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HELICOBACTER PYLORI VacA, A PARADIGM FOR TOXIN MULTIFUNCTIONALITY

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Abstract | Bacterial protein toxins alter eukaryotic cellular processes and enable bacteria to successfully colonize their hosts. In recent years, there has been increased recognition that many bacterial toxins are multifunctional proteins that can have pleiotropic effects on mammalian cells and tissues. In this review, we examine a multifunctional toxin (VacA) that is produced by the bacterium *Helicobacter pylori*. The actions of *H. pylori* VacA represent a paradigm for how bacterial secreted toxins contribute to colonization and virulence in multiple ways.

TOXIN MULTIFUNCTIONALITY
In this review, toxin multifunctionality is defined as the ability of a bacterial protein toxin to cause multiple different cellular effects.

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The importance of bacterial toxins in infectious diseases was recognized more than a century ago, when it was discovered that culture filtrates of many pathogens contain soluble factors that can damage host tissues[1]. Over the past century, more than one hundred different toxins that are produced by various bacterial species have been characterized. The cellular effects of these toxins range from cell death to a variety of non-lethal changes, including permeabilization of membranes, blockade of exocytosis, and alterations in cellular signal transduction, cellular cytoskeletal properties and cellular proliferation[2,3]. Thus, bacterial toxins can modulate many different aspects of eukaryotic cell physiology.

Bacterial toxins can interact with molecular targets on the surface of host cells or can utilize one of several pathways to enter cells and interact with intracellular targets (FIG. 1). Alteration of cellular functions by bacterial toxins typically involves a multistep intoxication process. For toxins that form pores in the plasma membrane, these steps include oligomerization of toxin monomers, insertion of the toxin into the membrane and formation of membrane channels[4,5]. For toxins that act inside cells, these steps include binding of the toxin to the cell surface, internalization and intracellular trafficking of the toxin, translocation of the toxin into the cytosol and enzymatic modification of a specific intracellular target[6]. Successful accomplishment of these multiple intoxication steps requires bacterial toxins to be versatile proteins with multiple activities. Many toxins that act intracellularly have a multidomain structural organization, with each domain capable of mediating a separate step of the intoxication process.

Some bacterial toxins produce a single cellular effect (such as cell death), whereas others can produce multiple cellular effects (BOX 1; TABLE 1). There are potentially several different mechanisms by which a bacterial toxin can produce pleiotropic cellular effects. One possibility is that a toxin could cause a single cellular alteration that results in multiple direct or indirect cellular consequences. Another possibility is that a toxin could enzymatically modify multiple target molecules within a cell or act at multiple sites within a cell. A toxin could possess multiple biochemical activities, each of which results in a different cellular effect. Finally, a toxin could produce disparate effects on different cell types, either through a single mechanism of action that results in different consequences in different cell types or through multiple cell-type-specific mechanisms of action. These mechanisms are not mutually exclusive, but illustrate the diversity of mechanisms that may account for TOXIN MULTIFUNCTIONALITY. On the basis of their ability to cause pleiotropic cellular effects, many bacterial toxins can be regarded as multifunctional proteins.

GASTRIC MUCUS LAYER
A thin layer of mucus covering gastric epithelial cells and consisting mainly of mucins (high-molecular-mass glycosylated proteins).

PEPTIC ULCER DISEASE
A disease that is characterized by ulcerative damage to the mucosal lining of the stomach or duodenum.

GASTRIC ADENOCARCINOMA
The most common form of stomach cancer. Two types can be differentiated based on histological features — intestinal and diffuse types.

VACUOLES
Large intracellular membrane-bound compartments.

The *Helicobacter pylori* vacuolating cytotoxin (VacA) represents an important paradigm for understanding the actions of multifunctional bacterial toxins. In this review, we will consider the wide range of cellular effects that have been attributed to VacA, as well as the functions of VacA *in vivo* that promote *H. pylori* colonization and persistence in the human host.

H. pylori VacA: structure and diversity

For many years, the human stomach was considered to be an inhospitable acidic environment in which bacteria could not grow. This view changed following the isolation of a novel Gram-negative bacterial species, designated *Helicobacter pylori*, from the human stomach in the early 1980s[7]. In the absence of antimicrobial therapy, *H. pylori* can persist in the human stomach for many decades or potentially for the entire lifetime of the host. *H. pylori* persistently colonizes about half of the world's human population[8–11]. Within the human stomach, *H. pylori* can be found either free-living in the GASTRIC MUCUS LAYER or attached to gastric epithelial cells. Localization of *H. pylori* in intracellular sites in epithelial cells or other cell types is relatively uncommon[12,13]. *H. pylori* colonization of the human stomach results in gastric inflammation, known as superficial gastritis. Most *H. pylori*-infected individuals remain asymptomatic, but the presence of *H. pylori* is a risk factor for the development of PEPTIC ULCER DISEASE and GASTRIC ADENOCARCINOMA (the second most common cause of cancer-related death worldwide)[8–11].

Soon after the discovery of *H. pylori* it was reported that a protein in *H. pylori* broth culture filtrates could cause the formation of large intracellular VACUOLES in cultured mammalian cells[14] (FIG. 2a). The *H. pylori* protein responsible for this effect (designated vacuolating cytotoxin or VacA) is encoded by a chromosomal gene known as *vacA*[15–18] (FIG. 3). The amino acid sequence of VacA does not show similarity to any other known bacterial or eukaryotic protein. Mature 88-kDa VacA toxin molecules are secreted as soluble proteins into the extracellular space[15], but can also remain localized on the surface of *H. pylori*[19]. The secreted toxin can assemble into water-soluble oligomeric structures[20–23], and can insert into planar lipid bilayers to form anion-selective membrane channels[24–27]. As will be discussed below, the capacity of VacA to form membrane channels is an important feature of its mechanism of action.

No high-resolution structural data are available for VacA, but insight has been gained by microscopic analysis of VacA oligomeric complexes (FIG. 4). On exposure to either acidic or alkaline pH conditions, these oligomeric complexes dissociate into monomeric components[21,28–30]. Water-soluble VacA oligomers are relatively inactive in assays of vacuolating cytotoxicity, but ACID OR ALKALINE ACTIVATION of these complexes markedly increases VacA activity[21,28–30]. Accordingly, VacA cytotoxicity probably requires an initial interaction of monomeric forms of VacA with cells, and subsequent oligomerization contributes to the ability of VacA to form channels in cell membranes.

