCACCTTCTTG-3') and B (5'-CCACAGTCATCAAAAAAGTCTATAGTTTAACTCCTCGTAAACATTTTTTCGA-3') for nt 3355–3988, and oligos C (5'-TCGAAAAATGTTTACGAGGAGTAACTATAGAACTTTTTTGTGATGACTGTGG-3') and D (5'-CGGAGCTCGGAATCGCTGTATGCAGGTATC-3') for nt 6998–8093, using genomic DNA as template and TripleMaster DNA polymerase (Eppendorf). The resultant PCR fragments were then ligated by PCR with primers A and D. The resultant product was ligated into pKAS32, an R6K suicide vector that expresses *rpsL* (Skorupski &

Taylor, 1996), transformed into CC118 λ pir (Herrero *et al.*, 1990) and mated into *V. cholerae* O395 Sm^R. Transconjugants were isolated on TCBS agar with $250\ \mu\text{g}$ carbenicillin ml^{-1} , recovered on LB agar and counterselected on LB agar with $1\ \text{mg}$ streptomycin ml^{-1} . Deletion of *tagA* (Δ *tagA*) was confirmed by PCR.

pBAD24 A^R (Guzman *et al.*, 1995) was a gift from Charles Cowles (University of Wisconsin, Madison, WI, USA). The entire *tagA* gene, including the signal sequence, was PCR-amplified from *V. cholerae* O1 genomic DNA using oligos E (5'-CGGGCTAGCTACGAGGAGTTAGTGGTGG-3') and F (5'-GCGGCATGCTCTATAGTTTATGGTAGTGCATCG-3') and cloned into pBAD24 using *NheI* and *SphI* (NEB) to create pTagA. The proteolytically inactive TagA(E433D) mutant was created by PCR mutagenesis using primer G (5'-CGAATGGTCTCATGATTTGGGACATAACTATGGATTGGGAC-3') and its complementary oligonucleotide to create pE433D. pTagA and pE433D were confirmed by DNA sequencing. As a control for attachment experiments, pBAD24 was transformed into *V. cholerae* O395 wt and Δ *tagA*.

For analysis of TagA expression, *V. cholerae* O395 wt, Δ *tagA* or the *toxR* null mutant (JJM43) were grown under ToxR-inducing conditions overnight (Taylor *et al.*, 1987). Cells were pelleted and proteins in culture supernatants were precipitated with 10 % trichloroacetic acid on ice. Samples were separated by SDS-PAGE and transferred to nitrocellulose. Protein expression was detected with polyclonal antiserum to a TagA peptide (aa 44–57, KKPSRPIIDEKNKG) raised in rabbits (Open Biosystems) and used at 1:2000 dilution.

Protein purification. pTagA and pE433D were transformed into *V. cholerae* O395 Δ *tagA*. Bacteria were grown to mid-exponential phase in LB (pH 6.5) with ampicillin, and protein expression was induced with 0.1 % arabinose for 24 h at 30°C . Culture supernatants were filtered and precipitated with ammonium sulfate (AMS). The 35–55 % AMS pellet was resuspended in Buffer A (20 mM Tris, pH 8.0, 50 mM NaCl). Soluble protein was applied to a Mono Q anion exchange column (GE Healthcare) equilibrated in Buffer A, and protein was eluted with a linear gradient with 20 mM Tris, pH 8.0, 1 M NaCl. Peak fractions were further purified by gel filtration using an S200 16/60 column (GE Healthcare) in 20 mM Tris, pH 8.0, 150 mM NaCl, and glycerol was added to 20 % prior to storage at -20°C .

Mucinase assays. Human saliva (10 %), bovine submaxillary mucin (0.04 %, BSM; Sigma) and porcine gastric mucin type II or III (0.04 %, PGM; Sigma) in PBS were treated with $2\ \mu\text{g}$ enzyme or equivalent volume PBS vehicle control overnight in a final volume of $100\ \mu\text{l}$ at room temperature. EDTA (50 mM) was used where indicated to inhibit metalloprotease activity. Samples were separated by electrophoresis through 1 % agarose with 0.1 % SDS at 23 V overnight in TAE buffer. Proteins were transferred to polyvinylidene difluoride (Amersham) and probed with 1:10 000 biotinylated wheatgerm agglutinin (WGA; US Biological) followed by horseradish peroxidase (HRP)-conjugated streptavidin (Bio-Rad), and detected by enhanced chemiluminescence (Thermo Fisher). Collection of human saliva samples was in accordance with a protocol approved by the University of Wisconsin Institutional Review Board. For experiments with cell-bound mucins, goblet-like LS174T cells (ATCC) were grown in Eagle's Minimum Essential Medium (Mediatech) with 10 % fetal bovine serum (Atlanta Biologicals). Confluent monolayers in 24-well plates were treated with $5\ \mu\text{g}$ enzyme for 3 h at 37°C , 5 % CO_2 . Cells were lysed and samples analysed as above.

