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FEMS Microbiology Reviews 26 (2002) 125–139

FEMS MICROBIOLOGY Reviews

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# *Vibrio cholerae* and cholera: out of the water and into the host

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Received 4 February 2002; accepted 11 February 2002

First published online 23 April 2002

**Abstract**

The facultative human pathogen *Vibrio cholerae* can be isolated from estuarine and aquatic environments. *V. cholerae* is well recognized and extensively studied as the causative agent of the human intestinal disease cholera. In former centuries cholera was a permanent threat even to the highly developed populations of Europe, North America, and the northern part of Asia. Today, cholera still remains a burden mainly for underdeveloped countries, which cannot afford to establish or to maintain necessary hygienic and medical facilities. Especially in these environments, cholera is responsible for significant mortality and economic damage. During the last three decades, intensive research has been undertaken to unravel the virulence properties and to study the epidemiology of this significant human pathogen. More recently, researchers have been elucidating the environmental lifestyle of *V. cholerae*. This review provides an overview of the current knowledge of both the host- and environment-specific physiological attributes of *V. cholerae*. 

*Keywords:* *Vibrio cholerae*; Cholera; Biofilm; Infection; Toxin; Colonization

**Contents**



## 1. Historical perspective

Accounts of a cholera-like disease go back to the times of Hippocrates and Buddha and perhaps even earlier [1]. However, the modern history of cholera began in 1817. At

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0168-6445/02/$22.00 
PII: S0168-6445(02)00091-8

that time an epidemic outbreak was reported in India, which subsequently spread across the Indian continent and was then defined as the first pandemic outbreak of cholera disease in southeast Asia. During the 19th century six cholera pandemics took place, ending in 1923 and affecting mostly the continents located in the southern hemisphere, as well as North America and Europe [1,2]. In 1961, the seventh pandemic began in Indonesia, then spread to the Indian subcontinent and the Middle East, then moved on to Africa in the 1970s and finally reached South America in the early 1990s [3–6].

The etiologic agents responsible for cholera and many other infectious diseases (e.g. tuberculosis, anthrax, diphtheria, tetanus and typhoid fever) were identified during the 19th century. In 1883 Robert Koch demonstrated that cholera is produced by a bacterium that he referred to as 'comma(-shaped) bacteria' [7], later designated *Vibrio cholerae*.

The early pandemics preceded Koch's discovery of the infectious cause of cholera, and thus have never been associated with a specific strain variant. The fifth and sixth pandemics, however, have been shown to have been caused by the *V. cholerae* serogroup O1 of biotype 'classical' (see below), while the seventh pandemic was caused by serogroup O1 biotype 'El Tor' [1]. In 1992, a serogroup conversion event(s) led to the emergence of the new *V. cholerae* serogroup O139, resulting in a large epidemic in Bangladesh and India [8–10]. Since then O139 strains are endemically found in these areas and cases have been reported all over south-east Asia. This epidemic has already been described as the eighth pandemic [6]; however, O1 El Tor strains also still persist in these areas.

As a result of continuing scientific and medical effort directed toward combating cholera, major improvements in medical treatment as well as a better understanding of the molecular processes involved in the virulence of *V. cholerae* have been achieved. However, despite all efforts, new variants of this pathogen are obviously still evolving that are able to circumvent established immunity and to resist clinical and hygienic prophylactics.

## 2. *V. cholerae* and its environment

*V. cholerae* is a motile, Gram-negative curved rod that belongs to the family Vibrionaceae. About 200 recognized O serogroups are known, however only serogroup O1 and the newly emerged O139 have been associated with severe disease and cholera pandemics. In contrast, intestinal and/or extraintestinal infections with non-O1 and -O139 serogroups or non-toxigenic O1 strains are rarely found and seem to be of little clinical significance [11–14].

It has been shown that *V. cholerae*, including serogroup O1 and O139 pandemic strains, exist as natural inhabitants of aquatic ecosystems [15–18], thus making them facultative human pathogens. Within the marine environment they attach to surfaces provided by plants, filamentous green algae, copepods (zooplankton), crustaceans, and insects [16,19]. Interestingly, there is a correlation of cholera outbreaks and the seasonal occurrence of algal blooms [16,18,20]; however, there is no direct evidence that such events lead to an enrichment of toxigenic *V. cholerae* strains responsible for cholera epidemics. Non-O1 and non-O139 strains are more frequently isolated from rivers and estuarine areas than O1 and O139 strains, and interestingly most environmental O1 strains are non-toxigenic [21]. This led to the hypothesis that environmental strains that acquire the respective virulence genes, which are harbored on genetic mobile elements (see below), may then be enriched by intestinal conditions [22,23]. Recent evidence [6] demonstrates that non-toxigenic environmental strains can be converted by phage transduction with cholera toxin (CT)-encoding phage CTXϕ, and this event could conceivably also take place in the gastrointestinal environment, yielding new detectable toxigenic strains.

*V. cholerae* O1 is found associated with marine organisms, and it has been shown that these strains can bind to chitin [24], and in addition can then acquire acid tolerance [25]. Recently, one factor involved in adherence to the chitin of zooplankton was identified as the mannose-sensitive hemagglutinin (MSHA) type IV pili. These pili are expressed and assembled by O1 El Tor and O139 strains; in contrast O1 classical strains carry the pili (*msh*) genes but do not assemble functional MSHA pili [26] (Fig. 1). Additional uncharacterized chitin binding proteins have been reported to be present in *V. cholerae* strains [27], and this has also been shown for other *Vibrio* species like *V. alginolyticus* [28]. *V. cholerae* also possess secreted chitinase enzyme(s) which are probably needed to utilize chitin (homopolymer of *N*-acetylglucosamine) as a carbon source [24,29]; five hypothetical *chi* gene products were identified in the genome sequence [30]). These findings are suggestive of a close association of *V. cholerae* with chitin structures in the environment (e.g. zooplankton such as copepods).

A recent report revealed that environmental non-O1 and non-O139 *V. cholerae* strains can also be isolated from chironomid egg masses (*Chironomus* sp., Diptera). These egg masses were collected from waste stabilization ponds and it was found that they also serve as a favorable rich nutrient niche for *V. cholerae* strains [31].