The mature, secreted 88-kDa toxin can undergo limited proteolytic cleavage to yield two fragments — p33 and p55 (REFS 17,31–34) (FIG. 3). These two fragments are considered to represent two domains or subunits of VacA. Several studies indicate that the p55 domain has an important role in binding of VacA to host cells[35–39]. About 422 amino acids at the amino-terminal end of VacA (which includes the p33 domain together with about 100 amino acids of the p55 domain) are sufficient to induce cell vacuolation if VacA is expressed intracellularly in transiently transfected cells[40–42]. The mature 88-kDa toxin is predicted to contain only one strongly hydrophobic region, which is located within the p33 domain near the N-terminus of VacA[43,44]. This region contains three tandem GXXXG motifs (defined by glycine residues at positions 14, 18, 22 and 26)[44–46], which are characteristic of transmembrane dimerization sequences. Mutagenesis of several residues within the N-terminal hydrophobic region of VacA (including Gly14 and Gly18), abolishes the

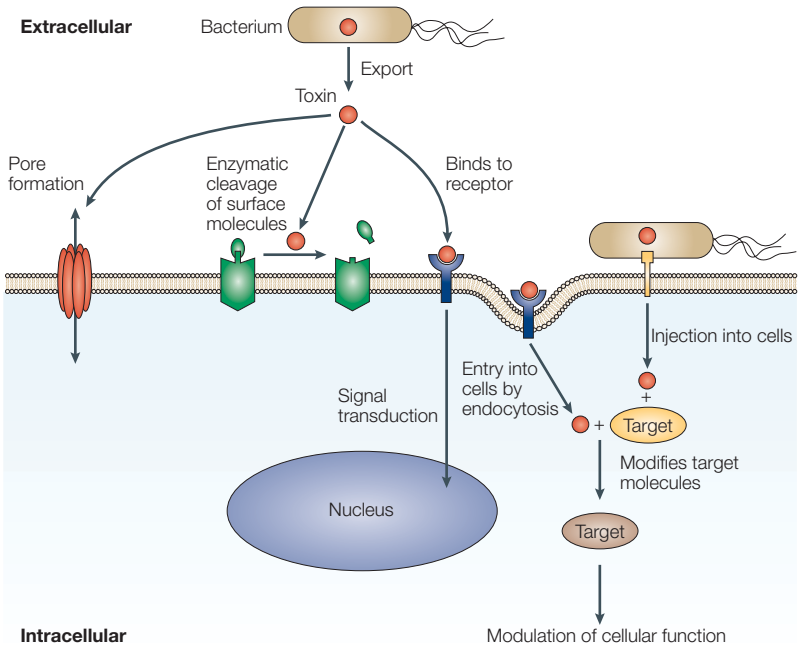

Figure 1 | **Mechanisms of cellular intoxication by bacterial protein toxins.** Pathogenic bacteria synthesize and release protein toxins that act on, or 'intoxicate', target cells in the host. There are many different variations in the mechanisms by which individual bacterial protein toxins intoxicate eukaryotic cells, but several paradigms can be described. Toxins that mainly interact with the plasma membrane of cells can be categorized into several groups. Members of one group insert into the plasma membrane to form pores or channels. Members of a second group are enzymes that catalyse the breakdown of components of the plasma membrane (or, sometimes, components of the extracellular matrix). In both cases, these toxins destabilize the plasma membrane and cause other changes in the cell. A third group of toxins bind to plasma-membrane receptors, resulting in the modulation of eukaryotic cellular signal-transduction pathways. Toxins with an intracellular site of action generally act on and modify specific intracellular eukaryotic targets by enzymatic mechanisms. Many of these toxins are quite potent, such that a single toxin molecule can alter normal cellular function. Some toxins that act intracellularly are secreted by bacteria into the extracellular environment, and must then bind to the mammalian cell surface and be transported into target cells to be effective. Other toxins that act intracellularly are injected directly from the bacterial cell into the eukaryotic cell through translocation conduits that resemble bacterial flagella or conjugative pilus machinery, specialized processes known as type III or type IV secretion pathways, respectively.

Box 1 | **Multifunctional bacterial toxins**

Two of the most extensively studied examples of multifunctional bacterial toxins are ExoS and ExoT from *Pseudomonas aeruginosa*[133]. These toxins have multiple effector domains, each of which is associated with a discrete activity and which is capable of producing a distinct effect on host cells. The amino-terminal domains of ExoS and ExoT are GTPase-activating proteins (GAPs) that target members of the Rho family of small GTPases, which regulate various features of the cellular cytoskeleton[134]. The carboxy-terminal domains are ADP-ribosyltransferases that modify multiple host proteins, including Ras and Ras-like GTPases[135]. The discrete effector domains of these toxins may retain functional activity if expressed as individual proteins. The RhoGAP activities of ExoS and ExoT are almost identical, whereas ExoS and ExoT ADP-ribosylate different substrates[133].

The *Bordetella pertussis* adenylate cyclase (CyaA) toxin represents another example of a multifunctional toxin comprised of multiple effector domains[136,137]. An N-terminal domain encodes adenylate cyclase and a C-terminal domain encodes a portion of the protein that can cause haemolysis of erythrocytes. The adenylate cyclase is activated by eukaryotic calmodulin and catalyses the synthesis of high levels of cyclic AMP. The haemolytic activity of this toxin is attributed to formation of cation-selective channels in cell membranes. CyaA contributes to early steps in colonization of the respiratory tract by *Bordetella pertussis* and has multiple effects on phagocytic cells, including impairing chemotaxis and oxidative responses and inducing apoptosis[137].

Another example of a multifunctional toxin is the binary toxin produced by *Bacillus anthracis*[124,125]. The B subunit of this toxin (protective antigen) can translocate two different A subunits (either a metalloproteinase known as lethal factor or an adenylate cyclase known as oedema factor) into host cells. On entry into host cells, lethal factor can cause many different effects, including alteration of dendritic-cell function, repression of glucocorticoid-receptor transactivation, lysis of macrophages and apoptosis[126–128].

These examples illustrate that although there are many variations in the structure and actions of multifunctional bacterial toxins, each multifunctional toxin is able to have many different effects on eukaryotic cells. Other examples of multifunctional toxins are listed in TABLE 1.

ability of VacA to form membrane channels in planar lipid bilayers and also abolishes vacuolating cytotoxin activity[32,44,47]. These data provide evidence that the unique hydrophobic region at the N-terminus of VacA has a role in membrane channel formation, and that membrane channel formation is required for VacA-induced cell vacuolation.

H. pylori strains isolated from unrelated humans exhibit a high level of genetic diversity, and intraspecies genetic recombination occurs commonly in *H. pylori*[48]. Several families of *vacA* alleles can be distinguished on the basis of diversity near the 5′ terminus of *vacA* (which is known as the s-region) and in the mid-region of the gene (known as the m-region)[49,50] (FIG. 3). VacA molecules of the s2 type are inactive in assays for vacuolating cytotoxicity[49,51–53], type s1/ml forms produce extensive vacuolation of many different cell types and type s1/m2 forms produce detectable vacuolation in a more limited range of cell types[36,37,54,55]. Most *in vitro* studies of VacA have been conducted using highly active s1/m1 forms of the toxin. Although the vacuolation of cultured mammalian cells has provided a convenient phenotype to study many properties of VacA, it seems likely that VacA contributes to *H. pylori* colonization and persistence *in vivo* via additional activities, as will be described below.

ACID OR ALKALINE ACTIVATION
Exposure of oligomeric VacA complexes to acidic or alkaline pH results in disassembly of oligomers and is associated with an increase in vacuolating cytotoxic activity.

VacA in *H. pylori* colonization of the stomach

Many, if not all, bacterial protein toxins contribute in some way to the ability of bacteria to colonize their hosts. Production of a particular toxin may be an absolute requirement for colonization to occur, or alternatively, toxin-producing bacteria may have a selective advantage for colonization compared with strains that do not produce a particular toxin. All strains of *H. pylori* that have been isolated from humans contain the *vacA* gene, which suggests that production of VacA is important for colonization or persistence of *H. pylori* in the human stomach. By contrast, *vacA* orthologues have not been detected in several species of *Helicobacter* that colonize non-human mammalian stomachs[18].