Protease assays. For MUC7 cleavage, $30\ \mu\text{l}$ whole human saliva was incubated with $2\ \mu\text{g}$ enzyme or PBS vehicle control for 90 min at 37°C . Samples were separated by SDS-PAGE, transferred to nitrocellulose and probed with 1:1000 rabbit anti-MUC7 (kind gift

from J. G. M. Bolscher, Academic Centre for Dentistry Amsterdam, The Netherlands) and anti-rabbit-HRP. For C1-INH cleavage, 1 µg C1-INH (Comptech) was treated with 1 µg enzyme in a final volume of 30 µl for 3 h at 37 °C. Samples were analysed as above using 1:2000 goat anti-C1-INH and anti-goat-HRP. For casein cleavage, 5 or 10 µg enzyme was assayed using the EnzChek green fluorescence protease assay kit (Molecular Probes/Invitrogen), according to the manufacturer's instructions.

Flow cytometry. The human T-lymphocyte Jurkat cell line was obtained from ATCC and maintained as per the supplier's instructions. Cells were suspended to 5×10^5 ml⁻¹ in RPMI with 5% fetal bovine serum and treated with 1 µg enzyme in a final volume of 500 µl for the indicated times at 37 °C, 5% CO₂. Cells were pelleted at 400 g for 5 min and blocked by addition of 0.05% BSA (Sigma) in Dulbecco's PBS with Ca²⁺/Mg²⁺. Samples were stained with phycoerythrin-conjugated anti-CD43 (clone L10; Invitrogen) and analysed by flow cytometry on an LSR II (Becton Dickinson).

Attachment assays. The HEp-2 human epithelial-like cell line was acquired from ATCC and maintained as per the supplier's instructions. Confluent monolayers (5×10^5) in 24-well tissue culture plates were used for attachment experiments and washed twice before use. Bacteria were grown to an OD₆₀₀ of 0.2–0.3, *tagA* expression was induced with 0.1% arabinose for 2 h, and bacteria were applied to monolayers for 30 min at 37 °C, 5% CO₂. Non-attached bacteria were removed by three PBS washes, HEp-2 cells were lysed in 1% Triton X-100, and recovered bacteria were enumerated by serial dilution and plating on LB agar with ampicillin. Percentage attachment was calculated as output c.f.u./input c.f.u. × 100%. Statistical analyses were performed using GraphPad Prism. For immunofluorescence analysis, HEp-2 cells were grown to confluent monolayers on Lab-Tek II chamber slides (Nalge Nunc International) and incubated with *V. cholerae* as above for 30 min, followed by washing to remove non-attached bacteria. Samples were fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Sigma), stained with 1:5000 anti-TcpA antibody (a kind gift of V. J. DiRita) followed by 1:200 Alexa-488 anti-rabbit IgG and 1:50 Alexa-594 phalloidin, and mounted in ProLong Gold antifade reagent with DAPI (Molecular Probes). Coverslips were imaged using a ×100 oil immersion lens on a Zeiss Axioplan III epifluorescence microscope.

RESULTS

Identification of TagA as a SLiMe

We previously identified and characterized StcE, a secreted protein of *E. coli* O157:H7, that has been identified as the prototype member of family M66 within the MEROPS protease database (<http://merops.sanger.ac.uk>; Rawlings *et al.*, 2010). Members of this family, which we refer to as the SLiMe family, share a conserved ~300 aa metalloprotease domain with a predicted zinc-dependent active site sequence of HEXGHXXXGXGH. SLiMe family members are found in several species of the *Gammaproteobacteria* in the families *Vibrionaceae*, *Enterobacteriaceae*, *Aeromonadaceae* and *Shewanellaceae*. BLAST analysis and CLUSTAL W alignment confirmed that *V. cholerae* TagA (RefSeq NP_230468.1) is a homologue of StcE. The two proteins share amino acid sequences with 37% similarity throughout the total protein and 41% identity within the central, 300 aa metalloprotease domain. In addition to TagA, *V. cholerae* encodes another

SLiMe, TagA-related protein (TARP; RefSeq NP_232548.1), on chromosome II. *Vibrio vulnificus* also encodes a TARP homologue on chromosome II, but lacks a *tagA* gene on chromosome I. Several other pathogenic and environmental vibrios, including *Vibrio parahaemolyticus* and *Vibrio fischeri*, do not encode any SLiMes. All currently sequenced *V. cholerae* isolates encode TARP, whereas only those possessing the VPI encode TagA. Because there is evidence that TagA is expressed during human infection (Bina *et al.*, 2003), we chose to focus our studies solely on the functions of this SLiMe family member in *V. cholerae*.

TagA is a secreted, ToxR-regulated protein

The *tagA* gene has a ToxT-dependent promoter (Withey & Dirita, 2005) and encodes a 981 aa polypeptide with a predicted molecular mass of 115 kDa. TagA has a leader peptidase II-dependent signal sequence, which predicts that the N-terminal cysteine of the processed protein will be lipidated. Following secretion across the inner membrane, TagA may be secreted extracellularly by a type II secretion system, similar to StcE or the lipoprotein pullulanase (d'Enfert *et al.*, 1987; Stathopoulos *et al.*, 2000), or may be associated with the outer membrane via the attached N-terminal lipid moiety (Bos *et al.*, 2007). We therefore examined TagA expression and secretion *in vitro* under ToxR-inducing conditions. A polyclonal antibody was produced to a peptide within the N-terminal region of TagA. Immunoblot analysis demonstrated that TagA was expressed by *V. cholerae* O395 under ToxR-inducing conditions and not in the ToxR null mutant strain JJM43 (Fig. 1a). An isogenic TagA deletion strain ($\Delta tagA$) was created using allelic exchange and used to confirm that the antibody specifically recognized TagA. Owing to a cross-reactive band of a very similar size, samples were electrophoresed until the 75 kDa marker was at the bottom of the acrylamide gel in order to resolve TagA. Natively expressed TagA was detectable in both the supernatant and cell pellet fractions (Fig. 1b). Overexpression of TagA in *V. cholerae* (see below) resulted in large amounts of TagA in the supernatant, further confirming that it is a secreted protein. The possibility remains that TagA is both secreted and cell-associated, and that some protein may bind back to the cell surface via its lipid moiety.

