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Vibrio cholerae CytR is a repressor of biofilm development

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Summary

Vibrio cholerae is both a human pathogen and a natural inhabitant of aquatic environments. In the aquatic environment, microorganisms are found attached to surfaces in structures known as biofilms. We have identified a transcriptional repressor in *V. cholerae* that inhibits exopolysaccharide synthesis and biofilm development. Our studies show that this repressor is the *V. cholerae* homologue of *Escherichia coli* CytR, a protein that represses nucleoside uptake and catabolism when nucleosides are scarce. We propose that the role of CytR in *V. cholerae* biofilm development is to co-ordinate bacterial biofilm accumulation with the presence of nucleosides. Thus, nucleosides may be a signal to planktonic cells to join the biofilm.

Introduction

Although surface adhesion or biofilm development is critical for the persistence and proliferation of all bacterial species in their natural habitats, the environmental signals that promote the surface-adherent mode of life are poorly understood. *Vibrio cholerae*, a native inhabitant of diverse aquatic environments and the human intestinal pathogen that causes the severe diarrhoeal disease cholera, is an ideal model for the study of biofilm development. Surface adhesion or biofilm development by *V. cholerae* is instrumental in both pathogenesis and colonization of aquatic environments. In the environment, *V. cholerae* has been identified on the surfaces of zooplankton, phytoplankton, crustaceans, insects and plants (Huq *et al.*, 1983; 1986; Tamplin *et al.*, 1990; Shukla *et al.*, 1995). Because zoo-plankton and phytoplankton blooms precede cholera outbreaks, the association of *V. cholerae* with these organisms is hypothesized to play a role in the epidemiology of disease (Colwell, 1996; Lobitz *et al.*, 2000). Thus, an understanding of the regulation of *V. cholerae* surface adhesion will not only increase our understanding of bacterial adaptation to aquatic habitats but may also suggest methods of improving our predictive models for cholera epidemics.

Vibrio cholerae biofilm development on abiotic surfaces has been described previously (Watnick and Kolter, 1999; Watnick *et al.*, 2001). In batch biofilm experiments, two stages in *V. cholerae* biofilm development are observed during the first 24 h of growth (Watnick *et al.*, 2001). In the initial period of 4–5 h, only transient association with the surface is observed. This association requires a functional polar flagellum. In the second stage of biofilm development, transient association with the surface continues, but now permanent immobilization and microcolony formation are observed. Permanent immobilization is dependent on the *vps* genes, which encode enzymes required for the synthesis of VPS, an exopolysaccharide produced by *V. cholerae* (Watnick and Kolter, 1999; Yildiz and Schoolnik, 1999).

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Studies of both *V. cholerae* and *Pseudomonas aeruginosa* suggest that increased exopolysaccharide synthesis alters both the course of biofilm development and the three-dimensional architecture of the mature biofilm (Wai *et al.*, 1998; Mizunoe *et al.*, 1999; Yildiz and Schoolnik, 1999; Hentzer *et al.*, 2001). In *V. cholerae*, spontaneously occurring 'rugose' variants have been isolated (White, 1938; Crutchley, 1968; Morris *et al.*, 1996; Wai *et al.*, 1998; Mizunoe *et al.*, 1999; Yildiz and Schoolnik, 1999). These variants display a rough colony morphology that is correlated with increased transcription of the *vps* genes and increased VPS synthesis (Wai *et al.*, 1998; Mizunoe *et al.*, 1999; Yildiz and Schoolnik, 1999; Yildiz *et al.*, 2001). Increased VPS synthesis by rugose variants also results in a higher profile biofilm than that formed by the parent *V. cholerae* strain (Yildiz and Schoolnik, 1999). The genetic basis of increased *vps* transcription in spontaneous rugose variants has not yet been ascertained, and one possibility is that these rugose variants represent a genotypically heterogeneous population.

The transcription profiles of biofilm-associated cells are quite different from their planktonic counterparts. Evidence from *P. aeruginosa* and *Escherichia coli* suggests that increased transcription of genes required for exopolysaccharide synthesis is a characteristic of cells that have been incorporated into the biofilm (Davies *et al.*, 1993; Prigent-Combaret *et al.*, 1999). More comprehensive genomics- and proteomics-based profiling of biofilm-associated cells is under way or completed in many bacterial backgrounds, and these studies are likely to yield a deeper understanding of the physiological state of the biofilm-associated cell (Sauer and Camper, 2001; Schoolnik *et al.*, 2001; Whiteley *et al.*, 2001; Sauer *et al.*, 2002; Zhu *et al.*, 2002). Complementary studies of the environmental signals and transcription factors that are operative in biofilm development will allow us to link signal transduction cascades with changes in gene transcription and protein expression within the biofilm.

Several environmental signals that influence bacterial biofilm development have been identified. These include quorum sensing, surface sensing and the nutritional content of the aquatic environment. In *P. aeruginosa*, autoinducer synthesis mutants form a flat, detergent-sensitive biofilm, suggesting that quorum sensing directs normal biofilm development (Davies *et al.*, 1998; Parsek and Greenberg, 1999). The quorum-sensing systems of *Burkholderia cepacia* and *V. cholerae* have also been implicated in biofilm development (Huber *et al.*, 2001; Zhu *et al.*, 2002). Another signal that activates biofilm development is contact with the substratum. In both *V. cholerae* and *P. aeruginosa*, there is evidence that exopolysaccharide synthesis is activated by mutation of the flagellar structural genes but not the flagellar motor (Garrett *et al.*, 1999; Watnick *et al.*, 2001). One possibility is that, as is true for the closely related organisms *Vibrio parahaemolyticus* and *Vibrio alginolyticus*, the flagellar motor of *V. cholerae* is able to sense and respond to changes in torque on the polar flagellum that occur when a surface is encountered (Belas *et al.*, 1986; McCarter *et al.*, 1988; Kawagishi *et al.*, 1996). For diverse bacteria, the nutritional composition of the aquatic environment is a key determinant of surface attachment (Bowden and Li, 1997; O'Toole and Kolter, 1998; Pratt and Kolter, 1998; Watnick *et al.*, 1999; Danese *et al.*, 2001). In particular, for many organisms, glucose and related carbohydrates greatly enhance exopolysaccharide production and, thus, biofilm development (Bryan *et al.*, 1986; Bonet *et al.*, 1993; Abbad Andaloussi *et al.*, 1995; Kimmel *et al.*, 1998; Degeest and De Vuyst, 1999; Looijesteijn *et al.*, 1999; Petry*et al.*, 2000; Degeest *et al.*, 2001; Mozzi *et al.*, 2001). Although transcription factors that regulate exopolysaccharide synthesis have been identified in various organisms, the environmental signals that these regulators sense is not known (Reeve *et al.*, 1997; Chapman and Kao, 1998; Yildiz *et al.*, 2001). This is the case for VpsR, the only known activator of *V. cholerae* biofilm development and VPS synthesis (Yildiz *et al.*, 2001). By sequence homology, VpsR is the response element in a two-component system. Although activation of *vps* gene transcription by VpsR has been demonstrated, the environmental signals that govern activation by VpsR have not yet been delineated.