## 3. Mechanisms involved in environmental survival

In general it is assumed that association of *V. cholerae* with zooplankton or egg masses may provide some protection from the relatively harsh environmental conditions faced in the aquatic habitats of planktonic bacterial cells. A variety of microorganisms, including *V. cholerae*, are able to build up large multicellular structures on solid

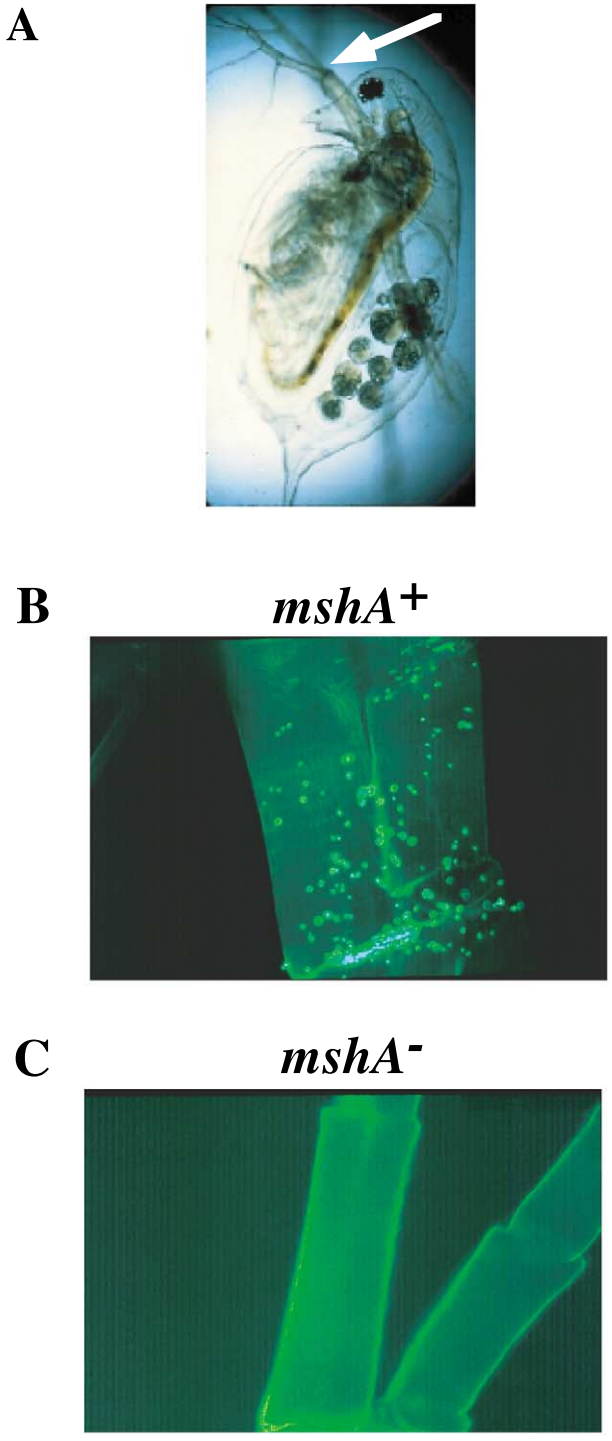


Fig. 1. MSHA-mediated attachment of *V. cholerae* to zooplankton. A: Shown is a light micrograph of an adult *Daphnia pulex* with a length of about 2.5 mm. The arrow indicates the position of the antenna structure, an exoskeleton structure which is shown at higher magnification in panels B and C. In panel B *D. pulex* was colonized by *V. cholerae* O139 (GFP-expressing); the attached bacteria are visualized as bright green spots. The *D. pulex* in panel C were incubated with GFP-expressing *V. cholerae* O139 *mshA* mutants, indicating a role for MSHA in zooplankton colonization. (Reprinted with permission from R. Taylor [26]).

surfaces known as biofilms. The biofilm provides a microenvironment that favors survival and persistence due to increased resistance to various stresses (e.g. chlorine, antibiotics).

It has been demonstrated that *V. cholerae* O1 El Tor and O139 are both able to form a three-dimensional biofilm an abiotic surfaces [32–34] (Fig. 2). Biofilm formation is likely to be important for the life cycle of *V. cholerae*, facilitating environmental persistence within natural aquatic habitats during interepidemic periods.

Biofilm formation by both O1 El Tor and O139 strains is dependent on the expression of an exopolysaccharide (VPS) [32,34,35]. Strains containing defects in one of the *vps* genes necessary for exopolysaccharide synthesis can colonize an abiotic surface but fail to make a three-dimensional biofilm, indicating that the exopolysaccharide is used as the 'glue' to build a mature biofilm [33,34]. Expression of the VPS exopolysaccharide causes a rugose colony phenotype, and provides enhanced chlorine resistance and also phage resistance [32,36,37]. Interestingly, expression of the VPS exopolysaccharide inhibits intestinal colonization in the infant mouse cholera model, suggesting that the expression of a factor that enhances environmental persistence actually decreases virulence [34]. Rugose colonies (i.e. expressing the VPS exopolysaccharide) appear in low numbers as phase variants, and their appearance increases during carbon starvation [32,38]. The lack of flagellar synthesis causes high-level VPS exopolysaccharide expression (at least in some strains, e.g. O139), indicating that flagellar synthesis is coupled to VPS expression. This effect is specifically due to the lack of a flagellum, rather than a lack of motility, suggesting that the loss of the flagellum may be a developmental cue during biofilm formation [34].

The VPS exopolysaccharide constituents have been identified, and galactose was identified among the carbohydrate residues [32,35]. Recent attempts to investigate the Gal pathway in *V. cholerae* prompted us to generate and characterize a *galU* mutant, encoding the UDP-glucose-4-epimerase. We found that the *galU* mutant has lost the ability to produce the VPS exopolysaccharide, and is defective for biofilm development [37]. These results are consistent with a requirement for the synthesis of UDP-galactose via UDP-glucose for the biosynthesis of the VPS exopolysaccharide.