TagA is a metal-dependent mucinase

In order to conduct functional protease assays, we attempted to purify TagA. For unknown reasons, the protein was not expressed well in numerous strains of *E. coli*, with or without a variety of epitope tags. We were able to overexpress TagA with arabinose induction from a *tagA* recombinant-based pBAD24 vector plasmid in the *V. cholerae* O395 background. The majority of overexpressed, recombinant TagA was present as a soluble form in the culture supernatant. Ammonium sulfate precipitation of filtered supernatants, followed by ion exchange and subsequent size exclusion chromatography, yielded highly purified TagA protein (data not shown).

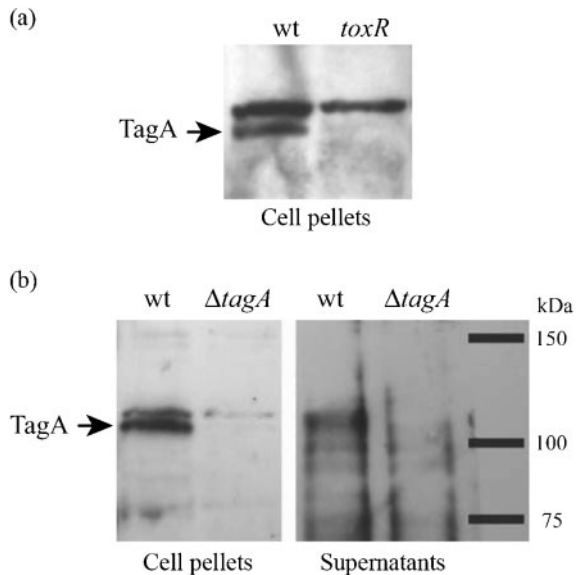


Fig. 1. TagA is secreted by *V. cholerae* in a ToxR-dependent manner. (a) *V. cholerae* O395 and the isogenic ToxR null mutant strain JJM43 were grown under ToxR-inducing conditions until late-exponential phase. Cells were pelleted, supernatants precipitated with TCA, and samples were analysed by SDS-PAGE followed by immunoblotting with a polyclonal antibody raised to an N-terminal peptide of TagA. (b) Experiments were performed as described for (a), comparing *V. cholerae* O395 wt with the $\Delta tagA$ strain.

StcE recognizes glycan-induced protein conformations in order to specifically cleave mucin-type glycoproteins. We hypothesize that SLiMe family members share a similar mucin-specific substrate profile. We therefore investigated potential mucinase activity of TagA. Solutions of crude purified BSM or PGM type II or III were treated with TagA. StcE served as a positive control, and EDTA was used to inhibit metalloprotease activity. Mucins were separated by agarose gel electrophoresis and probed with WGA, a lectin that recognizes the *N*-acetylglucosamine sugar linkage common to many glycoproteins. Mucins run as large, diffuse bands using this method as a result of their heterogeneity in size and glycosylations. Mucin cleavage, detected as clearance of these lectin-reactive bands, was evident in TagA- and StcE-treated samples, and was prevented by incubation with EDTA (Fig. 2a). In addition to reconstituted crude animal mucin preparations, we investigated cleavage of human-derived mucins *ex vivo* and *in vitro*. TagA cleaved mucins present in human saliva (Fig. 2b) and mucins that were attached to the LS174T human goblet-like cell line (Fig. 2c).

TagA is not a general protease

StcE uniquely cleaves proteins with mucin-type *O*-glycosylations (Grys *et al.*, 2006). With the demonstration that TagA cleaved mucins, we sought to confirm that it did

not exhibit general proteolytic activity toward non-mucin substrates. Gelatin zymography was performed with purified TagA, using trypsin as a positive control for proteolysis. Like StcE, TagA did not exhibit any proteolytic activity toward gelatin (data not shown). Furthermore, TagA did not cleave hide powder azure and casein, two common substrates used for measuring proteolytic activity (Fig. 2d and data not shown).

Enzymic activities of TagA and StcE are not identical

Our data suggest that TagA is a metal-dependent protease that shares a similar mucin substrate profile to StcE. We sought to further delineate the specific substrate profile by examining cleavage of CD43, MUC7 and C1-INH, known substrates of StcE. The cleavage of these substrates may contribute to the role of StcE in EHEC infection (Grys *et al.*, 2005; Lathem *et al.*, 2002; Szabady *et al.*, 2009). As a negative control, we purified a version of TagA with a single point mutation in the active site (E433D), a mutation that was previously demonstrated to inactivate StcE and other zinc metalloproteases (Lathem *et al.*, 2002). CD43 is a large anti-adhesive glycoprotein found on the surface of immune cells. TagA cleaved CD43 from the surface of Jurkat T cells and, similarly to StcE, degraded the *O*-glycosylated extracellular domain (Fig. 3a). Immunoblotting with an antibody to the CD43 N terminus revealed that TagA cleaved CD43 with less apparent efficiency than StcE (discussed further below). MUC7 is a 250 kDa glycoprotein found in human saliva. Saliva samples were treated with TagA and probed with a MUC7-specific antibody, demonstrating cleavage of MUC7 (Fig. 3b). Cleavage of MUC7 and CD43 was not observed with the TagA(E433D) mutant, confirming that this mutation inactivated the protease active site.