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In *E. coli*, CytR has been shown to repress nucleoside uptake and catabolism in nucleoside-poor environments. In this paper, we provide evidence that *V. cholerae* CytR plays a role not only in nucleoside catabolism but also in the control of biofilm development and, specifically, in the synthesis of the *V. cholerae* exopolysaccharide, VPS. Our studies link nucleoside concentrations to transcriptional regulation of exopolysaccharide synthesis and biofilm development by *V. cholerae*. Thus, we suggest that nucleosides may be a signal to planktonic cells to join the nascent biofilm.

Results

Identification of a 'super-biofilm' mutant

A library of mini-Tn*10* transposon-insertion mutants was constructed in the *V. cholerae* O139 strain MO10. This library was screened for mutants that formed increased or 'super' biofilms as described previously (Watnick and Kolter, 1999; Watnick *et al.*, 2001). The transposon-insertion junctions of identified mutants were amplified by arbitrary polymerase chain reaction (PCR) and sequenced (O'Toole *et al.*, 1999). Of the 6000 transposon-insertion mutants evaluated, three independent clones were found to be in the gene given the TIGR designation VC2677 (Heidelberg *et al.*, 2000). Further evaluation demonstrated that all these mutants displayed a rugose colony morphology and formed a biofilm containing approximately twice as many cells as a wild-type *V. cholerae* biofilm, as determined by crystal violet staining and quantification. These mutants also displayed normal motility on swarm agar (data not shown). A transposon-insertion mutant in the same gene of *V. cholerae* O1 El Tor demonstrated a similar increase in biofilm development (data not shown), suggesting that this type of regulation is not strain specific.

The 'super biofilm' mutant is an *E. coli* CytR homologue

Sequence analysis demonstrated that the amino acid sequence of the gene containing the transposon insertion is 65% identical and 81% similar to the CytR protein of *E. coli*. No other protein in the *V. cholerae* genome showed this extent of similarity to *E. coli* CytR. As shown in Fig. 1A, this homology extends throughout the protein. Furthermore, the genomic environments of the *E. coli* CytR gene and the *V. cholerae* biofilm repressor are strikingly similar as shown in Fig. 1B. Thus, we term our biofilm repressor *V. cholerae* CytR and present additional evidence below that this protein is, in fact, *V. cholerae* CytR.

Construction of a *V. cholerae cytR* deletion mutant, cloning of *V. cholerae cytR* and complementation of the mutant phenotype

To confirm that the phenotype demonstrated by the transposon-insertion mutant was indeed the result of a mutation in *cytR*, a strain carrying a complete deletion of the *cytR* gene was constructed (strain PW324 denoted Δ*cytR*). The planktonic growth curves of wild-type *V. cholerae* and the Δ*cytR* mutant in LB broth were measured and found to be essentially superimposable before the onset of biofilm development (data not shown). As shown in Fig. 2, the deletion mutant demonstrated a rugose colony morphology and 'super-biofilm' phenotype that was similar to that of the transposon-insertion mutant. Measurements of total protein in wild-type *V. cholerae* and Δ*cytR* mutant biofilms indicated an increase in cell mass within the Δ*cytR* mutant biofilm that was consistent with crystal violet quantification (data not shown). Furthermore, we constructed a Δ*cytR* mutant harbouring a deletion in a large *vps* operon containing six open reading frames (ORFs) including *vpsL* (Yildiz *et al.*, 2001; strain PW329 denoted Δ*cytR* Δ*vps*). The Δ*cytR* Δ*vps* mutant formed a smooth colony and did not accumulate on surfaces. This result suggests that the phenotype of the Δ*cytR* mutant results from derepression of exopolysaccharide synthesis rather than activation of a new mechanism of surface adhesion.

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We also confirmed the correlation of the Δ*cytR* mutation with the 'super-biofilm' phenotype by complementation with the *V. cholerae cytR* gene *in trans.* We amplified the *cytR* gene with its native promoter by PCR, sequenced the product fully and cloned the resulting PCR product into the moderate-copy-number plasmid pBR322. The cloned *cytR* promoter and coding sequence from the *V. cholerae* O139 strain, MO10, had a nucleotide sequence identical to that of the *V. cholerae* O1 El Tor strain N16961. As shown in Fig. 3, when the *cytR* gene was provided *in trans*, the Δ*cytR* mutant displayed a smooth colony morphology and biofilm accumulation similar to that of wild-type *V. cholerae*.

Vibrio cholerae CytR is able to regulate transcription of the *E. coli udp* gene, a member of the *E. coli* CytR regulon

When cytidine is scarce in the environment, the *E. coli* CytR protein represses the transcription of genes encoding proteins involved in nucleoside uptake and catabolism. One of these genes, *udp*, encodes uridine dephosphorylase, an enzyme involved in the conversion of uridine to ribose-1-phosphate and uracil (Barbier and Short, 1992; Brikun *et al.*, 1996; Neidhardt, 1996; Gavigan *et al.*, 1999). Uridine dephosphorylase is operative in the catabolism of both uridine and cytidine. To confirm that the gene we identified as *V. cholerae cytR* had a function similar to that of the *E. coli cytR* gene, we tested whether *V. cholerae cytR*, provided *in trans*, could repress transcription of the *udp* gene of *E. coli*. We compared transcription of a *udp–lacZ* fusion in a parent *E. coli* strain containing a wild-type copy of *cytR* as well as in a derivative *cytR* mutant transformed with either a control plasmid or a plasmid carrying the wild-type *V. cholerae cytR* gene (Barbier and Short, 1992). As shown in Fig. 4A, *udp–lacZ* transcription was derepressed in the *E. coli cytR* mutant. When the *V. cholerae cytR* gene was supplied *in trans* to an *E. coli cytR* mutant, *udp–lacZ* transcription was repressed.