The role of VacA in *H. pylori* colonization of the stomach has been examined in several experimental animal studies. *H. pylori vacA*-null mutant strains are capable of establishing gastric colonization of mice, gerbils and gnotobiotic piglets, which indicates that production of VacA is not an absolute requirement for gastric colonization in these animal models[56–60]. However, mixed infection studies indicate that a VacA-producing *H. pylori* strain has a selective advantage for colonization of the mouse stomach compared with an isogenic *vacA*-null mutant strain[59]. Moreover, immunization of mice with VacA confers protective immunity against subsequent challenge with *H. pylori*[61–63]. These studies provide evidence that expression of VacA contributes to the ability of *H. pylori* to colonize the stomach.

Persistent *H. pylori* infection results in inflammation of the gastric mucosa in humans and experimental animal models, and elicits a local and systemic humoral immune response. Nevertheless, *H. pylori* is able to resist clearance by the host immune defences[8–11]. The possibility that VacA facilitates *H. pylori* persistence has not yet been tested in animal models. However, as will be discussed below, *in vitro* studies indicate that VacA has several immunosuppressive properties. Therefore, it seems plausible that VacA might not only contribute to initial colonization of the stomach by *H. pylori*, but might also have a role in enabling *H. pylori* to persistently colonize human hosts.

Role of VacA in gastroduodenal disease

Strains of *H. pylori* that contain certain allelic forms of *vacA* are associated with an increased risk of symptomatic gastroduodenal disease compared with strains containing other allelic forms of *vacA*[49,50,64,65]. In particular, *H. pylori* strains that contain *vacA* alleles of the s1 type are associated with an increased risk for development of peptic ulcer disease and gastric cancer compared with strains containing *vacA* alleles of the s2 type[49,50,64,65]. These data can be correlated with the failure of type s2 forms of VacA to cause detectable cytotoxicity in *in vitro* assays[49,51–53]. In addition, strains containing type m1 *vacA* alleles are associated with an increased risk for development of gastric epithelial injury and gastric cancer compared with strains containing *vacA* alleles of the m2 type[65,66]. Although drawing conclusions from these epidemiological studies is limited by the possibility of numerous confounding variables, these data suggest that

Table 1 | **Examples of multifunctional toxins**

Toxin	Pathogen	Activities
ExoS	*Pseudomonas aeruginosa*	ADP-ribosylation, GTPase activation
ExoT	*Pseudomonas aeruginosa*	ADP-ribosylation, GTPase activation
Adenylate cyclase	*Bordetella pertussis*	Adenylate cyclase, haemolysis
Cholera toxin	*Vibrio cholerae*	ADP-ribosylation, immunomodulatory effects
Pertussis toxin	*Bordetella pertussis*	ADP-ribosylation, T cell mitogen, haemagglutination, signal transduction through inositol phosphate
Shiga toxin	*Shigella dysenteriae*	Inhibition of protein synthesis, induction of cytokine expression
Toxin A	*Clostridium difficile*	Actin depolymerization, mitochondrial alterations and apoptosis
Lethal toxin	*Bacillus anthracis*	Alterations in dendritic-cell function, apoptosis of various cell types, hypoxic tissue injury, repression of glucocorticoid receptor transactivation, lysis of macrophages
PMT	*Pasteurella multocida*	Cytotoxicity of various cell types, mitogenesis of various cell types, immunomodulatory effects
Aerolysin	*Aeromonas hydrophila*	Haemolysis, cell vacuolation
Alpha toxin	*Staphylococcus aureus*	Haemolysis, apoptosis
Enterotoxin	*Clostridium perfringens*	Cytotoxicity, loosening of tight junctions
Pneumolysin	*Streptococcus pneumonia*	Cytolysis, complement activation
RTX toxin	*Vibrio cholerae*	Cell rounding, actin crosslinking
VacA	*Helicobacter pylori*	Alterations in late endosomes, alterations in mitochondrial membrane permeability, inhibition of T-cell proliferation

certain forms of VacA contribute to the pathogenesis of *H. pylori*-associated peptic ulcer disease and gastric cancer.

To experimentally investigate whether the production of VacA contributes to gastric disease, several different approaches have been used. One approach has been to administer large quantities of the VacA protein directly into the stomachs of mice. This procedure causes gastric mucosal injury and gastric inflammation[17,67,68]. Another approach has been to infect animals with isogenic wild-type and *vacA*-null mutant strains and compare the disease pathologies produced by these strains. Using this approach, no effects of VacA on gastric histology have been detected in gnotobiotic piglets[56], but VacA contributes to the pathogenesis of gastric ulceration in gerbils[58].

VacA as a multifunctional cellular modulator

In an effort to understand how VacA contributes to *H. pylori* colonization of the stomach and development of gastroduodenal disease, the effects of VacA on human cells have been investigated *in vitro*. VacA is able to intoxicate a wide range of cell types, including gastric epithelial cells and several types of immune cells, resulting in an array of multiple different cellular alterations.

VacA as a modulator of epithelial cell function

VacA-induced effects on endosomal compartments. The first documented effect of VacA was its ability to induce cell vacuolation[14] (FIG. 2a). Many different types of cultured cells, including primary human gastric epithelial cells, transformed cell lines derived from multiple different types of human tissue and cell lines derived from multiple mammalian species, undergo vacuolation in response to VacA[14,36,69]. The intralumenal environment of VacA-induced vacuoles is acidic, and the vacuoles are endocytically active[70–73]. The membranes of these vacuoles are enriched in markers for late endocytic compartments, including Rab7, LAMP1 and Lgp110, but do not contain markers for early endocytic compartments[71,74,75]. These features provide evidence that the vacuoles are derived from late endocytic compartments. Vacuolated cells exclude trypan blue[14], which indicates that VacA-induced cell vacuolation is not a cytolethal phenomenon. A current model to explain the mechanism by which VacA induces vacuole formation proposes that VacA binds to the plasma membrane of cells, is internalized by cells, forms anion-selective channels in endosomal membranes and that vacuoles arise due to swelling of endosomal compartments[24,25,27,76] (FIG. 2b). Interactions of VacA with the plasma membrane and the process by which it enters cells are discussed further in subsequent sections of this review, and a detailed discussion of the cellular events leading to formation of intracellular vacuoles is included in BOX 2.

The formation of intracellular vacuoles in response to purified VacA is dependent on the presence of weak bases such as ammonium chloride[15,75,77]. In the absence of weak bases, VacA does not cause cell vacuolation, but still causes detectable alterations in late endocytic compartments. These alterations include inhibition of the intracellular degradation of epidermal growth factor[78], inhibition of procathepsin D maturation (which is associated with mis-targeting of procathepsin D outside the cell)[78], clustering of late endocytic compartments[75] and inhibition of antigen presentation[79].

APOPTOSIS
An active process of programmed cell death, characterized by cleavage of chromosomal DNA, chromatin condensation and fragmentation of the nucleus.

MAP KINASES
A family of mitogen-activated protein kinases that regulate cell growth and differentiation.