Unlike StcE, TagA was unable to cleave purified C1-INH (Fig. 3c). This finding indicates that despite sharing a conserved metalloprotease domain, the substrate specificities of TagA and StcE are not identical. We examined cleavage efficiency of TagA toward CD43, a substrate that it shares with StcE. Intact Jurkat cells were treated with similar amounts of TagA or StcE for various times, and CD43 on the surface was detected by flow cytometry. StcE cleaved nearly all of the CD43 on the surface after 30 min; in fact, reduction of CD43 on the surface was evident after 5 min of protease treatment (Fig. 4a and data not shown). In contrast, although TagA cleaved some CD43 after 30 min, substantial cleavage was not evident until 1–3 h, and remained incomplete after 3 h (Fig. 4b). TagA(E433D) served as a negative control and did not remove any CD43 from the cell surface (Fig. 4c). Our results indicate that TagA is less efficient at cleaving CD43 than StcE and, combined with the lack of cleavage of C1-INH, suggest that sequence divergence between StcE and TagA might confer differing substrate preferences despite a general similarity in substrate type.

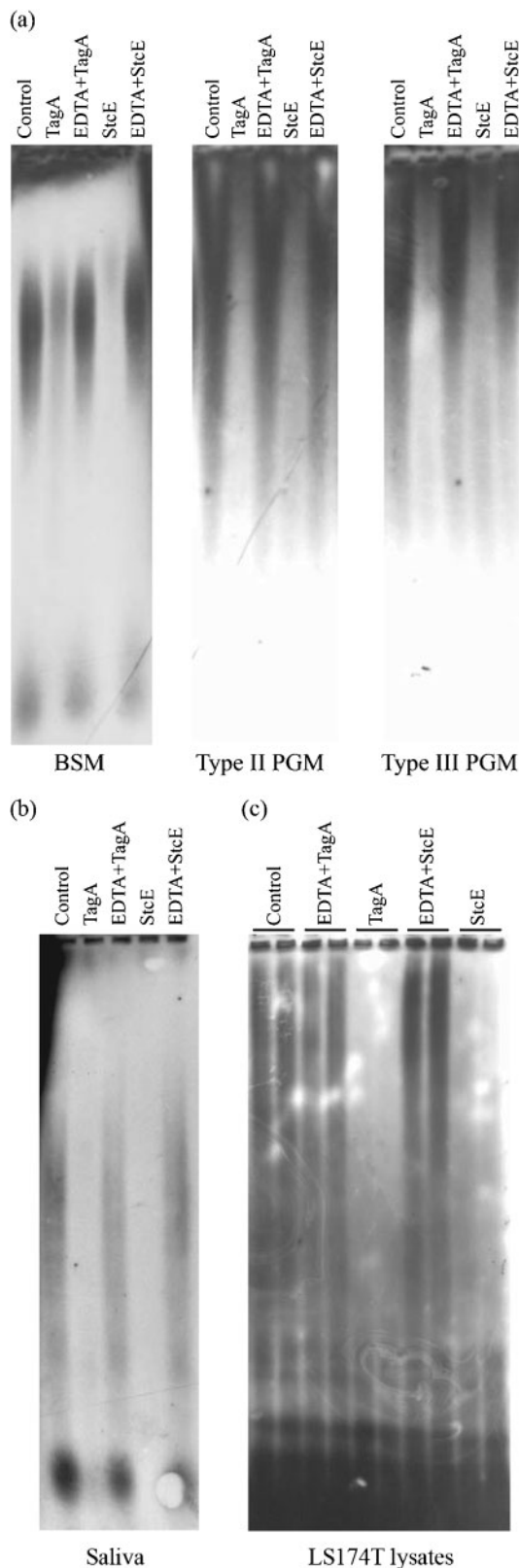


Fig. 2. Cleavage of mucins by TagA. (a) Crude purified BSM or PGM type II and III (0.04 % in PBS) were treated with 2 μ g TagA or StcE or buffer control overnight at room temperature. EDTA (50 mM) was included as a negative control to inhibit metalloprotease activity where indicated. Samples were separated by agarose gel electrophoresis, transferred to polyvinylidene difluoride and probed with WGA. (b) Saliva (10 %) was treated and probed as described for (a). (c) Differentiated goblet-like LS174T cells were treated with 5 μ g enzyme with or without EDTA for 3 h at 37 $^{\circ}$ C, 5 % CO_2 . Cells were lysed, and samples were analysed as described for (a).