Vibrio cholerae CytR regulates the *V. cholerae udp* gene in response to cytidine

We questioned whether CytR regulates nucleoside catabolism in *V. cholerae* as it does in *E. coli*. We identified an ORF in the *V. cholerae* genome (TIGR locus VC1034) whose putative protein product is 75% identical and 89% similar to the Udp protein of *E. coli* K-12. We constructed a *lacZ* fusion to the *V. cholerae udp* promoter and inserted this fusion into the *lacZ* locus of both wild-type *V. cholerae* and a Δ*cytR* mutant. We then assayed β-galactosidase activity in extracts of planktonic cells grown in LB broth alone as well as in LB broth with added cytidine, glucose and glycerol. In all the media used, the growth curves of wild-type *V. cholerae* and the Δ*cytR* mutant were similar (data not shown). As shown in Fig. 4B, when these strains were grown in LB broth, *udp–lacZ* transcription was greater in the *V. cholerae* Δ*cytR* mutant than in wild-type *V. cholerae*. As predicted, the addition of cytidine to LB broth increased *udp–lacZ* transcription in wild-type cells almost to the level of the *V. cholerae* Δ*cytR* mutant. The addition of glucose to the growth medium decreased the difference in *udp–lacZ* transcription between wild-type *V. cholerae* and a Δ*cytR* mutant slightly, whereas the addition of glycerol to the growth medium had no significant effect on *udp–lacZ* transcription in wild-type *V. cholerae* and Δ*cytR* mutant cells.

Phase-contrast microscopy of wild-type *V. cholerae* and Δ*cytR* mutant biofilms

Once the biofilm repressor was identified as the *V. cholerae* CytR protein, the mechanism of biofilm repression and its role in biofilm development was explored further. It has been shown previously that biofilm development by *V. cholerae* O139 consists of an initial phase of several hours when only transient surface association is seen. After this initial phase, immobilization and accumulation of the cells in a biofilm is observed. This step requires exopolysaccharide synthesis (Watnick *et al.*, 2001).

The colony morphology and biofilm phenotype of the *V. cholerae* Δ*cytR* mutant suggests that exopolysaccharide synthesis is derepressed in this mutant. We were interested in determining

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how the course of biofilm development was altered by this derepression. We hypothesized that increased exopolysaccharide synthesis might (i) shorten or eliminate the initial phase of transient attachment; (ii) increase deposition of bacteria on the surface during the biofilm accumulation phase; and/or (iii) alter the mature biofilm architecture. Video and time-lapse microscopy of the early stages of biofilm development demonstrated that the initial phase of transient attachment was indistinguishable from that of wild-type *V. cholerae* both in duration and in the types of interactions observed between the bacterium and the surface. In Fig. 5A, we show representative micrographs of early surface immobilization by wild-type *V. cholerae* and a Δ*cytR* mutant. These micrographs represent simultaneous experiments in which wells were incubated with similar numbers of either wild-type *V. cholerae* or Δ*cytR* mutant cells for 5 h at 27°C. These micrographs demonstrate that the early stages of biofilm development by wild-type *V. cholerae* and a Δ*cytR* mutant were indistinguishable. However, as shown in Fig. 5B, after 18 h of incubation with a surface, the biofilm formed by the *V. cholerae* Δ*cytR* mutant was confluent and dense, whereas the wild-type *V. cholerae* biofilm had not yet reached confluence. Thus, biofilm development by the Δ*cytR* mutant deviates from that by wild-type *V. cholerae* in the surface accumulation phase but not in the initial phase of transient association.

Three-dimensional architecture of *V. cholerae ΔcytR* mutant biofilms

Previous studies have suggested that bacterial variants or mutants that exhibit increased exopolysaccharide production may form three-dimensional biofilms with an altered architecture (Hentzer *et al.*, 2001). To determine whether the architecture of a *V. cholerae* Δ*cytR* mutant biofilm is grossly different from that of wild-type *V. cholerae*, we used confocal microscopy to visualize the three-dimensional structures of wild-type *V. cholerae* and Δ*cytR* mutant biofilms after 2 days of growth. As shown in Fig. 6, horizontal and vertical sections through both wild-type *V. cholerae* and Δ*cytR* mutant biofilms demonstrate pillars of bacteria with water channels between. In contrast to the wild-type *V. cholerae* biofilm, however, the water channels observed in the Δ*cytR* mutant biofilm are much narrower, and the bacterial pillars are almost confluent. Thus, the density of cells in the Δ*cytR* mutant biofilm is much greater than that in wild-type biofilms.

Reporter measurements of *vps* gene transcription

To investigate further the observation that a *cytR* mutation affects the surface accumulation phase of biofilm development, we measured transcription of one of the identified *vps* operons in the planktonic and biofilm-associated phases. We inserted a *lacZ* fusion to the *vpsL* promoter into the chromosomal *lacZ* gene of wild-type *V. cholerae* and a Δ*cytR* mutant. *vpsL* is the first gene in one of two large putative operons involved in VPS synthesis (Yildiz *et al.*, 2001). *vpsL–lacZ* transcription was measured in planktonic and biofilm-associated wild-type *V. cholerae* and Δ*cytR* mutant cells. As shown in Fig. 7, transcription of *vpsL–lacZ* in both wild-type *V. cholerae* and Δ*cytR* mutant planktonic cells was low. However, *vpsL–lacZ* transcription in biofilm-associated cells was seven and 14 times, respectively, that in planktonic cells with the same genetic background. Furthermore, *vpsL–lacZ* transcription in the *V. cholerae* Δ*cytR* biofilm was three times that of *vpsL–lacZ* transcription in the wild-type *V. cholerae* biofilm.