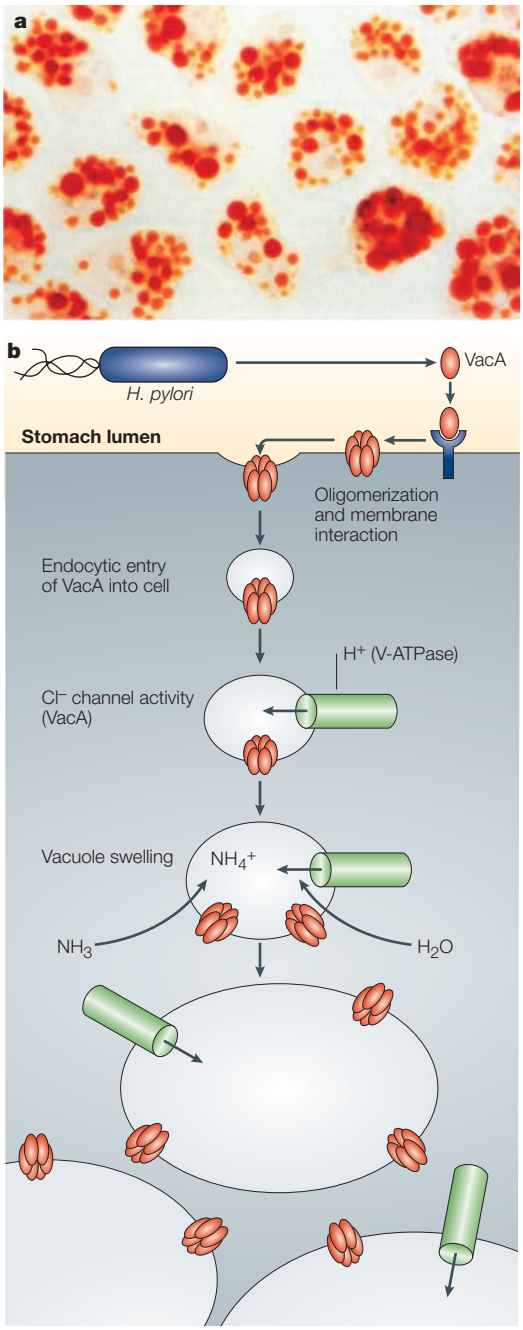

Figure 2 | **Cellular vacuolation induced by VacA.** **a** | Addition of VacA to many different cell types in the presence of weak bases results in the formation of large intracellular vacuoles. This image shows HeLa cells incubated with VacA and stained with neutral red dye[70]. **b** | Schematic illustration of a model for the mechanism of VacA-induced vacuole formation[24,25,27,76]. After binding of VacA to the cell surface, VacA is internalized and forms anion-selective VacA channels in the membranes of late endocytic compartments. Conductance of chloride through these channels results in an increased intralumenal chloride concentration. To compensate for this increased anion concentration, vacuolar ATPase activity increases, resulting in increased proton pumping and a reduction in intralumenal pH. Membrane-permeant weak bases such as ammonia diffuse into late endocytic compartments and become protonated and trapped in these compartments. Osmotic swelling of these compartments ultimately results in cell vacuolation.

VacA-induced effects on mitochondria. In addition to its effects on late endocytic compartments, VacA causes alterations in mitochondria, such as reduction of the mitochondrial transmembrane potential and release of cytochrome *c* — changes that are consistent with an alteration of mitochondrial membrane permeability[80–83]. In addition, VacA-induced modifications to mitochondria have been reported to result in a reduction in cellular ATP concentrations and impaired cell-cycle progression[80]. These effects have been detected after the addition of VacA to the surface of cells, and after intracellular expression of VacA in transiently transfected cells[80–83]. Expression of VacA in transiently transfected cells results in cleavage of poly(ADP-ribose) polymerase (PARP), which is an indication of caspase-3 activation[81]. Co-transfection with DNA encoding Bcl2, which blocks mitochondria-dependent apoptotic signals, inhibits VacA-induced PARP cleavage[81]. Release of cytochrome *c* from mitochondria and activation of the executioner caspase-3 are pro-apoptotic phenomena and, accordingly, prolonged exposure of AGS gastric cells to exogenously added VacA induces APOPTOSIS[84,85].

The development of mitochondrial alterations in response to exogenously added VacA requires longer time periods and higher doses than are required for VacA-induced cell vacuolation[83]. It is possible that VacA-induced mitochondrial alterations might be an indirect consequence of VacA cytotoxicity. However, several studies have reported localization of VacA to the mitochondria[81,82], which suggests that VacA acts directly on mitochondria. An N-terminal fragment of VacA expressed directly within mammalian cells and full-length fluorescently labelled VacA applied exogenously to cells have each been reported to localize to mitochondria[81,82]. In addition, VacA is translocated into isolated yeast mitochondria *in vitro*[81]. The changes in mitochondrial membrane permeability that are observed might result from activation of endogenous channels found in mitochondria. However, mutant forms of VacA that are defective in the capacity to form membrane channels fail to cause cytochrome *c* release, and NPPB, an agent that blocks formation of VacA membrane channels, inhibits VacA-induced cytochrome *c* release[82,83]. Therefore, it seems likely that VacA-induced effects on mitochondria may be dependent on the formation of VacA channels in mitochondrial membranes.

VacA-induced effects on cellular signal-transduction pathways. The effects of VacA on late endosomal compartments and mitochondria are detectable within several hours of the addition of VacA to cells. By contrast, VacA causes several other cellular effects that occur much more rapidly. Within 10 minutes of addition of VacA to a human gastric adenocarcinoma cell line (AZ-521), two classes of mitogen-activated protein kinases (MAP KINASES) (p38 and ERK1/2) and the activating transcription factor 2 (ATF2) signalling pathway are activated[86]. VacA has also been reported to induce activation of p38 in several other types of cells[87]. An

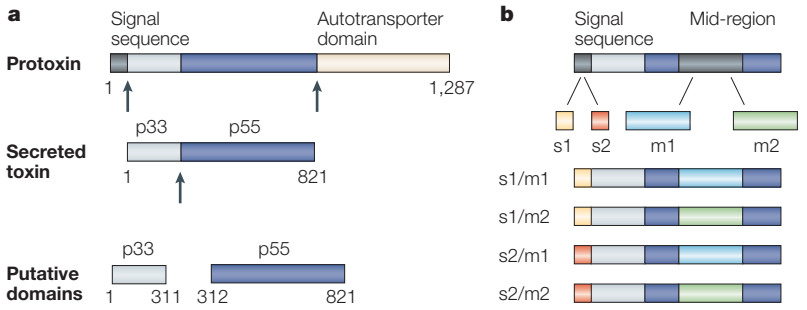

Figure 3 | ***vacA* gene structure and allelic diversity. a** | The amino-terminal signal sequence and carboxy-terminal domain are cleaved from the 140-kDa VacA protoxin to yield an 88-kDa mature toxin that is secreted into the extracellular space via an autotransporter mechanism[16–18]. The structure of the *vacA* gene and the secretion and proteolytic processing of the VacA protein are characteristic of a family of autotransporter proteins secreted by Gram-negative bacteria. Proteins of this family are able to mediate their own secretion, without the actions of additional bacterial proteins. A 33-kDa C-terminal β-barrel domain of VacA is predicted to insert into the outer membrane and form a channel, through which the mature VacA toxin is secreted[17]. The capacity of the VacA C-terminal β-domain to function in this manner has been experimentally confirmed by demonstrating that this region can transport a foreign passenger protein (cholera toxin B subunit) to the *H. pylori* surface[147]. Arrows indicate sites of proteolytic cleavage. **b** | There is a high level of diversity among *vacA* alleles from different *H. pylori* strains[49,50]. Allelic diversity is particularly striking near the 5′ terminus of *vacA* (the s-region) and in the mid-region of the gene (the m-region). Two main families of s-region sequences (s1 and s2) and two main families of m-region sequences (m1 and m2) have been described[49]. Type s1 and s2 VacA proteins differ in the site at which N-terminal signal sequences are cleaved, and consequently, the mature, secreted type s2 VacA toxin contains a 12-amino-acid hydrophilic extension at its N-terminus that is absent from type s1 VacA toxins[49,51–53]. VacA toxins that contain this 12-amino-acid hydrophilic extension fail to induce cell vacuolation *in vitro*[49,51,52]. The amino acid sequences of type m1 and m2 VacA proteins are ~65% identical within a region comprising 250 amino acids[36,49]. Subtypes of *vacA* alleles (for example, s1a, s1b, s1c, m1a, m1b, m2a and m2b) have been described, and certain subtypes have a geographically restricted distribution[50]. Mosaic forms of *vacA* are thought to arise via homologous recombination among *vacA* alleles from different strains.