contribution in an *in vitro* model of *V. cholerae* attachment to human epithelial-like cells (HEp-2) that has been used previously to identify *V. cholerae* adherence factors (Sperandio *et al.*, 1995). Wild-type *V. cholerae* O395 and the isogenic Δ tagA mutant strain were allowed to attach to HEp-2 monolayers, washed and the attached bacteria enumerated. Of the input *V. cholerae* cells, 3–15 % adhered to the HEp-2 cells, consistent with previously reported attachment figures in this model (Sperandio *et al.*, 1995). The loss of TagA expression had no significant effect on *V. cholerae* attachment. However, overexpression of proteolytically inactive TagA(E433D), but not TagA, resulted in a significant 70 % increase in attachment of *V. cholerae* to the cultured epithelial cells (Fig. 5a). We did not observe any qualitative differences in protein expression or bacterial autoaggregation between the TagA and TagA(E433D) overexpression strains. Immunofluorescence microscopy confirmed the increased attachment of the TagA(E433D) overexpression strain (Fig. 5c). A possible explanation for the differential attachment observed is that TagA normally binds and cleaves an unidentified epithelial cell surface protein, but in the absence of proteolysis there remains a receptor–ligand interaction that results in increased attachment by the TagA(E433D) overexpression strain. To test this hypothesis, we pretreated HEp-2 cells with purified active TagA. This treatment reduced the hyper-attachment of the TagA(E433D) overexpression strain (Fig. 5b), suggesting that active TagA cleaved the binding partner of the TagA(E433D) mutant protein and eliminated this receptor–ligand interaction. The addition of purified TagA had no effect on the attachment of *V. cholerae* Δ tagA cells not overexpressing the TagA(E433D) mutant. These assays were repeated in the mucus-producing cell line LS174T with similar results (data not shown). We were unable to identify binding partner(s) for the TagA(E433D) mutant using immunoprecipitation and mass spectrometry, possibly as a result of interference by protein glycosylation with mass spectrometric identification.

DISCUSSION

We have demonstrated that TagA, a homologue of *E. coli* O157:H7 StcE and a member of the SLiMe family, is expressed and secreted by *V. cholerae* under ToxR-dependent

Secretion of a mucinase during infection with *V. cholerae* could promote intestinal colonization. With the demonstration of TagA mucinase activity, we next evaluated its