Discussion

We have identified a repressor of exopolysaccharide synthesis and biofilm development in *V. cholerae* and have shown that this repressor is the *V. cholerae* CytR protein. The *E. coli* CytR protein is a member of the LacI family of repressors. These repressors respond to increased concentrations of effector molecules by lifting repression of the catabolic pathways relevant to these effector molecules. The previously defined function of CytR in *E. coli* and other Gram-negative bacteria was repression of the transcription of genes encoding proteins involved in

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nucleoside uptake and catabolism in response to low nucleoside concentrations (Neidhardt, 1996; Thomsen *et al.*, 1999). Our experiments demonstrate that *V. cholerae* CytR functions in a manner similar to that of *E. coli* CytR. When provided *in trans*, *V. cholerae* CytR is able to complement an *E. coli cytR* mutant. The *udp* gene product, encoding uridine dephosphorylase, is involved in cytidine and uridine catabolism and is regulated by CytR in *E. coli*. We have shown that *V. cholerae* CytR represses transcription of the *V. cholerae udp* gene in response to low environmental cytidine concentrations. Thus, we provide strong evidence that the mechanism of regulation and binding site specificity of *V. cholerae* CytR is similar to that of *E. coli* CytR and that *V. cholerae* CytR is also involved in the repression of nucleoside catabolism in response to low nucleoside concentrations.

We draw two conclusions from our studies of *vpsL* transcription in wild-type *V. cholerae* and Δ*cytR* mutant planktonic and biofilm-associated cells. The first is that, in both planktonic and biofilm-associated cells, *V. cholerae* CytR regulates VPS synthesis at the level of *vps* gene transcription. Furthermore, for wild-type *V. cholerae*, *vpsL* transcription in the biofilm is greater than *vpsL* transcription in the planktonic phase. We hypothesize that, as has been observed for other Gram-negative bacteria, surface contact activates transcription of exopolysaccharide synthesis genes in wild-type *V. cholerae* (Davies *et al.*, 1993; Prigent-Combaret *et al.*, 1999; Sauer and Camper, 2001; Whiteley *et al.*, 2001; Sauer *et al.*, 2002). Because surface contact activation of *vpsL* is also observed in Δ*cytR* mutant cells, we conclude that CytR is not a component of the surface-sensing signal transduction cascade. Instead, an independent regulatory circuit must be operative in surface sensing.

Previous studies suggest that wild-type *V. cholerae* biofilm development includes a period of transient surface association followed by surface immobilization and accumulation (Watnick and Kolter, 1999; Watnick *et al.*, 2001). The initial period of transient surface association by the *V. cholerae* Δ*cytR* mutant is indistinguishable from that of wild-type *V. cholerae* in both its character and duration. Transient surface association has previously been postulated to be important for surface sensing (Watnick *et al.*, 2001). The observation that transient surface association is unaltered in biofilm development by the *V. cholerae* Δ*cytR* mutant is consistent with the conclusion stated above that surface contact activation, which occurs during the period of transient surface activation, is functional in the *V. cholerae* Δ*cytR* mutant. The period of biofilm accumulation is altered, however, in a *V. cholerae* Δ*cytR* mutant. Once biofilm accumulation by the *V. cholerae* Δ*cytR* mutant begins, it proceeds rapidly and produces a structure in which cells are much more densely packed together than they are in the wild-type *V. cholerae* biofilm. We hypothesize therefore that signals that maintain water channels between the pillars of the biofilm, such as those involved in quorum sensing, are over-ridden in the Δ*cytR* mutant.

To the well-characterized roles of the CytR protein in repression of nucleoside uptake and catabolism, we have now added a novel role for *V. cholerae* CytR in the repression of an anabolic process, namely synthesis of the *V. cholerae* exopolysaccharide VPS. The co-regulation of a catabolic process and an anabolic process is particularly thought provoking. One possibility is that derepression of exopolysaccharide synthesis gene transcription in a *V. cholerae* Δ*cytR* mutant is simply the indirect result of increased uptake and catabolism of nucleosides, leading to increased carbon and energy stores within the cell. This seems unlikely given the growth medium used in our experiments. The primary carbon and energy sources in LB broth are amino acids, and nucleosides represent ≈ = 0.1% of the dry weight of amino acids in LB. Thus, the carbon and energy stores of a bacterium growing in LB broth would not be significantly altered by derepression of nucleoside uptake and catabolism. Rather, we propose that elevated intracellular cytidine levels may be a signal for surface immobilization and that this signal is transduced by the *V. cholerae* CytR protein.

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Our studies build a bridge between increased levels of cytidine and synthesis of an extracellular polysaccharide matrix leading to surface attachment. These findings are particularly intriguing in view of a recent report that DNA is an important component of the extracellular matrix of *P. aeruginosa* biofilms and may actually be required for initial biofilm development (Whitchurch *et al.*, 2002). Although it seems improbable that nucleosides would be present in abundance in the aqueous phase of an aquatic environment, we hypothesize that DNA and its catabolites may accumulate within the extracellular matrix of the biofilm. Thus, nucleoside concentrations in and around biofilms may be increased, providing an environmental signal that recruits new bacteria into the biofilm.

Experimental procedures

Bacterial strains and media

The bacterial strains and plasmids used in this study are listed in Table 1. All experiments used Luria–Bertani (LB) broth. Cytidine, glucose and glycerol were used at concentrations of 3 g l^{-1} where noted. The wild-type *V. cholerae* strain, MO10, is resistant to streptomycin, as are all mutants derived from this strain. Thus, most biofilm experiments were performed in LB broth supplemented with 100 μg ml^{-1} streptomycin. Furthermore, biofilm experiments involving strains harbouring a plasmid encoding ampicillin resistance were performed in LB broth supplemented with 150 μg ml^{-1} ampicillin. Procedures used to construct strains and plasmids for this study are detailed below.

Construction of *Δvps* and *ΔcytR* mutants

A VPS locus containing two large operons required for VPS synthesis has been identified. (Yildiz *et al.*, 2001). One of these, containing *vpsL* as well as five additional putative ORFs, was selected for deletion. A 400 bp fragment located just upstream of the start codon of *vpsL* and a 346 bp fragment located 184 bp downstream from the start codon of the sixth ORF were amplified by PCR using the primer pairs P62, P63 and P64, P65 respectively (see Table 2). Primers P63 and P64 included a complementary 15 bp sequence at their 3′ and 5′ ends respectively. These two fragments were joined using the splicing by overlap extension (SOE) technique, resulting in the construction of a fragment with a 6 kb deletion in the *vpsL* operon (Horton *et al.*, 1990; Lefebvre *et al.*, 1995). The fragment containing the deletion was purified and ligated into pCR2.1TOPO (Invitrogen). This fragment was then removed from pCR2.1TOPO by digestion with *Spe*I and *Xho*I and ligated into pWM91 to create the suicide plasmid pAJH5. This plasmid was used to create *vpsL* operon deletions in the relevant strains by double homologous recombination and sucrose selection as described previously (Donnenberg and Kaper, 1991).