inhibitor of p38 kinase activity (SB203580) does not block VacA-induced vacuolation or VacA-induced cytochrome *c* release, which indicates that VacA-induced activation of the p38/ATF-2 signalling pathway is independent of the effects of VacA on late endocytic compartments and mitochondria[86]. The cell-surface receptors for VacA that are involved in activating these signalling pathways have not yet been characterized, and the functional consequences of these signalling events are not yet well understood.

Within 30–60 minutes of the binding of VacA to BHK-21 cells expressing receptor protein tyrosine phosphatase β (RPTPβ, also known as Ptprz), tyrosine phosphorylation of G-protein-coupled receptor kinase interactor (Git1) can be detected[67]. In another model system, binding of VacA to a mast cell line (RBL-2H3 cells) results in a rapid change in cytosolic calcium concentrations[88]. The effects of VacA on mast cells do not require acid activation of purified oligomeric VacA[88], which contrasts with the requirement of acid activation for cell-vacuolating activity of VacA. In contrast to effects of VacA on endocytic compartments and mitochondria, the relatively rapid cellular responses to VacA described above are likely to be due to the binding of VacA to specific cell-surface components, without a requirement for internalization of the toxin.

PARACELLULAR EPITHELIAL PERMEABILITY
Diffusion across a polarized epithelial monolayer via the junctions betwen adjacent cells.

VacA-induced effects on epithelial monolayer permeability. VacA reduces the transepithelial electric resistance (TER) of monolayers formed by several different types of polarized epithelial cells[89], an effect that is attributable to increased PARACELLULAR EPITHELIAL PERMEABILITY of the monolayers to molecules and ions of low molecular mass. It is proposed that selective permeabilization of epithelial monolayers by VacA results in the release of molecules such as Fe^{3+}, Ni^{2+}, sugars and amino acids, which might support the growth of *H. pylori* in the gastric mucus layer[89]. As yet, the mechanisms by which VacA alters paracellular permeability are not well understood. This activity does not require acid activation of purified oligomeric VacA and is not inhibited by bafilomycin A1 (REF. 89). In addition to altering paracellular permeability, VacA also increases the transepithelial flux of certain molecules, including urea and bicarbonate[27,90–92]. The release of the latter factors from cells is attributed to the formation of VacA channels in the plasma membrane[27].

VacA as a modulator of immune cell function

Effects of VacA on phagocytosis and antigen presentation. Two reports have provided evidence that VacA contributes to the formation of large vesicular compartments (known as megasomes) in *H. pylori*-infected macrophages by stimulating homotypic phagosome fusion[93,94]. VacA promotes recruitment and retention of the tryptophan–aspartate-containing coat protein (TACO or coronin 1) by phagosomes, thereby disrupting phagosome maturation[93]. By altering various properties of phagosomes, VacA might impair phagocytic killing of *H. pylori*. Notably, one report did not detect any effects of VacA on intracellular survival of *H. pylori* in human monocytes[95].

VacA has been reported to interfere with antigen presentation by B lymphocytes[79]. In one model system, VacA affected proteolytic processing of tetanus toxoid and inhibited the invariant chain (Ii)-dependent pathway of antigen presentation that is mediated by newly synthesized major histocompatibility complex (MHC) class II molecules[79]. It seems likely that these effects of VacA on phagocytic cells and antigen-presenting cells are due to VacA-induced effects on endocytic compartments.

Effects of VacA on T lymphocytes. VacA has multiple effects on T lymphocytes[87,96,97]. When added to cultured Jurkat T cells, VacA inhibits the production of interleukin 2 (IL2) (which is required for T-cell viability and proliferation) and downregulates surface expression of IL2 receptor-α[87,96,97]. These effects are attributable to the ability of VacA to inhibit activation of nuclear factor of activated T cells (NFAT) — a transcription factor that acts as a global regulator of immune response genes and which is required for optimal T-cell activation[87,96]. The mechanism by which VacA inhibits NFAT activation might involve blocking the influx of calcium from the extracellular milieu, thereby inhibiting the activity of the Ca^{2+}-calmodulin-dependent phosphatase calcineurin

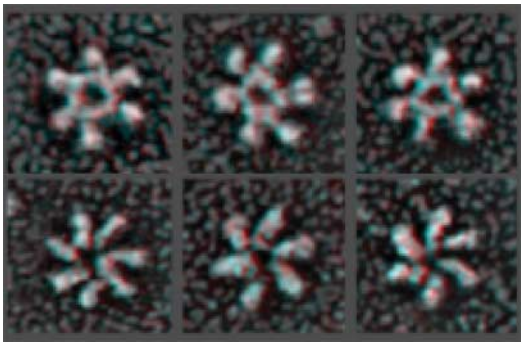

Figure 4 | **Water-soluble oligomeric structures formed by VacA, imaged by deep-etch electron microscopy.** VacA can assemble into bilayered structures (top row) or single-layered structures (bottom row)[20–23]. The diameter of these oligomeric structures is ~30 nm.

(which dephosphorylates NFAT)[87,96]. Intoxication of Jurkat T cells by VacA alters the expression of more than 100 genes, including many that are also affected by FK506 (Tacrolimus) and cyclosporine A[96], which are two immunosuppressive drugs that inhibit calcineurin activity. Other effects of VacA on T lymphocytes include activation of intracellular signalling through MAP kinases (such as MKK3/6 and p38) and the Rac-specific nucleotide exchange factor Vav[87]. Studies with primary human $CD4^+$ T cells indicate that VacA can inhibit proliferation of activated T cells via a mechanism that is independent of the effect of VacA on NFAT activation and IL2 expression[97]. The anti-proliferative effects of VacA might provide a mechanism by which the clonal expansion of T cells that have already been activated by *H. pylori* antigens can be inhibited.

Pro-inflammatory effects of VacA. The effects of VacA on T lymphocytes described above are expected to result in localized immunosuppression. However, VacA is also reported to induce pro-inflammatory effects. VacA stimulates the production of PRO-INFLAMMATORY CYTOKINES (such as TNFα and IL6) by mast cells and induces chemotaxis and degranulation of these cells[68,88]. Moreover, VacA treatment stimulates expression of cyclooxygenase-2, a pro-inflammatory enzyme, in neutrophils and macrophages[87]. Thus, the effects of VacA on immune cells are complex and are characterized by both immunostimulatory and immunosuppressive actions.