In addition to the VPS exopolysaccharide, *V. cholerae* O1 El Tor requires the MSHA type IV pilus to form a normal biofilm [33], but the O139 strain does not [34]. The *V. cholerae* O1 El Tor also requires flagellar motility for biofilm formation, while non-flagellated O139 strains can eventually form three-dimensional biofilms, apparently because of high level VPS exopolysaccharide expression [33,34]. Watnick et al. have postulated a three-step model in *V. cholerae* biofilm formation (see Fig. 2). Flagellar-driven motility is important for the initial attachment step, followed by microcolony formation assisted by the

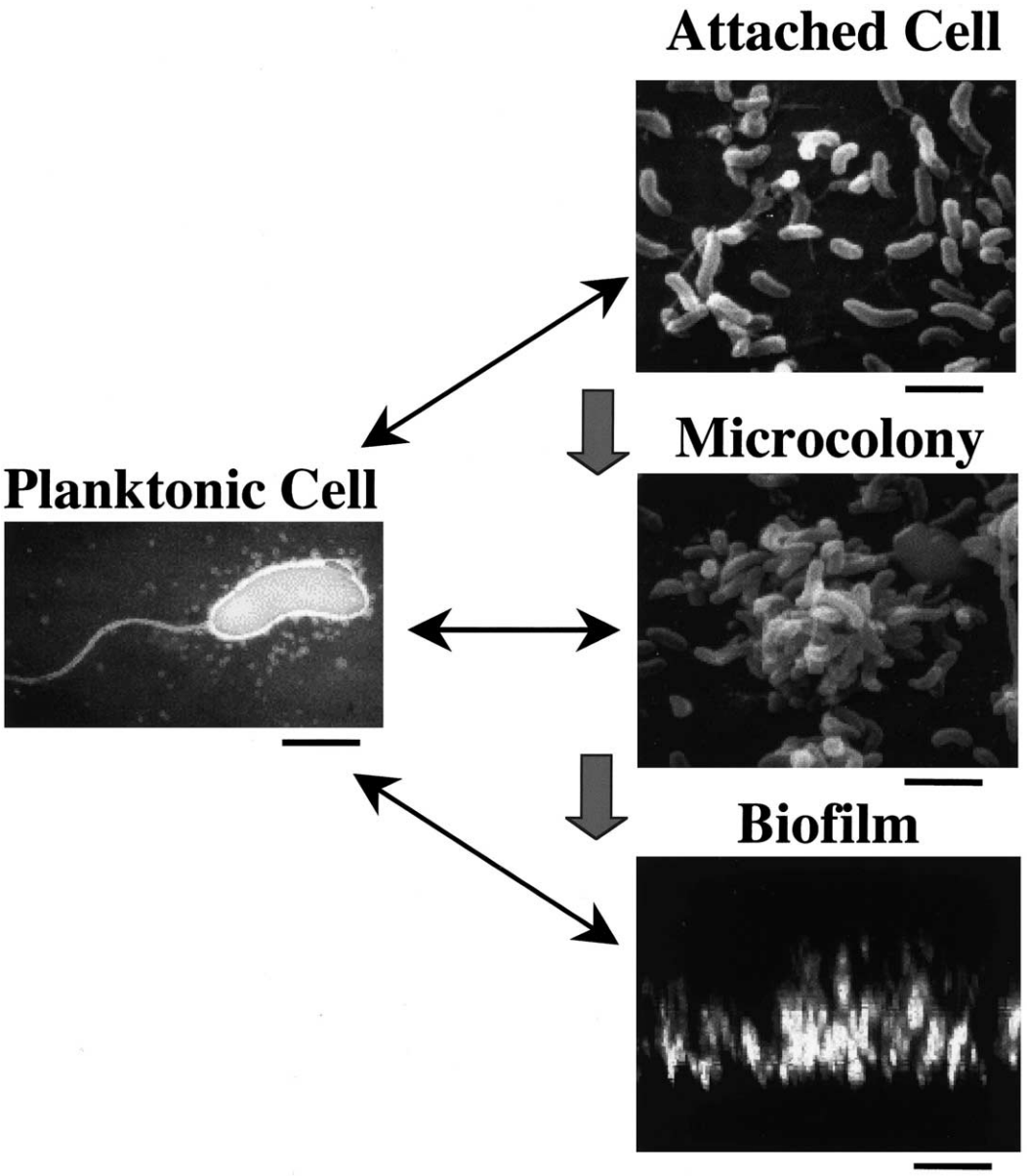


Fig. 2. Steps in *V. cholerae* biofilm formation. The planktonic bacterium was visualized by transmission electron microscopy (bar = 1 μm), the attached cells and microcolony were visualized by scanning electron microscopy (bar = 2 μm), and the biofilm micrograph represents a vertical section through a 20-μm biofilm taken by confocal scanning laser microscopy (bar = 10 μm). (Reprinted with permission from P. Watnick [150]).

MSHA pili. The final step is VPS exopolysaccharide expression, which allows the three-dimensional architecture of the mature biofilm. A non-flagellated O139 strain bypasses the need for motility presumably because it already expresses VPS exopolysaccharide, which allows for microcolonies to form in solution that eventually settle onto the solid surface. It is not clear why the O139 strain does not require MSHA pili, but it may perhaps be due to the fact that O139 strains are encapsulated, unlike the O1 El Tor (see below).

In addition to biofilm formation, *V. cholerae* possess the remarkable ability of being able to switch into a viable, but non-culturable (VNC) state in response to nutrient deprivation [39,40]. Such a dormant state could serve as a survival strategy, which can be resuscitated upon receiving the appropriate signals, e.g. extreme change of environmental conditions such as the transition from the aquatic system upon ingestion into the human intestinal environment [6,39]. During interepidemic periods, epidemic *V. cholerae* strains have been postulated to exist in the VNC form, with unknown environmental conditions serving to resuscitate these cells back to free-living, virulent organisms [18,41]. If this ecological niche serves as a common reservoir for toxigenic *V. cholerae* then such strains should be highly related. In contrast to this assumption, isolated epidemic strains are quite different, based on ribotyping and different phenotypic and genetic properties (as reviewed recently [6]), hence a clonal origin of a VNC

reservoir seems less likely. In general, the existence of the VNC state is in debate, since there is no direct conclusive information about the underlying molecular processes or the genetic factors involved.

## 4. Transmission and identification of toxigenic *V. cholerae*

Poor sanitation practices in highly populated areas harboring endemic toxigenic strains are the source of occasional outbreaks due to contamination of drinking water and/or improper food preparation. Contaminated water with free-living *V. cholerae* cells are probably the main origin of epidemics, followed to a lesser extent by contaminated food, especially seafood products like oysters, crabs, and shellfish (for review see [42,43]).

For example, a massive outbreak of O1 El Tor *V. cholerae*-mediated cholera took place in the South American continent and caused over 750 000 cases and 6500 deaths [44]. The exact origin of the epidemic El Tor strain remains unknown, but the epidemic originated on the west coast of Peru. The spread of the epidemic strain was mainly caused by contaminated drinking water supplies, followed by fecal contamination of the marine environment, which led to contamination of seafood [45].

There have been reports of bottled water contamination with toxigenic *V. cholerae*. The evidence suggested that carbonated water was safer than non-carbonated water, due to the organism's poor acid tolerance [46]. Subsequently, pH neutral food was found to serve as a good carrier for toxigenic *V. cholerae* strains [47]. In fact, it was shown that toxigenic O1 *V. cholerae* can survive refrigeration and freezing in food supplies shipped internationally, therefore an epidemic strain may travel far from its original endemic location [48].