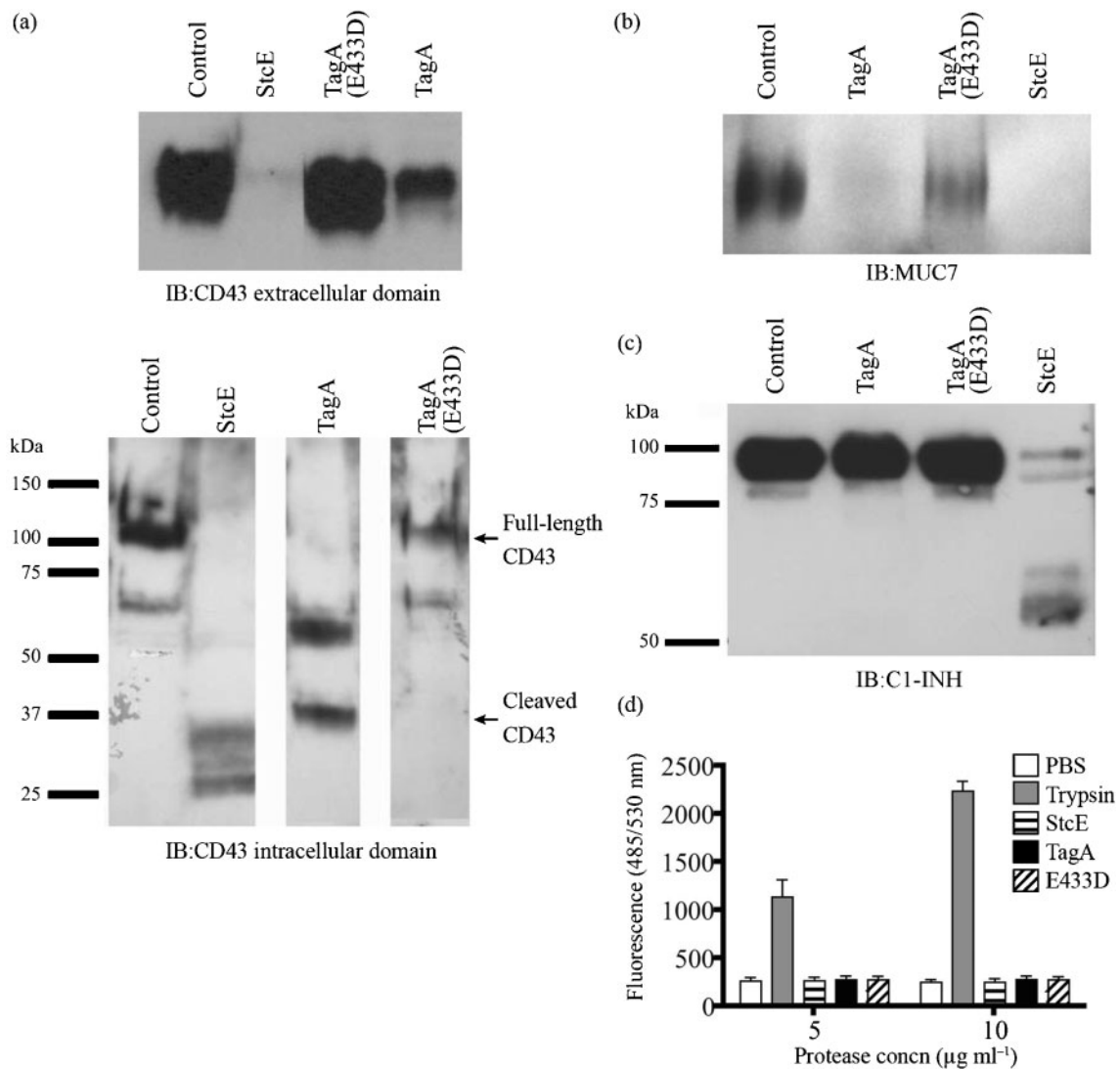


Fig. 3. Comparisons of TagA and StcE substrate profiles. (a) Intact Jurkat cells (1×10^6) were treated with $1 \mu\text{g}$ TagA, TagA(E433D), StcE or PBS control at 37°C , 5% CO_2 for 30 min. Samples were separated by SDS-PAGE and immunoblotted (IB) for the CD43 extracellular or intracellular domain. (b) Human saliva was treated with control or $2 \mu\text{g}$ enzyme at 37°C for 90 min, separated by SDS-PAGE, and immunoblotted for MUC7. (c) Purified C1-INH ($1 \mu\text{g}$) was treated with control or $1 \mu\text{g}$ enzyme at 37°C for 3 h, separated by SDS-PAGE and immunoblotted for C1-INH. (d) Purified enzymes (5 or $10 \mu\text{g}$) were evaluated for cleavage of fluorescent BODIPY-FL casein substrate using the EnzChek protease assay kit. Data are means \pm SEM of two experiments.

conditions. Purified TagA cleaves CD43, MUC7, crude BSM and PGM, and LS174T goblet cell surface mucin, but does not digest C1-INH, hide azure powder, casein or gelatin. Substrate cleavage is prevented by EDTA or by a single glutamic acid to aspartic acid substitution in the consensus metalloprotease active site. We have thus shown that TagA is a metalloprotease similar to StcE, which cleaves specific mucins but does not exhibit general proteolysis. TagA activity stands in clear contrast to the classic *V. cholerae* mucinase, HA/P. HA/P is a broad-spectrum protease that cleaves numerous non-mucin substrates (Finkelstein *et al.*, 1983; Häse & Finkelstein, 1990). HA/P is also regulated by

quorum sensing through HapR and is repressed under conditions that induce the ToxR virulence regulon (Zhu *et al.*, 2002), whereas TagA is activated by ToxR (Taylor *et al.*, 1987).

TagA is homologous to StcE, but exhibits a distinct proteolytic activity and is therefore a unique member of the StcE-like metalloprotease family. *Aeromonas hydrophila* TagA is the only other SLiMe protein whose activity has been characterized. This protein cleaves C1-INH to the same-sized product as the StcE-cleaved product (Pillai *et al.*, 2006), whereas TagA failed to cleave C1-INH. TagA