Mechanistic basis for multiple VacA activities

As described above, VacA has been reported to have many different effects on mammalian cells. It should be noted that not all of the reported actions of VacA have been rigorously evaluated and confirmed in multiple independent laboratories. However, there has been sufficient reproducibility to conclude that VacA can cause a panoply of diverse effects. In this section, we discuss mechanistic features that could account for the considerable variation in VacA cellular effects.

Interactions of VacA with the cell surface. For most bacterial toxins, cellular intoxication is initiated by binding of the toxin to a plasma-membrane component that functions as a receptor for the toxin. Several bacterial toxins bind specifically to a single receptor on the surface of host cells. By contrast, VacA has been reported to bind to multiple cell-surface components, including RPTPβ[30,67], RPTPα[98], various lipids[24,29,99], the epidermal growth factor (EGF) receptor[100] and heparan sulphate[101]. Determining which VacA binding interactions are required for VacA-induced cellular alterations has been a challenging area of study[102–104].

So far, binding of VacA to RPTPβ has been studied in most detail. RPTPβ antisense oligonucleotides inhibit VacA-induced vacuolation of AZ-521 gastric cells, and cells that are resistant to the cytotoxic effects of VacA become sensitive when they are transfected with plasmids encoding RPTPβ[105,106]. These data indicate that RPTPβ has an important role in the process of VacA-induced vacuolation. Binding of VacA to RPTPβ might have other functional consequences besides vacuolation, including activation of signalling pathways that result in tyrosine phosphorylation of Git1 (REF. 67). Another reported consequence of VacA binding to RPTPβ is detachment of primary murine gastric epithelial cells from a reconstituted basement membrane[67]. When administered intragastrically, VacA causes gastric injury in wild-type mice but not in RPTPβ-null mutant mice[67], which indicates that interaction of VacA with RPTPβ on the surface of gastric epithelial cells is mechanistically important in VacA-induced gastric injury. Interestingly, VacA causes vacuolation of $RPTP\beta^{-/-}$ gastric epithelial cells and G401 human kidney cells, which do not express RPTPβ[67,98]. Treatment of G401 cells with RPTPα antisense oligonucleotides inhibits VacA-induced vacuolation[98]. So, the data indicate that either RPTPβ or RPTPα could be functionally important receptors for VacA in various cell types.

On contact with cells, acid-activated VacA localizes to detergent-resistant membrane fractions of the plasma membrane[107–111], which are the biochemical equivalent of LIPID RAFTS. Whether or not the cell-surface receptors for VacA described above also localize to lipid rafts is currently not known. The importance of VacA interactions with lipid rafts is suggested by the fact that disruption of lipid rafts by cholesterol depletion results in inhibition of VacA-induced cellular vacuolation[104,107–109].

Most studies of VacA binding to cells have analysed type s1/m1 forms of VacA, which have the greatest levels of vacuolating toxin activity in many cell types. Interestingly, the cell-binding patterns of type s1/m1 and s1/m2 VacA proteins are quite different, and these two types of VacA proteins have different cell-type specificities in assays for vacuolating toxin activity[36,37,54,55]. It seems likely that m1 and m2 types of VacA toxins might target different components of the plasma membrane.

In summary, the interactions of VacA with the cell surface are complicated by the potential for this toxin to interact with multiple cell-surface components, and by

PRO-INFLAMMATORY CYTOKINES
Secreted proteins that regulate the inflammatory response.

LIPID RAFTS
Membrane microdomains enriched in cholesterol, sphingolipids and GPI-anchored proteins.

the potential for VacA to interact with cells either as a monomer or as an oligomer. Further complexity arises because the relevant cell-surface receptors for VacA may vary among different cell types. In addition, type m1 and type m2 VacA proteins might preferentially bind to different cell-surface components. The complexity of VacA interactions with the cell surface is likely to be an important factor that contributes to the diverse cellular activities attributed to VacA.

Membrane insertion and internalization of VacA. Following binding of VacA to the cell surface, at least some of the bound VacA molecules insert into the plasma membrane to form membrane channels. Expression of GPI-ANCHORED PROTEINS on the cell surface seems to be important for this process[111]. The structure of VacA membrane channels is predicted to be similar to the structure of water-soluble VacA oligomers[23,24]. Notably, water-soluble VacA oligomers can be formed by a variable number of monomeric components[23]. If there is similar variation in the composition of membrane-associated oligomeric channels formed by VacA, this would be expected to result in variation in the properties of these membrane channels, potentially associated with different cellular effects.

In addition to forming plasma-membrane channels, bound VacA molecules can undergo internalization into the cell. The process by which VacA is internalized has not yet been characterized in detail, but it is known to be temperature-, energy- and actin-dependent but clathrin-independent [35,103,104,107–109,111]. Interaction of VacA with lipid rafts has an important role in VacA internalization and intracellular trafficking[104,107–109]. Internalized VacA has been localized to multiple intracellular sites, including endosomal compartments, the large intracellular vacuoles that form as a consequence of VacA intoxication, and the inner mitochondrial membrane[75,81–83,112,113]. The mechanisms by which VacA undergoes sorting and trafficking to multiple intracellular sites are poorly understood. In particular, the intracellular pathway that is used by VacA for trafficking from the cell surface to mitochondria has not yet been characterized, and there are few, if any, reported examples of such a cellular protein-trafficking pathway. Localization of VacA in multiple cellular sites could be an important factor contributing to the different activities described for this toxin.

Membrane channel-dependent and channel-independent actions of VacA. Many of the cellular effects of VacA, both in epithelial cells and T lymphocytes, can be attributed to the ability of this toxin to insert into membranes and form anion-selective channels[24–27,111,114]. Several effects of VacA, including leakage of ions and other small molecules from gastric epithelial cells[27,90,92], result from the formation of VacA channels in the plasma membrane. VacA-induced cell vacuolation can be attributed to VacA channel formation in the membranes of late endocytic compartments[24,25,27,76] and VacA-induced alterations in mitochondrial membrane permeability can be attributed to VacA channel formation in mitochondrial membranes[82,83]. Thus, the formation of VacA channels at different cellular sites might account for several different VacA activities.

Although many cellular effects of VacA are attributable to membrane channel formation in various cellular locations, several VacA effects are probably the consequences of channel-independent actions. For example, activation of p38 and Git1 after VacA interaction with gastric epithelial cells[67,86] and the rapid changes in calcium concentration that follow VacA interaction with mast cells[88] may result from VacA interactions with cell-surface receptors and the activation of specific signal-transduction pathways, without a requirement for membrane channel formation. VacA-induced activation of intracellular signalling in T cells can also occur by a channel-independent mechanism[87]. Yeast two-hybrid studies have demonstrated that VacA can interact with several intracellular proteins in cells, including VIP54 (a protein associated with intermediate filaments)[115] and RACK1 (a receptor for protein kinase C)[116]. The functional significance of these VacA interactions has not yet been investigated, but such interactions might form the basis for channel-independent intracellular actions of the toxin. We speculate that the full range of cellular activities of many toxins, including VacA, has not yet been defined.

VacA actions *in vitro* and *in vivo*

VacA modulates mammalian cells in an extraordinary number of ways, each of which could potentially contribute to *H. pylori* colonization, persistence or gastroduodenal disease (FIG. 5). One of the exciting challenges at present is to understand which cellular modulatory activities of VacA contribute to specific stages of the infectious process. Data addressing this topic are sparse, but several hypotheses can be proposed.