Δ*cytR* mutants were constructed in a manner similar to that described for the construction of Δ*vps* mutants. Briefly, the primer pairs P38, P39 and P40, P41 were used to amplify a 564 bp fragment immediately upstream of the *cytR* gene and a 507 bp fragment beginning 23 bp downstream from the putative *cytR* stop codon. These fragments were ligated using the SOE technique and used to create a deletion in the *V. cholerae cytR* gene, resulting in strain PW324.

Construction of pAJH3 for complementation studies

In order to determine whether we could complement the phenotype of our Δ*cytR* mutant with the *cytR* gene provided *in trans*, we cloned *cytR* into the cloning vector pBR322. The *cytR* gene is 309 bp downstream of the *priA* gene. Thus, it is most probably transcribed by a promoter positioned directly upstream of its own coding sequence. The primers P55 and P56, which are positioned 20 bp upstream of the stop codon of *priA* and 80 bp downstream of the stop codon of *cytR*, respectively, were used to amplify the *cytR* gene promoter and coding sequence. The amplification product was ligated into pCR2.1-TOPO (Invitrogen) to yield pCR2.1-TOPO::

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cytR. This plasmid was digested with *Eco*RI, and the fragment containing the *cytR* gene was purified and ligated into pBR322.

Construction of chromosomally based operon fusions of the *vpsL* promoter (*vpsLp*)and the *udp1* promoter (*udp1p*) to *lacZ*

The intergenic region between the divergently transcribed genes, VC0933 and VC0934, was amplified by PCR using the primer pair P75 and P76. This produced a DNA fragment (*vpsLp*) including 31 bp of the coding region of VC0933 and 140 bp of the coding region of VC0934. The primers were designed with stop codons at either end of the fragment to avoid the generation of chimeric proteins. *vpsLp* was recovered by cloning into pCR2.1-TOPO (Invitrogen) to create PCR2.-TOPO::*vpsLp*. PCR2.-TOPO::*vpsLp* and pUJ10 were digested with *Xho*I and *Bam*HI. The resulting *vpsLp* promoter fragment and the pUJ10 fragment harbouring a promoterless *lacZ* gene were purified and ligated to yield a plasmid-based fusion of *vpsLp* to the *lacZ* gene. The resulting plasmid and p6891 multiple cloning site (MCS) were digested with *Not*I. The liberated *vpsLp–lacZ* fusion was purified and ligated with p6891MCS to yield the *vpsL–lacZ* fusion inserted between two homologous fragments of the *V. cholerae lacZ* gene and oriented opposite to the direction of transcription of the wild-type *V. cholerae lacZ* gene. The promoter region of this construct was fully sequenced to confirm the absence of mutations introduced by PCR or cloning. This fusion was crossed into the chromosomal locus of the *V. cholerae lacZ* gene of relevant strains as described previously (Watnick *et al.*, 2001).

The chromosomal *udp1p–lacZ* fusion was constructed as described for the *vpsLp–lacZ* fusion except that the region between the putative ORFs with the TIGR designation VC1033 and VC1034 was amplified by PCR using the primer pair P95 and P96.

Transposon mutagenesis and screen for biofilm-altered mutants

Transposon mutagenesis and the screen for biofilm-altered mutants were performed as described previously (Watnick and Kolter, 1999). Briefly, wild-type *V. cholerae* and the *E. coli* strain β2155(pBSL180) were crossed on an LB agar plate supplemented with 0.3 mM diaminopimelic acid for 2 h at 37°C. Transposon-insertion mutants were selected on LB agar plates containing kanamycin. The resulting *V. cholerae* transposon-insertion mutants were replica plated onto LB agar plates and then transferred to LB-filled polyvinylchloride microtitre dish wells using a multipronged device. The bacteria were incubated in these microtitre dish wells at 27°C for 18 h. Wells were then rinsed and stained with crystal violet. Wells that demonstrated an enhanced crystal violet ring were stored at −80°C in LB broth supplemented with 15% glycerol.

Biofilm assays

For observation and quantification of biofilms by crystal violet staining, biofilms were formed either in wells of sterile polystyrene microtitre dishes or in borosilicate glass tubes. Very little difference in bacterial surface accumulation was observed between these two surfaces. Again, biofilms were incubated at 27°C for 18 h. Quantification of surface-adherent cells was measured by crystal violet staining as described previously (Watnick *et al.*, 2001). Briefly, biofilms were stained by incubation with a 1 mg ml^{-1} aqueous solution of crystal violet for 6 min. Dimethyl sulphoxide (DMSO) was then added immediately to disrupt the biofilm, and an OD_{595} of the resulting solution was measured.

Phase-contrast and confocal microscopy

Biofilms intended for observation by time-lapse phase-contrast microscopy were formed in 24-well polystyrene microtitre dishes. Wells were filled with 300 μl of LB broth, which allowed

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adequate aeration of the base of the well and, thus, the formation of a robust biofilm. Biofilm development on the bottoms of wells was recorded either at discrete times, by video microscopy, or at intervals by time-lapse microscopy using an Eclipse TE-200 inverted phase-contrast microscope (Nikon) equipped with an Orca digital CCD camera (Hamamatsu) and VVM-D1 shutter drivers (Uniblitz). A computer equipped with METAMORPH imaging software (Universal Imaging) was used for image acquisition and processing.

For confocal microscopy, bacteria harbouring the plasmid pSMC2 were incubated with coverslips placed vertically in Falcon tubes containing LB broth supplemented with ampicillin as described previously (Watnick *et al.*, 2001). For these experiments, the medium was changed daily. Confocal micrographs were obtained using an Odyssey confocal microscope (Noran) equipped with a graphics work station.