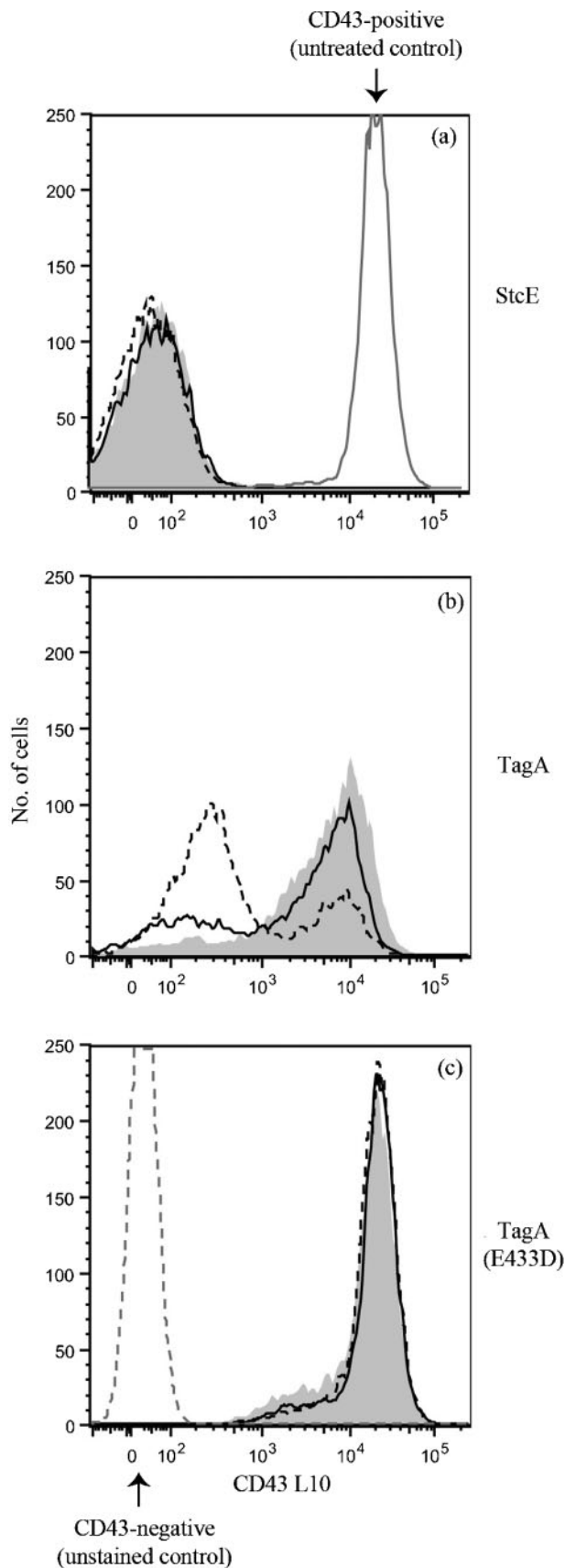


Fig. 4. TagA and StcE exhibit differing cleavage efficiencies toward CD43. Intact Jurkat cells (5×10^5) were treated with control or 1 μg enzyme at 37 $^\circ\text{C}$, 5% CO_2 for 30 min (grey shading), 1 h (solid line) or 3 h (dotted line) as indicated. The presence of the CD43 extracellular domain on the cell surface was detected by flow cytometry. (a) StcE, (b) TagA and (c) E433D. Data are from a representative of three independent experiments.

also demonstrated reduced cleavage efficiency toward CD43, a substrate that it shares with StcE. *A. hydrophila* TagA and EHEC StcE share 68% sequence similarity, as compared to 35% for *V. cholerae* TagA and StcE. This suggests that divergent sequence elements of these proteins might dictate specificity for different substrates.

Our results further suggest that the homologous members of the SLiMe family are restricted to proteolytic cleavage of substrates within the general class of mucin-like O-linked glycoproteins. TagA did not cleave gelatin, which is non-glycosylated, or a fluorogenic casein substrate, which has non-mucin O-glycosylations. Previous research suggests that StcE recognizes an extended random coil structure in the protein backbone that is induced by dense O-glycan attachments, a structure common to mucins and mucin-like glycoproteins (L. Walters & others, unpublished data). We have been unable to identify specific conserved amino acid sequences or glycan linkages that confer recognition and cleavage by StcE. If TagA also recognizes the extended random coil structure, why does StcE cleave C1-INH and TagA does not? We hypothesize that among SLiMe family members with different enzymic efficiencies, their ability to cleave may depend on the length and glycosylation density of the mucin-like region within the substrate. C1-INH has a relatively short mucin-like region that is cleaved by StcE (Lathem *et al.*, 2004), whereas nearly the entire 250 aa extracellular domain of CD43 is O-glycosylated. The intestinal mucins possess variable numbers of tandem O-glycosylated repeats and can form polymers up to 1 MDa in size (Thornton & Sheehan, 2004). It appears that the more efficient StcE mucinase can cleave proteins such as C1-INH with relatively short mucin-like regions, whereas less efficient enzymes like TagA require more extensive mucin-like regions. We have shown that StcE is more efficient at cleaving MUC7 than C1-INH, supporting the hypothesis that the size of the mucin region may contribute to protease activity (Grys *et al.*, 2006). The structural and sequence determinants of enzyme efficiency and specificity of the SLiMe proteins remain to be investigated. Determination of crystal structures and synthesis of recombinant chimeric enzymes could be useful tools to investigate these questions.

Many of the bacteria that encode SLiMes cause disease in mucin-rich environments such as the human intestine, or fish skin in the case of *A. hydrophila*, and the SLiMe mucinase activity may be useful in promoting bacterial colonization and disease in their respective hosts (Austin *et al.*, 2005; Beaz Hidalgo *et al.*, 2008; Thompson *et al.*,