Isolation of *V. cholerae* from the extraintestinal environment or stool samples of patients can be achieved by taking advantage of the organism's fast generation time, and tolerance to alkaline conditions (pH 8.5). Thus, isolation generally includes growth in alkaline peptone water medium, which provides a competitive advantage to Vibrionaceae. Also frequently used are blood -and chocolate-based or Müller–Hinton and MacConkey agar media. Subsequently, a selective growth medium and identification scheme routinely used for Vibrionaceae is growth on thiosulfate–citrate–bile salts–sucrose medium. This medium is highly selective for *Vibrio* spp., and the ability of the organism to ferment the sucrose in this medium (e.g. *V. cholerae*) results in yellow colonies. Additional testing of oxidase activity will lead to the identification of *Vibrio* spp. (for additional information see [49]). In contrast to the halophilicity of other *Vibrio* species, *V. cholerae* is one of the few Vibrionaceae that can grow in the absence of NaCl, so this is a further defining characteristic.

Once a strain has been identified as *V. cholerae* by these and several additional tests, typing of *V. cholerae* is

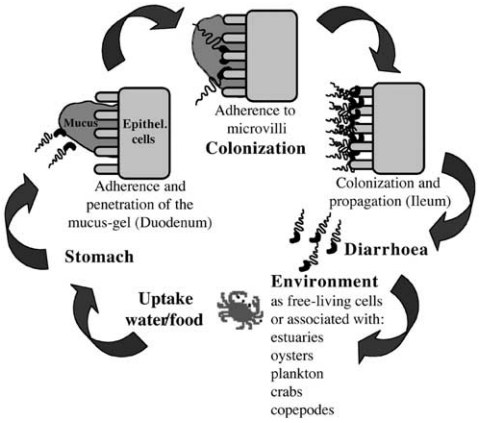


Fig. 3. Infection cycle of *V. cholerae*. Shown is a simplified depiction of the infection cycle of the cholera-causing bacteria *V. cholerae*.

achieved by slide agglutination with O1 and/or O139 antisera. This differentiates the strain as either O1, O139, or non-O1/O139, based on the structure of the lipopolysaccharide and capsule for O139 (Fig. 4). Mostly the serogroups O1 and O139 are associated with epidemic disease and further classification is necessary. In case of the O1 serogroup, three different serotypes or antigen variations were recognized termed 'Inaba', 'Ogawa', and a minor serotype 'Hikojima' [50]. In addition, O1 strains can be classified in two different biotypes, called O1 'El Tor' and O1 'classical' determined by physiological properties, such as polymyxin B resistance, number of CT-encoding genes, hemolysin activity and the presence of the mannose-sensitive hemagglutinin (for review see [51]).

## 5. *V. cholerae* infections and the outcome of cholera

Infection normally starts with the oral ingestion of food or water contaminated with *V. cholerae* (Fig. 3). Subsequently the bacteria must pass through and survive the gastric acid barrier of the stomach, then penetrate the mucus lining that coats the intestinal epithelia. In human volunteer studies, the infectious dose was determined to be fairly high, and varied depending on the inocula conditions (ranging from $10^6$ to $10^{11}$ colony-forming units) [52]. This high dose is probably needed because of the acid sensitivity of *V. cholerae* cells, which are exposed to low pH in the gastric compartment [53]. The surviving bacteria adhere to and colonize the intestinal epithelial cells, eventually producing the CT and causing cholera symptoms [54]. The primary site of *V. cholerae* colonization is the small intestine. During the transition from the aquatic environment to the human body the bacterial cells are exposed to a series of changes, such as temperature, acidity, and osmolarity. They also must survive in the intestinal environment, which contains growth inhibitory substances like bile salts and organic acids, and also factors of the innate immune system, such as complement secreted by intestinal epithelial cells [55] and defensins

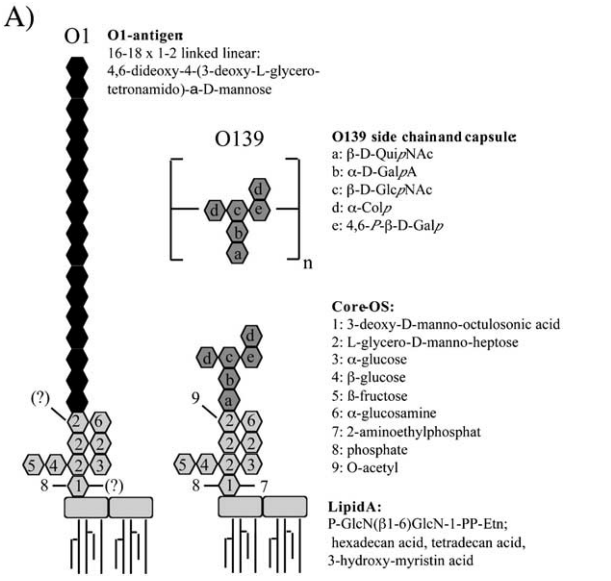


Fig. 4. LPS and capsule structure of serogroup O1 and O139 *V. cholerae*. The deduced structure of *V. cholerae* serogroup O1 (left) and O139 (right); shown are the different O antigen and capsule structures, and the similar core-OS and lipid A structures [139,151] (carbohydrate and fatty acid compositions are indicated). The few chemical modifications found in O139 but not O1 core-OS are indicated with '?'. The O-antigen of *V. cholerae* O1 consists of a homopolymer of approximately 18 perosamine (4-$NH_2$-4,6-dideoxymannose) residues consisting of dideoxy-phosphomannose substituted with tetronate [152]. The O-antigen biosynthesis is catalyzed by the gene products encoded by the *rfb* gene cluster (see recent review [153]). *V. cholerae* O139 O-antigen has a different carbohydrate composition, which is also presented as a capsule [138,139].

produced by Paneth cells [56]. Therefore, *V. cholerae* has developed the ability to survive, colonize, and express virulence factors such as CT in spite of, and possibly in response to, harsh environmental conditions.