H. pylori lives in the gastric mucus layer and interacts with the apical surface of gastric epithelial cells. It seems likely that these cells are one of the main targets of VacA. The presence of VacA on the surface of *H. pylori*[19] indicates that one function of VacA might be as an adhesin, mediating binding of the bacteria to gastric epithelial cells. VacA may also alter the surface properties of gastric epithelial cells or alter the secretion of various factors by these cells[78] in a manner that stimulates *H. pylori* adherence or growth. One study has suggested that the effects of VacA on endocytic compartments might promote intracellular survival of *H. pylori* in gastric epithelial cells[12], but the results of another study did not confirm this result[13]. Another important function of VacA might be to compromise the integrity of the gastric epithelial barrier, thereby releasing nutrients that are required for *H. pylori* growth in the gastric mucus layer. VacA can stimulate nutrient release via several mechanisms, including increasing paracellular permeability of the gastric epithelial monolayer[89], permeabilizing the plasma membrane of gastric epithelial cells[27,90–92], inhibiting epithelial cell proliferation[117], causing apoptosis of gastric epithelial cells[81–83,85] or causing direct damage to the gastric epithelial monolayer[17,67]. Permeabilization of

GPI-ANCHORED PROTEINS
Proteins that are anchored to a lipid bilayer by a glycosylphosphatidylinositol (GPI) moiety.

Box 2 | **Mechanism of cell vacuolation induced by VacA**

The membranes of VacA-induced cell vacuoles contain Rab7 and other markers for late endocytic compartments[71,74,75]. A current model for VacA-induced vacuolation proposes that VacA is internalized by cells and forms membrane channels in the membranes of late endocytic compartments (FIG. 2). In support of this model, VacA has been found to localize to the membranes of VacA-induced vacuoles[112,113]. Intracellular expression of VacA in transiently transfected cells results in cell vacuolation[40,42], which provides additional evidence that it can act at an intracellular site. An inhibitor of VacA channel formation, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), blocks the ability of VacA to cause cell vacuolation[27,114], and VacA mutant toxins that lack the ability to form membrane channels also lack the ability to cause cell vacuolation, regardless of whether they are applied to the surface of cells or expressed intracellularly in transiently transfected cells[44,47]. Interestingly, a mutant toxin that lacks the ability to form membrane channels can block the activity of wild-type VacA in a dominant-negative fashion[43]. This dominant-negative phenotype is associated with the formation of mixed oligomeric structures that comprise both wild-type and mutant VacA[51]. The ability of a dominant-negative mutant toxin to block wild-type VacA activity provides further evidence that an oligomeric form of VacA is required for VacA cytotoxicity.

Multiple cellular factors, including vacuolar ATPase, Rab7, Rac1, syntaxin 7 and dynamin, are reported to be required for VacA-induced vacuolation[75,138–144]. These factors might be required for VacA internalization or the process of vesicle swelling. Overexpression of PIKfyve kinase has been reported to inhibit VacA-induced vacuolation, which indicates that VacA-induced cellular alterations might be related to changes in cellular phosphatidylinositol metabolism[145].

An important biophysical question surrounding the process of VacA-induced vacuole formation concerns the source of the membrane from which intracellular vacuoles are derived. Massive swelling of pre-existing vesicular compartments might be expected to result in lysis of these compartments if an additional source of membrane were not available. One possibility is that VacA-induced vacuoles might form as the result of fusion of multiple smaller endocytic compartments. In support of this view, the SNARE protein syntaxin 7, which is involved in intracellular membrane-fusion events, has been localized to the membranes of VacA-induced vacuoles, and intracellular expression of a dominant-negative mutant form of syntaxin 7 blocks VacA-induced vacuolation[143]. Another possibility is that vacuoles could arise from late endosomes without a requirement for fusion of different compartments via a process involving fusion of late endosomal internal membranes with the late endosomal limiting membrane[146]. Further studies are needed to clarify the role of membrane-fusion events in VacA-induced cell vacuolation.

gastric epithelial cells by VacA results in the release of urea, which is the substrate for urease (an enzyme that is required for *H. pylori* colonization of the stomach)[90], and also results in the release of bicarbonate ions, thereby buffering the acidic pH of the gastric mucus layer[27,91]. Besides affecting gastric epithelial cells, it seems possible that VacA can affect other cell types that are found in the gastric mucosa, including acid-secreting parietal cells[118] and pepsin-secreting chief cells.

Several lines of evidence indicate that VacA produces pro-inflammatory effects[17,67,68,87]. VacA-mediated inflammation may result in numerous alterations to the gastric epithelial layer, and thereby support *H. pylori* colonization. A controlled level of gastric mucosal inflammation might benefit *H. pylori*, but at the same time, *H. pylori* uses multiple mechanisms to evade clearance by the host immune response. By interfering with phagosome maturation in phagocytic cells, VacA might enable *H. pylori* to resist phagocytic killing[93]. The effects of VacA on antigen presentation might also enable *H. pylori* to resist immune clearance[79]. Finally, by inhibiting activation and proliferation of T lymphocytes[87,96,97], it seems likely that VacA can induce a localized immunosuppression, and thereby contribute to the ability of *H. pylori* to persistently colonize the stomach.

Toxin multifunctionality

Similar to VacA, many other bacterial toxins are able to affect mammalian cells in multiple different ways (TABLE 1). A common property of several of these multifunctional toxins is the ability to cause distinct cellular effects depending on whether the toxin acts at the cell surface or an intracellular site. For example, *Staphylococcus aureus* alpha toxin (classified as a 'channel-forming' toxin) inserts into the plasma membrane to form membrane channels but also targets mitochondria[119]. These different sites of action of alpha toxin are associated with different cellular responses — membrane permeabilization and apoptosis, respectively. It is likely that several enzymatically active toxins also cause cellular effects at both the cell surface and intracellular sites. In the case of cholera toxin and pertussis toxin, it is possible to distinguish between effects that are produced by the enzymatically active A subunits (which act as intracellular ADP ribosyltransferases), and effects produced by the B subunits (which mediate toxin binding to cells). Specifically, the pentameric B subunits of cholera toxin modulate signalling pathways in various types of immune cells[120], and the pentameric B subunits of pertussis toxin act as a mitogen for T lymphocytes[121,122].