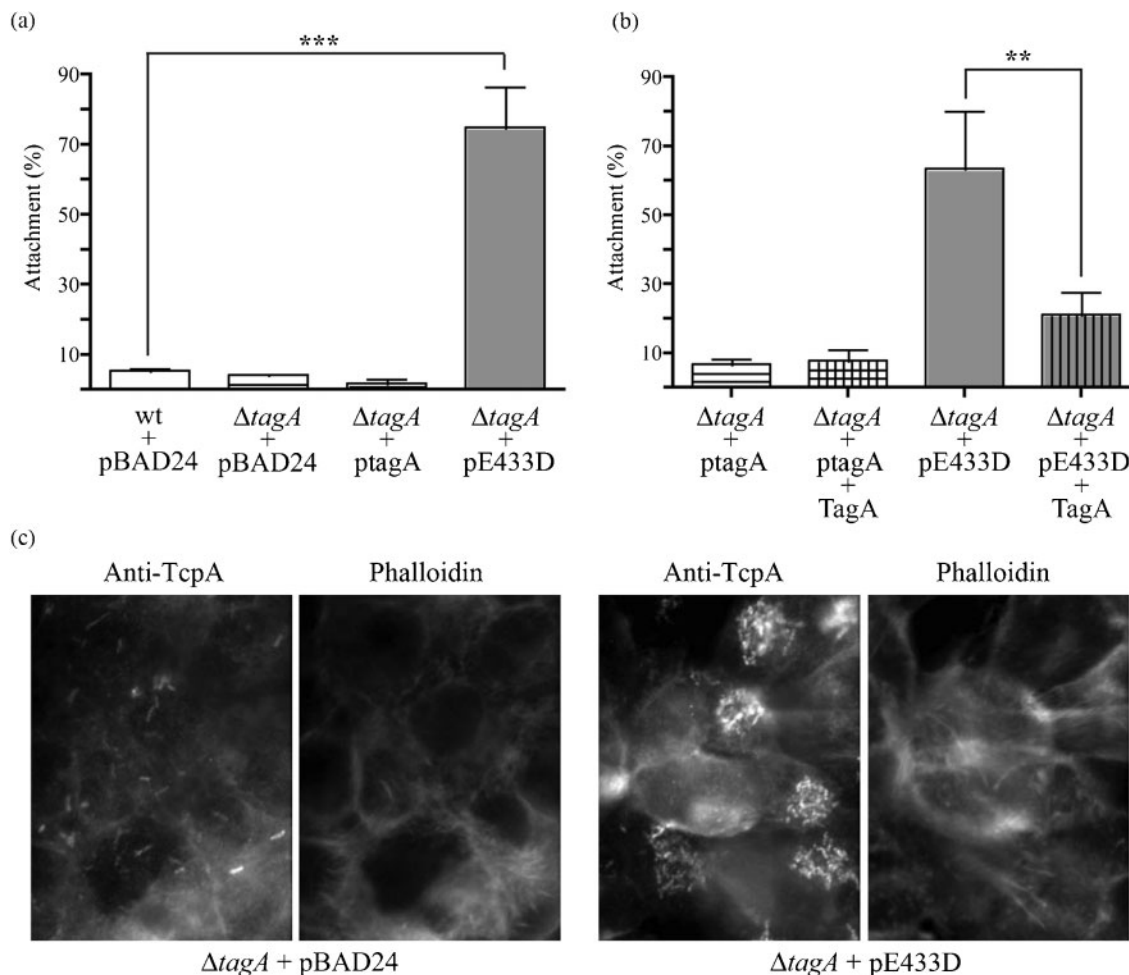


Fig. 5. Attachment of *V. cholerae* TagA mutants to HEp-2 cells. (a) Exponential-phase *V. cholerae* O395, $\Delta tagA$ or $\Delta tagA$ complemented with pBAD24 encoding TagA (ptagA) or TagA(E433D) (pE433D) were incubated with HEp-2 monolayers for 30 min at 37 °C, 5% CO₂. wt and $\Delta tagA$ contained pBAD24 alone as a control. Monolayers were washed and attached bacteria were enumerated by serial dilution and plating. Percentage attachment was calculated as output c.f.u./input c.f.u.×100. Data are from a representative of at least three independent experiments, and are presented as means ± SD ($n=3$). Statistical analysis was performed using GraphPad Prism and one-way ANOVA with the Bonferroni post test; ***, $P<0.001$. (b) Experiments were performed as described for (a), except that HEp-2 monolayers were first treated with 10 μ g purified TagA for 2 h and washed prior to adding bacteria. Data were analysed as above; **, $P<0.01$. (c) HEp-2 monolayers were grown on coverslips and attachment assays were performed as described. Samples were fixed and stained for immunofluorescence with rabbit anti-TcpA to stain *V. cholerae*, and with phalloidin to label HEp-2 actin.

2004, 2006). *A. hydrophila* TagA promotes virulence in a mouse peritonitis model (Pillai *et al.*, 2006), and StcE promotes the intimate attachment of *E. coli* O157:H7 required for human colonization (Grys *et al.*, 2005). TagA is co-expressed with known virulence factors of *V. cholerae* and is expressed *in vivo* during human infection (Bina *et al.*, 2003). *V. cholerae* colonization of the human intestine occurs in the context of a mucus layer. Although TCP is thought to promote attachment by causing bacterial self-aggregation, a direct receptor–ligand interaction with host cells has not been demonstrated for *V. cholerae*. We hypothesized that TagA may contribute to *V. cholerae* attachment by clearing the mucus layer because TagA

demonstrably cleaves crude mucin preparations and the surface mucus of goblet-like intestinal cells. However, we were unable to demonstrate *in vitro* a defect in attachment by bacteria lacking *tagA*. The attachment of *V. cholerae* to HEp-2 cells in culture may have insufficient sensitivity to measure the effects of mucin cleavage in an *in vivo* setting, a more dynamic environment accompanied by peristalsis of the small intestine. Preliminary attachment experiments with the mucus-producing LS174T cell line yielded similar results, further suggesting that more complex assays might be required to fully understand the role of TagA *in vivo*. StcE does not affect overall adherence of *E. coli* O157:H7 to epithelial cells, but specifically increases the ability of

the bacteria to attach intimately and form pedestals (Grys *et al.*, 2005).

TagA may play a role in attachment not revealed by our assay, or it may be important for other aspects of *V. cholerae* colonization and disease. The observation that overexpression of a non-proteolytic mutant of TagA leads to a large increase in *V. cholerae* attachment suggests that TagA can mediate an interaction with the host cell surface. The location of the *tagA* gene within the VPI and the fact that TagA is positively co-regulated with TCP and other ToxR targets provide circumstantial evidence that TagA may play a role in *V. cholerae* disease. The possibility remains that TagA may act similarly to the HA/P mucinase in promoting detachment of *V. cholerae* cells, therefore aiding transmission to the environment. Alternatively, TagA may modulate the immune response to *V. cholerae* by interacting with CD43 or other immune cell surface mucins, as we have recently demonstrated for StcE (Szabady *et al.*, 2009). Another possibility is that TagA plays a role promoting survival and growth in the non-human environment for this pathogen. Such a role is a current area of investigation in our laboratory.

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