After *V. cholerae* secretes the CT, the symptoms of cholera will occur. Cholera is reflected by massive watery diarrhea of such prodigious volume that hypotensive shock and death can occur within 12 h of the first symptoms [52]. The rate of body fluid loss is greatest in the jejunum with up to 20 l per day in adults [57]. This tremendous water loss is mainly mediated by the action of the CT (for review see [58]). CT is a hetero-oligomer consisting of one A subunit in association with five B subunits. The B subunit is responsible for specific binding to the $GM_1$ ganglioside receptor of epithelial cells [59,60]. Upon binding, the A subunit is translocated into the host cell cytosol, where it is activated by thiol dependent reduction, probably by thiol:protein disulfide oxidoreductases [61]. Only the resulting nicked $A_1$ subunit possesses an ADP-ribosylating activity that targets the host cell G-protein Gsα. ADP-ribosylated Gsα in turn permanently activates adenylate cyclase activity, leading to increased levels of intracellular cAMP. cAMP inhibits active sodium chloride absorption and increases chloride and bicarbonate secretion (for review see [58,62]). This results in passive water loss, leading to a marked decrease in intravascular volume, hypotension and hypoperfusion of critical organs, and in severe cases death ensues with a high mortality rate ($>20\%$) (for review see [52]).

## 6. Dissecting the molecular aspects of *V. cholerae* infections

Due to extensive molecular and genetic analyses, the important virulence factors and the virulence-responsive regulatory systems of *V. cholerae* have been elucidated [62–64]. Although no animal model replicates all aspects of *V. cholerae* infection in humans, several animal models have been extremely useful in the study of cholera pathogenesis. Two particular animal models have been widely used. The infant mouse colonization assay has accurately predicted the colonization behavior of *V. cholerae* strains in human volunteer studies, while the rabbit ileal loop model has been useful to measure the effect of CT in vivo, namely fluid accumulation within the intestine.

Recently the complete genome sequence of the two chromosomes of *V. cholerae* O1 El Tor was made available to the scientific community [30], which will help to facilitate the further dissection of cholera pathogenesis. Cholera is the result of a multifactorial process, consisting of *V. cholerae* colonization, coordinate expression of virulence factors, and toxin action. *V. cholerae* also acquire virulence genes in a step-by-step process, in which a pathogenicity island and a temperate bacteriophage play important roles. In the following paragraphs, an overview will be provided of recent discoveries and an outlook toward future developments.

### 6.1. Colonization factors

Several *V. cholerae* gene products have been shown to be important for colonization of the small intestine, primarily in the infant mouse and the adult rabbit model of cholera. These include the toxin-coregulated pili (TCP) [65], accessory colonization factors (ACFs) [66], mannose–fucose-resistant hemagglutinin [67], TolC [68], regulatory proteins (e.g. ToxR/ToxS, TcpP/TcpH, and ToxT) [65,69,70], outer membrane porins [71], biotin and purine biosynthetic genes [72], an iron-regulated OMP protein IrgA, [73], the O-antigen of the lipopolysaccharide (LPS) [74–76] and attributes of the LPS core region [37].

The TCP, a type IV pilus, is considered to be the most important colonization factor because it has been demonstrated to be required for intestinal colonization both in animal models and in human volunteer studies [65,77]. The TCP is thought to be a polymer composed of a single structural subunit, TcpA, and also serves as the receptor for the CTXϕ bacteriophage (see below; [23,78]). The genes required for TCP synthesis, including *tcpA*, as well as the *acf* genes and the genes encoding the transcriptional activators ToxT and TcpP (see below), are located on a ~40-kb *Vibrio* pathogenicity island (VPI [22,79]). Despite

its essential role in colonization, TCP appears to not directly bind to intestinal epithelia, but rather seems to facilitate microcolony formation (i.e. bacterial–bacterial interaction) on the epithelial cell surface [80]. Other factor(s) may be involved in adhesion to the intestinal epithelium, however, no specific *V. cholerae* adhesins or specific mucosal receptors have been identified yet.

### 6.2. The CT-converting phage CTXϕ

CT, while being critical for the symptoms of cholera, appears to play no direct role in intestinal colonization [65]. However, experimental data obtained from deletion analysis of *ctxAB* mutants suggests that CT activity may enhance growth in the intestinal environment through its destruction of epithelial cells [81]. In contrast, there is no experimental evidence showing any benefit of CT activity for *V. cholerae* outside its human host. The genes encoding CT are actually encoded within a single-stranded filamentous phage, termed CTXϕ [23]. The phage contains two regions, 'RS2' and the 'core'. RS2 encodes genes involved in integration, replication and regulation of CTXϕ, whereas the core region is composed of the CT operon (*ctxAB*), as well as the *zot*, *ace*, *cep*, and *orfU* genes (Table 1) [82]. *zot* and *ace* were originally believed to encode additional toxins [83,84], but are now recognized along with *orfU* and *cep* as genes involved in CTXϕ morphogenesis (Table 1) [23]. The CTXϕ is frequently found integrated into the chromosome in tandem arrays of prophage DNA, and this arrangement is important for excision and transmission of the virion [85]. The CTXϕ integrates into the *attRS* element located in the large *V. cholerae* chromosome; the CTXϕ can also replicate as a double-stranded RF form in the cytoplasm [23]. Interestingly, in the RF form CT expression is dependent on phage induction and independent of the virulence gene expression cascade (see below) [86].

### 6.3. Surface compounds

LPS represents the most abundant of exposed molecules in the outer membrane of Gram-negative bacteria, and contributes to barrier function [87,88]. During infection, *V. cholerae* cells are exposed to a series of changes, such as temperature, acidity, osmolarity and exposure to antibacterial agents and innate immune system components. The outer membrane effectively prevents the entry of noxious compounds into the cell, helps to evade recognition by host agents, and may facilitate colonization. LPS and capsule structures represent important virulence factors and typing determinants of pathogenic *V. cholerae* strains (Fig. 4). Mutant analysis for O1 and O139 strains lacking O-antigen as well as O139 strains lacking the capsule showed that they are significantly attenuated for intestinal colonization in the mouse model [37,76,89–92]. Moreover, mutations that affect the biosynthesis of LPS core oligosaccharide (core-OS) also reduce intestinal colonization [37,93]. Recently, we have found that the core-OS-encoding gene clusters (*wav* genes) of various environmental and clinical *V. cholerae* isolates have varying genes and organization, and can be differentiated into distinctive subgroups. One particular *wav* gene cluster is highly associated with pathogenic isolates, suggesting a contribution of this particular core-OS to virulence [94].