β-Galactosidase measurements

For measurements of gene transcription by the *vpsLp* promoter in both planktonic and biofilm-associated cells, strains of interest were incubated in 96-well plates at 27°C for 18 h. The planktonic cells from four wells were combined in a separate tube, wells were rinsed with LB broth, and biofilms from these four wells were removed by mechanical disruption and pooled. All experiments were done in triplicate. Planktonic and biofilm-associated cells were gently pelleted and then resuspended in 1.5 ml of Z-buffer (Miller, 1992). Borosilicate beads (1 mm; BioSpec) were added to the cell suspension, and this suspension was agitated using a mini-Beadbeater (BioSpec) for 10 s at 2500 r.p.m. to separate cell aggregates. Although this agitation step was specifically included to disperse biofilm-associated cells, planktonic and biofilm-associated cells were treated similarly. Cells were then lysed by three freeze–thaw cycles at −80°C. A sample of this lysate (200 µl) was set aside for subsequent protein determination using the Coomassie Plus protein assay (Pierce), and ONPG (Sigma), a colorimetric substrate for β-galactosidase, was added to 1 ml of the remaining mixture. All samples were incubated for 18.5 h at 37°C to allow a yellow colour to develop, samples were centrifuged to remove cell debris, and the OD_{405} of each sample was measured (model 550 microplate reader; Bio-Rad). To obtain relative β-galactosidase activity measurements for each sample, the OD_{405} was multiplied by 1000 and divided by the calculated protein concentration.

For all measurements of *udp–lacZ* transcription in *E. coli* and *V. cholerae* strains, relevant cells were grown overnight in LB broth at 37°C. Cells were lysed by three freeze–thaw cycles at −80°C. Subsequent measurements of β-galactosidase activity followed the protocol above.

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References

Abbad Andaloussi S, Talbaoui H, Marczak R, Bonaly R. Isolation and characterization of exocellular polysaccharides produced by *Bifidobacterium longum*. Appl Microbiol Biotechnol 1995;43:995–1000. [PubMed: 8590666]

Alexeyev MF, Shokolenko IN. Mini-Tn*10* transposon derivatives for insertion mutagenesis and gene delivery into the chromosome of gram-negative bacteria. Gene 1995;160:59–62. [PubMed: 7628717]

Barbier CS, Short SA. Amino acid substitutions in the CytR repressor which alter its capacity to regulate gene expression. J Bacteriol 1992;174:2881–2890. [PubMed: 1569019]

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Belas R, Simon M, Silverman M. Regulation of lateral flagella gene transcription in *Vibrio parahaemolyticus*. J Bacteriol 1986;167:210–218. [PubMed: 3013835]

Bloemberg GV, O'Toole GA, Lugtenberg BJ, Kolter R. Green fluorescent protein as a marker for *Pseudomonas* spp. Appl Environ Microbiol 1997;63:4543–4551. [PubMed: 9361441]

Bonet R, Simon-Pujol MD, Congregado F. Effects of nutrients on exopolysaccharide production and surface properties of *Aeromonas salmonicida*. Appl Environ Microbiol 1993;59:2437–2441. [PubMed: 8368833]

Bowden GH, Li YH. Nutritional influences on biofilm development. Adv Dent Res 1997;11:81–99. [PubMed: 9524446]

Brikun I, Suziedelis K, Stemmann O, Zhong R, Alikhanian L, Linkova E, et al. Analysis of CRP-CytR interactions at the *Escherichia coli udp* promoter. J Bacteriol 1996;178:1614–1622. [PubMed: 8626289]

Bryan BA, Linhardt RJ, Daniels L. Variation in composition and yield of exopolysaccharides produced by *Klebsiella* sp. strain K32 and *Acinetobacter calcoaceticus* BD4. Appl Environ Microbiol 1986;51:1304–1308. [PubMed: 3729401]

Butterton JR, Beattie DT, Gardel CL, Carroll PA, Hyman T, Killeen KP, et al. Heterologous antigen expression in *Vibrio cholerae* vector strains. Infect Immun 1995;63:2689–2696. [PubMed: 7790086]

Chapman MR, Kao CC. EpsR modulates production of extracellular polysaccharides in the bacterial wilt pathogen *Ralstonia (Pseudomonas) solanacearum*. J Bacteriol 1998;180:27–34. [PubMed: 9422588]

Colwell RR. Global climate and infectious disease: the cholera paradigm. Science 1996;274:2025–2031. [PubMed: 8953025]

Crutchley MJ. Rugose forms of an El Tor vibrio. J Gen Microbiol 1968;50:vii. [PubMed: 5652087]

Danese PN, Pratt LA, Kolter R. Biofilm formation as a developmental process. Methods Enzymol 2001;336:19–26. [PubMed: 11398398]

Davies DG, Chakrabarty AM, Geesey GG. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. Appl Environ Microbiol 1993;59:1181–1186. [PubMed: 8476292]

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 1998;280:295–298. [PubMed: 9535661]

Degeest B, De Vuyst L. Indication that the nitrogen source influences both amount and size of exopolysaccharides produced by *Streptococcus thermophilus* LY03 and modelling of the bacterial growth and exopolysaccharide production in a complex medium. Appl Environ Microbiol 1999;65:2863–2870. [PubMed: 10388677]

Degeest B, Janssens B, De Vuyst L. Exopolysaccharide (EPS) biosynthesis by *Lactobacillus sakei* 0–1: production kinetics, enzyme activities and EPS yields. J Appl Microbiol 2001;91:470–477. [PubMed: 11556912]

Dehio C, Meyer M. Maintenance of broad-host-range incompatibility group P and group Q plasmids and transposition of Tn5 in *Bartonella henselae* following conjugal plasmid transfer from *Escherichia coli*. J Bacteriol 1997;179:538–540. [PubMed: 8990308]

Donnenberg MS, Kaper JB. Construction of an eae deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect Immun 1991;58:4310–4317. [PubMed: 1937792]

Garrett ES, Perlegas D, Wozniak DJ. Negative control of flagellum synthesis in *Pseudomonas aeruginosa* is modulated by the alternative sigma factor AlgT (AlgU). J Bacteriol 1999;181:7401–7404. [PubMed: 10572149]

Gavigan SA, Nguyen T, Nguyen N, Senear DF. Role of multiple CytR binding sites on cooperativity, competition, and induction at the *Escherichia coli udp* promoter. J Biol Chem 1999;274:16010–16019. [PubMed: 10347150]

Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ, et al. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature 2000;406:477–483. [PubMed: 10952301]

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Hentzer M, Teitzel GM, Balzer GJ, Heydorn A, Molin S, Givskov M, Parsek MR. Alginate over-production affects *Pseudomonas aeruginosa* biofilm structure and function. J Bacteriol 2001;183:5395–5401. [PubMed: 11514525]

Horton RM, Cai ZL, Ho SN, Pease LR. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. Biotechniques 1990;8:528–535. [PubMed: 2357375]