Similar to VacA, several multifunctional toxins have disparate effects on different cell types. For example, *Aeromonas hydrophila* aerolysin (which is a pore-forming toxin) causes lysis of erythrocytes, and induces vacuolation of epithelial cells[123]. *Bacillus anthracis* toxin, which consists of lethal factor and protective antigen, causes apoptosis of endothelial cells, impaired function of dendritic cells and lysis of macrophages[124–128]. Shiga toxin 2 promotes the death of vascular endothelial cells[129], while stimulating a pro-inflammatory response in neutrophils and macrophages[130]. So, the actions of an individual toxin can vary markedly depending on the type of cell to which it is targeted. A common feature of many multifunctional

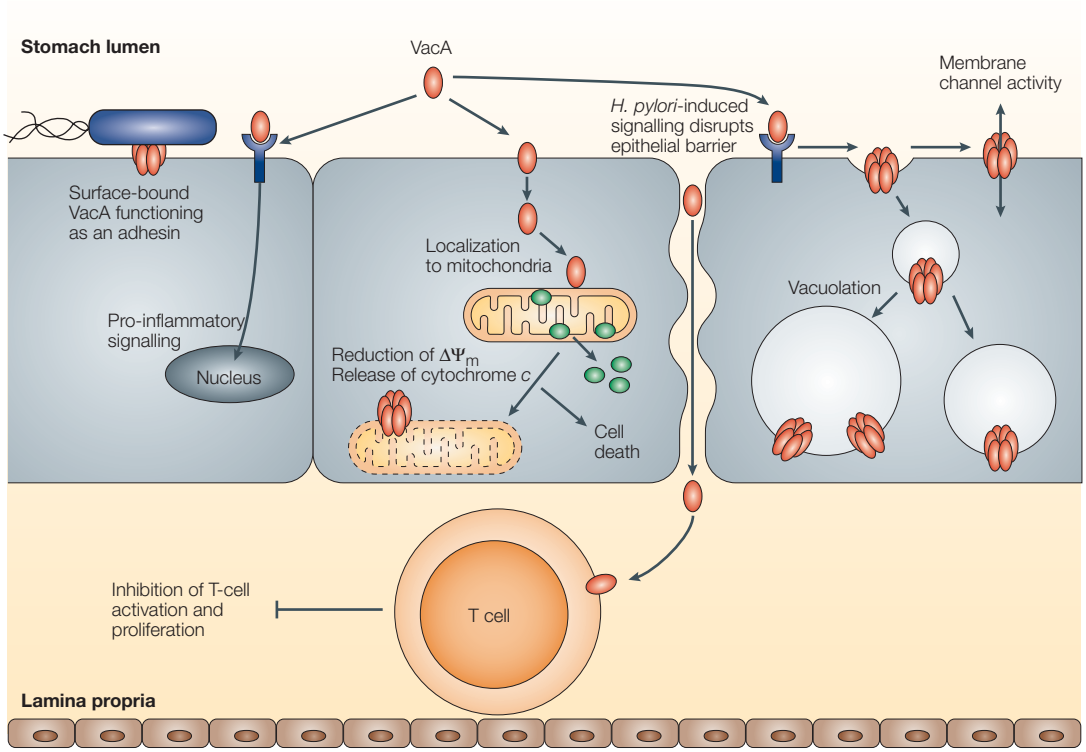

Figure 5 | **Multiple actions of VacA contribute to *H. pylori* colonization of the stomach.** Effects of VacA on gastric epithelial cells include alterations in mitochondrial membrane permeability and apoptosis, stimulation of pro-inflammatory signalling, increased permeability of the plasma membrane and alterations in endocytic compartments. Multiple *H. pylori* factors, including CagA, disrupt the gastric epithelial barrier and might thereby facilitate passage of VacA through the epithelial layer[148,149]. Within the lamina propria, VacA interferes with the activation and proliferation of T lymphocytes. Many of these effects of VacA are attributable to the formation of VacA membrane channels. $\Delta\Psi_m$ indicates mitochondrial transmembrane potential.

toxins seems to be the ability to induce one set of effects on cells that are encountered at early stages of infection (for example, epithelial cells found on mucosal surfaces), and a different set of effects on immune cells.

What can VacA teach us about the underlying basis of toxin multifunctionality? Perhaps one of the most striking clues is the ability of VacA to interact with multiple components on the surfaces of different mammalian cells. The ability of a toxin to engage multiple different receptors is expected to have important functional implications. First, targeting different receptors might allow a toxin to function at the cell surface (via its interaction with one receptor) and to be internalized by cells (via its interaction with another receptor). Second, binding to different cell-surface receptors might be linked to differential sorting of toxin molecules on the cell surface, entry of toxin molecules into cells via multiple different pathways or intracellular trafficking of toxin molecules via different routes. Consequent localization of toxin molecules at multiple intracellular sites could affect the actions of the toxin within a cell. Finally, targeting multiple receptors might allow a toxin to interact in unique ways with multiple different cell types. Interaction of a toxin with multiple different cell-surface receptors may allow a single biochemical activity (for example, channel formation) to be delivered to multiple cell types in a fairly nonspecific manner. Alternatively, it seems likely that binding of a toxin to cell-type-specific receptors could allow targeting of selected effector functions to specific cell types.

The underlying process by which VacA interacts with multiple components of the plasma membrane is poorly understood. One possibility is that VacA has multiple receptor-binding sites, each of which is optimized for binding to a different receptor. VacA oligomers might have a greater number of receptor-binding sites than VacA monomers, analogous to the multiple receptor-binding sites of several other oligomeric toxins[131,132]. For example, Shiga toxin has three different binding sites in each of its five cell-interaction domains — a total of 15 potential binding sites per oligomeric structure[131]. Therefore, an intriguing possibility is that, on encountering host cells, VacA might crosslink different receptors in a combinatorial fashion, resulting in the induction of several signalling pathways or several internalization pathways for entry of VacA into the cell.

In general, the identification of cell-surface receptors to which bacterial toxins bind is a difficult process, and relatively few TOXIN RECEPTORS have been identified and fully characterized. As yet it is not known whether the ability to bind to multiple cell-surface receptors is a property shared by many multifunctional toxins. It will be important in future studies to characterize further the full repertoire of cell-surface receptors to which

TOXIN RECEPTOR
A component of the eukaryotic cell surface to which a bacterial toxin can bind.

bacterial toxins bind, and to investigate the role that binding to multiple toxin receptors might have in the activities of multifunctional bacterial toxins.

Concluding remarks and perspectives

Bacterial toxins function at the interface between the prokaryotic and eukaryotic kingdoms by modulating eukaryotic cells in a manner that promotes colonization and persistence of bacteria within eukaryotic hosts. By producing toxins with multifunctional properties, bacteria are able to use a single protein to produce a range of effects at different sites in the host, depending on which cell types and tissues are targeted. Consolidating multiple functions within a single protein has several advantages to the bacteria, including conservation of genome space, conservation of energy and resources that would be needed to regulate, produce and secrete multiple gene products, and stoichiometric, spatial, and temporal control of multiple host-modulating activities.

VacA has been reviewed as a paradigm for toxins that have multiple activities *in vitro* and that can contribute to bacterial pathogenesis in several ways. For VacA, as well as other multifunctional bacterial protein toxins, a challenging issue is to identify which activities are relevant for specific stages of the infectious process. A current model proposes that VacA and other multifunctional toxins have an important role in remodelling host tissues to enable initial bacterial colonization of the host, and that these toxins also modulate the properties of cells that are involved in innate and adaptive immunity, thereby allowing the pathogens to evade host immune defences.

Further understanding of the role of bacterial toxins *in vivo* will require new and innovative approaches. One of the least-understood aspects of toxin biology is how the expression of bacterial toxins is regulated in response to specific environmental conditions encountered within the host. New imaging techniques will facilitate study of the temporal and spatial patterns of toxin expression, especially in chronic infections such as *H. pylori*. In addition, analysis of the expression patterns of cell-surface receptors for toxins *in vivo* will be crucial for understanding the importance of specific toxin activities in the host. Finally, the use of microdissection techniques will make it possible to monitor the effects of toxins on specific cell types at varying distances from the site of infection. These future studies are expected to lead to important new insights into the role of toxins as factors that promote bacterial colonization of the host and that enable bacteria to evade host immune defences.

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Competing interests statement

The author declares competing financial interests: see Web version for details.

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