In addition to LPS, the outer membrane proteins

Table 1
Relevant genes associated with virulence and environmental properties in *V. cholerae*

| Gene name | Function | Location | Virulence (v)/environmental (e) |
|---|---|---|---|
| *ace* | M13 gene VI homologue formerly 'accessory enterotoxin' | CTXϕ phage | v |
| *acfABCD* | accessory colonization factors, function unknown | VPI | v |
| *aldA* | aldehyde dehydrogenase ToxT-activated | VPI | ? |
| *aphAB* | regulatory proteins | chromosome | v |
| *cep* | M13 gene VIII homologue formerly 'core-encoded pili' | CTXϕ phage | v |
| *chi* genes | chitinase homologues | chromosome | e |
| *ctxAB* | CT subunits A, B | CTXϕ phage | v |
| *flrC* | flagellar transcriptional regulator | chromosome | v/e |
| *irgA* | iron-regulated outer membrane protein | chromosome | v |
| *msh* genes | type IV pili (mannose-sensitive hemagglutinin) | chromosome | e |
| *ompU*, *T* | outer membrane porins | chromosome | v/e |
| *orfU* | M13 gene III homologue | CTXϕ phage | v |
| *rfb* genes | O-antigen biosynthesis | chromosome | v/e |
| *rst* genes | regulation, integration, replication | CTXϕ phage | v |
| *rtxA* | 'repeats in toxin' toxin, cross-links cellular actin | chromosome | ? |
| *tagA* | ToxT-activated gene | VPI | ? |
| *tcpA* | toxin co-regulated pili major subunit (type IV pili) | VPI | v |
| *tcpPH* | transmembrane regulatory proteins | VPI | v |
| *toxRS* | transmembrane regulatory proteins | chromosome | v/e |
| *toxT* | virulence transcriptional activator | VPI | v |
| *wav* genes | LPS core oligosaccharide synthesis | chromosome | v/e |
| *zot* | M13 gene I homologue formerly 'zonula occludens toxin' | CTXϕ phage | v |
| *vps* genes | exopolysaccharide synthesis | chromosome | e |

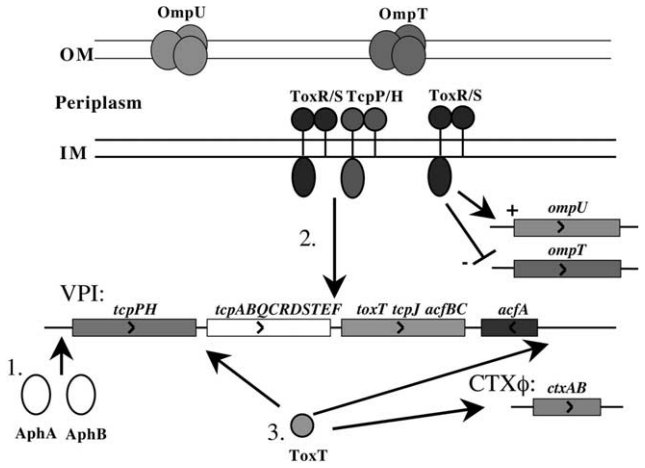


Fig. 5. The regulatory cascade that controls *ctx* and *tcp* transcription. Induction of CT and TCP expression in *V. cholerae* occurs in three basic steps (at least in vitro): (1) AphA and AphB activate transcription of *tcpPH*, (2) TcpPH and ToxRS (localized in the cytoplasmic membrane; 'IM') activate transcription of *toxT*, and (3) ToxT activates transcription of the genes encoding TCP (*tcp*) and CT (*ctx*), as well as additional genes (*acfA*, *tagA*, *aldA*, *tcpI*). Arrows within genes indicate direction of transcription. Within the laboratory, inducing environmental signals primarily stimulate step 1, while some repressing signals affect step 3. ToxRS alone modulates outer membrane porin expression by activating the transcription of *ompU* ('+') and repressing the transcription of *ompT* ('−'). OmpU (shown as a porin trimer in the outer membrane; 'OM') is predicted to be less permissive for passage of certain substances like bile through the outer membrane, compared to OmpT. The *tcp*, *toxT*, and *acf* genes are located on a horizontally acquired pathogenicity island (VPI; not drawn completely nor to scale). The ToxT-activated *tcpI*, *tagA* and *aldA* genes lie upstream of *tcpPH* within the VPI. The *ctx* genes are found on the lysogenic filamentous bacteriophage CTXϕ which integrates into the *attRS* site in the chromosome. The *toxRS*, *ompU*, *ompT*, *aphA* and *aphB* genes reside in the ancestral *Vibrio* chromosome.

OmpU and OmpT contribute to *V. cholerae* virulence. These porins were suspected to be involved in virulence because their expression is regulated by ToxR, which also regulates CT and TCP (see below) [95]; ToxR activates the transcription of *ompU* and represses the transcription of *ompT* [96–98]. OmpU is more protective (compared to OmpT) against the bactericidal effects of bile salts and other anionic detergents [99,100]. Switching the ToxR-dependent regulation of these porins (i.e. ToxR activation of *ompT* and repression of *ompU*) leads to increased bile sensitivity, reduced CT and TCP expression and reduced intestinal colonization [71]. These results can be explained by the differential permeabilities of the porins affecting the flux of molecules (e.g. bile salts) across the outer membrane and thereby altering the induction of the virulence cascade (see below). Recent evidence supports OmpT having a higher permeability than OmpU to negatively charged molecules such as bile salts [101]. *V. cholerae* is also able to mount an organic acid tolerance response, and this has been shown to be mediated by OmpU [102–104].

Flagellar-dependent motility has been linked to *V. cholerae* virulence, but the connection between motility and pathogenesis has been elusive. Non-motile mutants were shown to have decreased virulence in some animal models but not others [105–107]. Importantly, in human volunteer studies motility appears to play a role in virulence [108,109]. Recent studies suggest that the transcriptional activity of a flagellar regulatory protein, FlrC, affects the ability of *V. cholerae* to colonize the intestine, indicating a possible link between virulence and motility [110]. Normal transcription of *ctx* within the intestine is dependent on motility and chemotaxis, suggesting that *V. cholerae* must navigate to the correct site within the intestine before inducing virulence factor expression [111].

### 6.4. Additional virulence factors

A variety of additional putative virulence factors have been characterized that either do not affect colonization or have not been evaluated for effects on colonization; these factors may be involved in other aspects of cholera pathogenesis. These include protein factors such as neuroaminidases, secreted proteases, hemagglutinin (HA) protease and a hemolysin–cytolysin (as reviewed by Kaper et al. [62]). Recently, a gene encoding a 'repeats in toxin' (RTX)-type protein, encoded by *rtxA*, was discovered linked to the CT prophage [112]. The RTX toxin exhibits cross-linking activity on cellular actin leading to cell rounding in tissue culture, and G-actin appears to be the toxin target [113]. The role of this toxin in cholera is unclear because classical strains appear to lack functional RTX.