Huber B, Riedel K, Hentzer M, Heydorn A, Gotschlich A, Givskov M, et al. The cep quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. Microbiology 2001;147:2517–2528. [PubMed: 11535791]

Huq A, Small EB, West PA, Huq MI, Rahman R, Colwell RR. Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. Appl Environ Microbiol 1983;45:275–283. [PubMed: 6337551]

Huq A, Huq SA, Grimes DJ, O'Brien M, Chu KH, Capuzzo JM, Colwell RR. Colonization of the gut of the blue crab (*Callinectes sapidus*) by *Vibrio cholerae*. Appl Environ Microbiol 1986;52:586–588. [PubMed: 3767362]

Kawagishi I, Imagawa M, Imae Y, McCarter L, Homma M. The sodium-driven polar flagellar motor of marine *Vibrio* as the mechanosensor that regulates lateral flagellar expression. Mol Microbiol 1996;20:693–699. [PubMed: 8793868]

Kimmel SA, Roberts RF, Ziegler GR. Optimization of exopolysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* RR grown in a semidefined medium. Appl Environ Microbiol 1998;64:659–664. [PubMed: 9464404]

Lefebvre B, Formstecher P, Lefebvre P. Improvement of the gene splicing overlap (SOE) method. Biotechniques 1995;19:186–188. [PubMed: 8527132]

Lobitz B, Beck L, Huq A, Wood B, Fuchs G, Faruque ASG, Colwell R. Climate and infectious disease: use of remote sensing for detection of *Vibrio cholerae* by indirect measurement. Proc Natl Acad Sci USA 2000;97:1438–1443. [PubMed: 10677480]

Looijesteijn PJ, Boels IC, Kleerebezem M, Hugenholtz J. Regulation of exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* by the sugar source. Appl Environ Microbiol 1999;65:5003–5008. [PubMed: 10543815]

McCarter L, Hilmen M, Silverman M. Flagellar dynamometer controls swarmer cell differentiation of *Vibrio parahaemolyticus*. Cell 1988;54:345–351. [PubMed: 3396074]

Metcalf WW, Jiang W, Daniels LL, Kim SK, Haldimann A, Wanner BL. Conditionally replicative and conjugative plasmids carrying *lacZ* alpha for cloning, mutagenesis, and allele replacement in bacteria. Plasmid 1996;35:1–13. [PubMed: 8693022]

Miller, JH. A Short Course in Bacterial Genetics. Plainview, NY: Cold Spring Harbor Laboratory Press; 1992.

Miller VL, Mekalanos JJ. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires toxR. J Bacteriol 1988;170:2575–2583. [PubMed: 2836362]

Mizunoe Y, Wai SN, Takade A, Yoshida SI. Isolation and characterization of rugose form of *Vibrio cholerae* O139 strain MO10. Infect Immun 1999;67:958–963. [PubMed: 9916115]

Morris JG Jr, Sztein MB, Rice EW, Nataro JP, Losonsky GA, Panigrahi PO, et al. *Vibrio cholerae* O1 can assume a chlorine-resistant rugose survival form that is virulent for humans. J Infect Dis 1996;174:1364–1368. [PubMed: 8940236]

Mozzi F, Rollan G, de Giori GS, Font de Valdez G. Effect of galactose and glucose on the exopolysaccharide production and the activities of biosynthetic enzymes in *Lactobacillus casei* CRL 87. J Appl Microbiol 2001;91:160–167. [PubMed: 11442726]

Neidhardt, FC., editor. Escherichia coli and Salmonella. Washington, DC: American Society for Microbiology Press; 1996.

O'Toole GA, Kolter R. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol 1998;28:449–461. [PubMed: 9632250]

O'Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R. Genetic approaches to study of biofilms. Methods Enzymol 1999;310:91–109. [PubMed: 10547784]

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Parsek MR, Greenberg EP. Quorum sensing signals in development of *Pseudomonas aeruginosa* biofilms. Methods Enzymol 1999;310:43–55. [PubMed: 10547781]

Petry S, Furlan S, Crepeau MJ, Cerning J, Desmazeaud M. Factors affecting exocellular polysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* grown in a chemically defined medium. Appl Environ Microbiol 2000;66:3427–3431. [PubMed: 10919802]

Pratt LA, Kolter R. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol Microbiol 1998;30:285–293. [PubMed: 9791174]

Prigent-Combaret C, Vidal O, Dorel C, Lejeune P. Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. J Bacteriol 1999;181:5993–6002. [PubMed: 10498711]

Reeve WG, Dilworth MJ, Tiwari RP, Glenn AR. Regulation of exopolysaccharide production in *Rhizobium leguminosarum* biovar *viciae* WSM710 involves *exoR*. Microbiology 1997;143:1951–1958. [PubMed: 9202471]

Sauer K, Camper AK. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. J Bacteriol 2001;183:6579–6589. [PubMed: 11673428]

Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm. J Bacteriol 2002;184:1140–1154. [PubMed: 11807075]

Schoolnik GK, Voskuil MI, Schnappinger D, Yildiz FH, Meibom K, Dolganov NA, et al. Whole genome DNA microarray expression analysis of biofilm development by *Vibrio cholerae* O1, E1 Tor. Methods Enzymol 2001;336:3–18. [PubMed: 11398407]

Shukla BN, Singh DV, Sanyal SC. Attachment of non-culturable toxigenic *Vibrio cholerae* O1 and non-O1 and *Aeromonas* spp. to the aquatic arthropod *Gerris spinolae* and plants in the River Ganga, Varanasi. FEMS Immunol Med Microbiol 1995;12:113–120. [PubMed: 8589660]

Tamplin ML, Gauzens AL, Huq A, Sack DA, Colwell RR. Attachment of *Vibrio cholerae* serogroup O1 to zooplankton and phytoplankton of Bangladesh waters. Appl Environ Microbiol 1990;56:1977–1980. [PubMed: 2383016]

Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673–4680. [PubMed: 7984417]

Thomsen LE, Pedersen M, Norregaard-Madsen M, Valentin-Hansen P, Kallipolitis BH. Protein-ligand interaction: grafting of the uridine-specific determinants from the CytR regulator of *Salmonella typhimurium* to *Escherichia coli* CytR. J Mol Biol 1999;288:165–175. [PubMed: 10329134]