## 7. Transcriptional regulation of virulence factor expression

Extensive study of the molecular basis of cholera pathogenesis has revealed that expression of the most important virulence factors, namely CT and TCP, is coordinated by a unique regulatory system (Fig. 5). This regulatory system consists of three different transcriptional activators, two (ToxT and TcpP) located within the VPI, and the third, ToxR, located within the ancestral *Vibrio* chromosome [22,79,114]. The AraC-like transcriptional activator ToxT directly activates both the *ctx* and *tcp* gene clusters, as well as additional genes (e.g. *aldA*, *tagA*) [115,116]. *V. cholerae* strains lacking ToxT make no CT or TCP and are avirulent [70]. Transcription of the *toxT* gene is controlled by the two transmembrane transcriptional activators ToxR and TcpP [117,118]. Recent studies suggest that both proteins bind to the *toxT* promoter, with ToxR binding distally and acting as a 'scaffold' protein that allows TcpP binding adjacent to the RNA polymerase binding site [119].

The expression of CT and TCP is induced within the intestinal environment, and only occurs under specific culture medium compositions (e.g. peptone yeast NaCl, $NaHCO_3$; thiosulfate citrate yeast; casamino acids yeast extract peptone; and yeast extract peptone water me-

dium). Likewise, transcription of *toxT* occurs only under these same virulence-'inducing' conditions, consistent with ToxT expression controlling virulence factor induction [120]. Environmental signals such as osmolarity, pH and temperature affect *toxT* transcription in vitro, but the true intestinal signals that stimulate its transcription in vivo remain unknown. The signals that stimulate *toxT* transcription actually appear to stimulate *tcpP* transcription [121], and thus this virulence-regulatory cascade is initiated by TcpP expression, which is regulated by two additional activators, AphA and AphB [122,123]. Recent evidence has demonstrated that these two activators bind to the *tcpP* promoter and synergistically activate its transcription [124]. Expression of AphA and AphB, whose genes appear to be located in the ancestral *Vibrio* chromosome, is independent of the in vitro virulence factor-inducing conditions [123], and so the inducing environmental conditions likely modulate their activity. *aphA* and *aphB* mutants are defective for *tcpP* and *toxT* transcription, and subsequent intestinal colonization and CT expression, consistent with these gene products occupying the top level of the known virulence cascade [124]; it is not yet known what other genes are regulated by AphA and AphB besides *tcpP* on the VPI. Levels of ToxR protein, like AphA and AphB, also appear to remain constant under various in vitro conditions [120], consistent with ToxR continuously occupying the *toxT* promoter and acting as a 'scaffold' protein that facilitates TcpP binding once it is expressed, leading to *toxT* transcription and subsequent virulence factor expression. This implies that ToxR continuously tethers the *toxT* promoter to the cytoplasmic membrane, but as yet there is no direct evidence for this.

Although ToxT appears to be synthesized in a transcriptionally active form, certain environmental signals (e.g. bile, temperature) can inhibit ToxT transcriptional activity [125]. This observation led to the suggestion that the virulence cascade occurs in two temporally and spatially distinct steps within the intestinal environment: first *toxT* is transcribed within the lumen of the intestine in the presence of bile but the ToxT protein remains in an inactive state, until the bacteria penetrate the mucus lining and arrive at the cell surface, where lower bile concentrations would allow for ToxT-dependent expression of CT and TCP.

Camilli and coworkers have shown that *ctx* and *tcp* transcription within the intestine differs from their transcription under laboratory 'inducing' conditions. Most interestingly, *ctx* transcription was dependent on TCP expression within the intestine (unlike in the laboratory) [93], suggesting that the bacteria must colonize the intestinal cell surface to receive a specific signal (perhaps lower bile concentrations?) that leads to robust CT and TCP expression. Also interesting to note is that full *ctx* transcription within the intestine required *toxR* but not *tcpP* (unlike in the laboratory), suggesting that the true *V. cholerae* virulence cascade may differ significantly from that

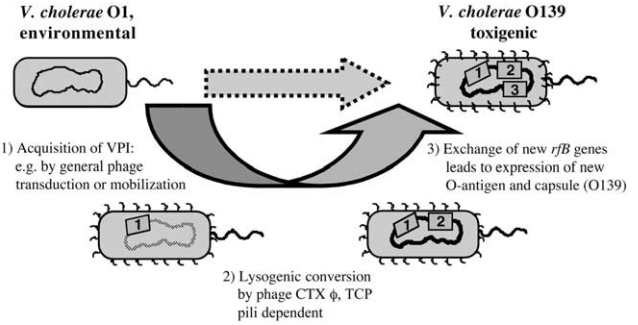


Fig. 6. Horizontal gene transfer events important for *V. cholerae* virulence. Shown are the possible events of horizontal gene transfer, which led to the acquisition of important virulence factor-encoding genes. See text for more information.

elucidated through in vitro laboratory experiments, and thus demonstrating the importance of studying pathogenesis in the context of a living host rather than in a test tube.

## 8. Evolution and transmission of virulence factors

It has become clear that *V. cholerae* virulence is the result of a series of horizontal gene transfers that allowed a benign marine bacterium to rapidly evolve into a dangerous human pathogen (summarized in Fig. 6). This transition can be achieved by the acquisition of two transmissible elements, the VPI, which contains the TCP cluster necessary for intestinal colonization, and phage CTXϕ, which encodes the CT that leads to the symptoms of cholera. The VPI, which is found in pathogenic strains and sporadically in environmental strains [22,126], has been suggested to be a filamentous bacteriophage [127]. This element can clearly move by some means from strain to strain, perhaps through phage transduction events or comobilization along with the self-transmissible replicon SXT ([128]; M. Waldor, personal communication). After *V. cholerae* acquires the VPI and expresses TcpA, it becomes susceptible to CTXϕ transduction, because this phage uses the TcpA as its receptor [23]. The CTXϕ has been found in environmental (non-O1) *V. cholerae* as well as in *V. mimicus* strains [129,130]. Because *tcp* expression is induced in the small intestine, toxigenic conversion by CTXϕ can occur there also. It is noteworthy that the *ctx* genes on CTXϕ are transcribed by the same regulatory factors that are responsible for TCP expression, and thus the phage appears to be 'choosing' a bacterium that is able to express CT.

The regulatory cascade responsible for CT and TCP expression (described above) is a fascinating mixture of ancestral and recently acquired elements. AphA and AphB, which appear to initiate the virulence cascade by stimulating *tcpP* transcription, are probably located in the ancestral *Vibrio* chromosome, and yet they both recognize and activate the *tcpP* promoter, located on the VPI. TcpP

then interacts with ToxR, which is encoded in the ancestral *Vibrio* genome, to activate the transcription of the *toxT* gene, located on the VPI. Finally ToxT activates the transcription of *tcp* and *acf* genes, located on the VPI, and the *ctx* genes, located on the CTXϕ. One can hypothesize that the elements located on the recently acquired VPI are usurping specific *Vibrio* regulatory factors because they respond to signals relevant to the intestinal environment. Presumably these complex interactions allow the integration of multiple environmental signals into the virulence cascade.

*V. cholerae* continues to evolve as a human pathogen. For example, the new serogroup O139 emerged as a result of newly acquired LPS biosynthesis-encoding genes (*rfb*). The high level of relatedness between O139 and O1 El Tor strains [131–133] indicates that epidemic O139 strains are derived from O1 El Tor strains, mainly through the acquisition of the genes encoding synthesis of the O139 antigen [89,134,135]. It appears that the genes encoding the O1 antigen have been replaced by genes with characteristic features of group 4 capsule gene clusters [136,137], leading to the synthesis of the LPS-linked O139 antigen and an unlinked O139 antigen capsule [138,139]. This new variant was able to circumvent established immunity in human populations that had been previously exposed to O1, and has led to large outbreaks in 1992/93 in Bangladesh and India. Considering that other non-O1 and non-O139 environmental *V. cholerae* strains have been found with both VPI and CTXϕ, it is still not clear why only those two serogroups have been associated with pandemic disease.

## 9. Control of cholera: conceptions of cholera vaccines

Beside hygienic and sanitary control measures and cholera surveillance, one of the main efforts at combating cholera epidemics is directed towards the development and use of modern vaccine strategies (for an overview see Tacket et al. [140]). Cholera is predicted to have a high potential for successful prevention by vaccination [141]. Analysis of a large number of Indian residents demonstrated that a primary episode of cholera disease provided significant protection against a second episode [142]. Efficient protection is dependent on the biotype: infection with the classical biotype shows more conserved protection against different serotypes (Inaba, Ogawa, and Hikojima) of classical strains, and El Tor-derived protection is more labile against different El Tor isolates [143]. It was also found that naturally acquired immunity lasts for at least 3 years, whereas longer immunity depends on the individual [141]. Antigenic components that contribute to the protective immune response are CT, LPS, flagella, fimbriae and outer membrane proteins. Immunity induced by anti-CT alone, however, is short-lived and not as effective as antibody responses to the whole bacterium [143].

In former vaccination strategies, acellular (inactivated holotoxins) or whole cell (phenol-inactivated) preparations only provided short-lived protection and proved to be reactogenic (i.e. causing some symptoms of disease). For example, in a large clinical trial in Bangladesh (reviewed in [144]), a vaccine consisting of a mixture of CT subunit B and killed whole bacterial cell cocktails or killed cells alone conferred about 85% and 58% protection, respectively, against cholera for the first 6 months and declined over 36 months to about 50% for both vaccine types.

Modern cholera vaccine development, based on attenuated live vaccine strains (for review see [143]), takes several biological parameters into account. (i) What type of immunity needs to be stimulated to provide protection against cholera? (ii) What virulence factors must be removed from the vaccine strain to eliminate disease symptoms? (iii) What factors have to be expressed to achieve efficient colonization and protective immunity? And finally, (iv) how can the reacquisition of virulence factors by horizontal transfer (phage conversion) be prevented in a live vaccine strain? For the future development of live attenuated oral vaccine strategies these questions are of fundamental importance since the maintenance of intestinal colonization enhances the risk of acquiring phage-transmitted CT.

In current approaches these questions are addressed in the following manner. (i) Colonization of *V. cholerae* occurs in the upper small intestine and is sensed by the local mucosal immune system. Because the organisms are non-invasive, the transcytotic activity of the M cells is of foremost importance to present antigens to the underlying immune cells of the lymph system. If specific IgA production is induced, the secretion of these antibodies into the intestinal lumen ensures the immune response against the colonizing strain. For this reason an oral vaccination strategy with a live strain, which mimics natural infection, is important to induce intestinal mucosal immunity. (ii) Because the primary symptoms of cholera are caused by CT, it was originally assumed that *V. cholerae* deleted for *ctx* (or just the toxic *ctxA* portion) would make a good vaccine. However, such strains still caused significant disease symptoms (reactogenic) in humans, indicating the presence of additional virulence factors [145–147]. Therefore, investigations are under way to address the contribution of other factors to reactogenicity, e.g. the HA protease [148]. (iii) The expression of TCP provides efficient colonization and the overexpression of the CT B subunit (the non-toxic portion) seems to produce good antigenicity, although the issue of reactogenicity still needs to be resolved. (iv) Since TCP is both critical for colonization and also serves as the CTXϕ receptor, a *ctx* vaccine strain could undergo toxigenic conversion and thus the question of vaccine safety has become an important issue. Waldor and colleagues have described a clever technique to protect live vaccines against toxigenic conversion by the CTXϕ, namely the provision of the CTXϕ repressor protein

RstR within the vaccine strain, which provides immunity against subsequent CTXϕ infection [149].

## 10. Conclusions

It is beyond the scope of this review to comprehensively cover all the research into CT, toxin regulation and *V. cholerae* pathogenesis. The characterization of the CT itself had a huge impact on the understanding of basic mechanisms of cellular signaling and of the function of a class of cell surface glycoconjugates known as gangliosides. During the past decade of cholera research two important findings have illuminated the complexity of *V. cholerae*: first, the characterization of a novel serogroup (O139) which circumvented established immunity against O1, and second, the discovery that CT genes are encoded on a bacteriophage genome. These two characteristics of *V. cholerae* make vaccine development more complicated, and illustrate how bacterial pathogens can rapidly evolve through the acquisition of horizontally transferred genetic elements.

Recently, researchers have begun to appreciate and study the environmental interepidemic persistence mode of *V. cholerae*. Our understanding of biofilm formation and the VNC state is still in its infancy, but this environmental phase of the *V. cholerae* infectious cycle is critical for the establishment of endemic pathogenic strains in various water sources, and serves as the inoculum that initiates cholera pandemics. Also, horizontal gene transfer that leads to new epidemic strains likely occurs in this environment, and therefore cannot be ignored. A better understanding of biofilm formation and the VNC state should lead to the identification of strategies to forecast and prevent cholera outbreaks and hopefully eradicate *V. cholerae* from community water supplies in developing countries.

## Acknowledgements

For contributing to this review we thank Ronald Taylor and Paula Watnick. We also thank Stefan Schlör for critical reading of the manuscript. Our part of the work was founded by the BMBF Grant 01KI8906 and NIH AI43486.

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