Wai SN, Mizunoe Y, Takade A, Kawabata SI, Yoshida SI. *Vibrio cholerae* O1 strain TSI-4 produces the exopolysaccharide materials that determine colony morphology, stress resistance, and biofilm formation. Appl Environ Microbiol 1998;64:3648–3655. [PubMed: 9758780]

Waldor MK, Mekalanos JJ. ToxR regulates virulence gene expression in non-O1 strains of *Vibrio cholerae* that cause epidemic cholera. Infect Immun 1994;62:72–78. [PubMed: 7903285]

Watnick PI, Kolter R. Steps in the development of a *Vibrio cholerae* biofilm. Mol Microbiol 1999;34:586–595. [PubMed: 10564499]

Watnick PI, Fullner KJ, Kolter R. A role for the mannose-sensitive hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. J Bacteriol 1999;181:3606–3609. [PubMed: 10348878]

Watnick PI, Lauriano CM, Klose KE, Croal L, Kolter R. Absence of a flagellum leads to altered colony morphology, biofilm development, and virulence in *V. cholerae* O139. Mol Microbiol 2001;39:223–235. [PubMed: 11136445]

Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. Extracellular DNA required for bacterial biofilm formation. Science 2002;295:1487. [PubMed: 11859186]

White PB. The rugose variant of vibrios. J Pathol Bacteriol 1938;46:1–6.

Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, Greenberg EP. Gene expression in *Pseudomonas aeruginosa* biofilms. Nature 2001;413:860–864. [PubMed: 11677611]

Yildiz FH, Schoolnik GK. *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. Proc Natl Acad Sci USA 1999;96:4028–4033. [PubMed: 10097157]

Yildiz FH, Dolganov NA, Schoolnik GK. VpsR, a member of the response regulators of the two-component regulatory systems, is required for expression of vps biosynthesis genes and EPS (ETr)-

associated phenotypes in *Vibrio cholerae* O1 El Tor. J Bacteriol 2001;183:1716–1726. [PubMed: 11160103]

Zhu J, Miller MB, Vance RE, Dziejman M, Bassler BL, Mekalanos JJ. Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. Proc Natl Acad Sci USA 2002;99:3129–3134. [PubMed: 11854465]

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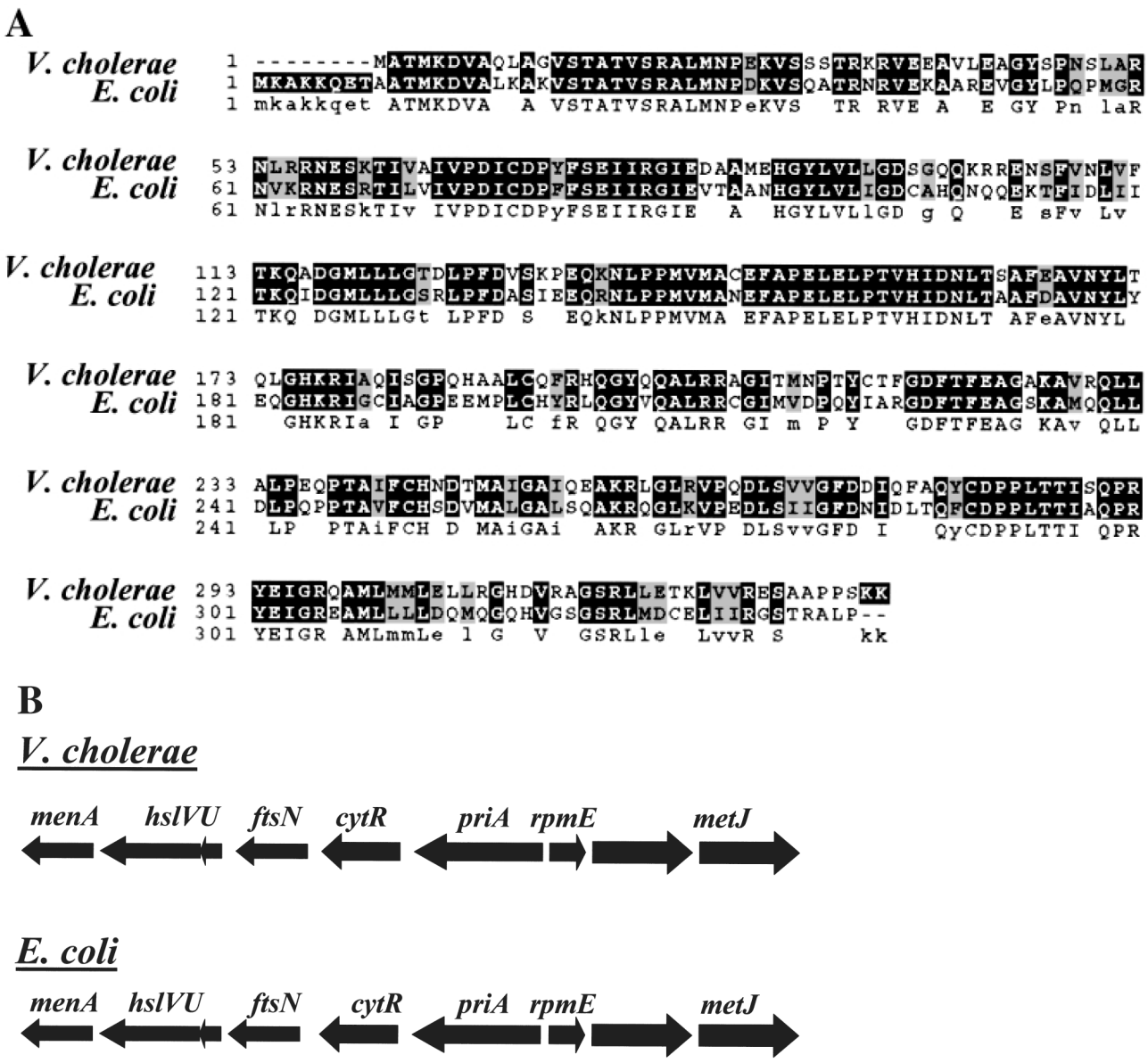

Figure 1.

A comparison of the *V. cholerae* O1 El Tor CytR amino acid sequence and genomic environment with that of *E. coli* K-12 CytR.

A. Alignment of the amino acid sequences of *V. cholerae* and *E. coli* CytR. CLUSTALW was used to produce the sequence alignment (Thompson *et al.*, 1994).

B. Genomic environment of *V. cholerae* O1 El Tor *cytR* and *E. coli* K-12 *cytR*.

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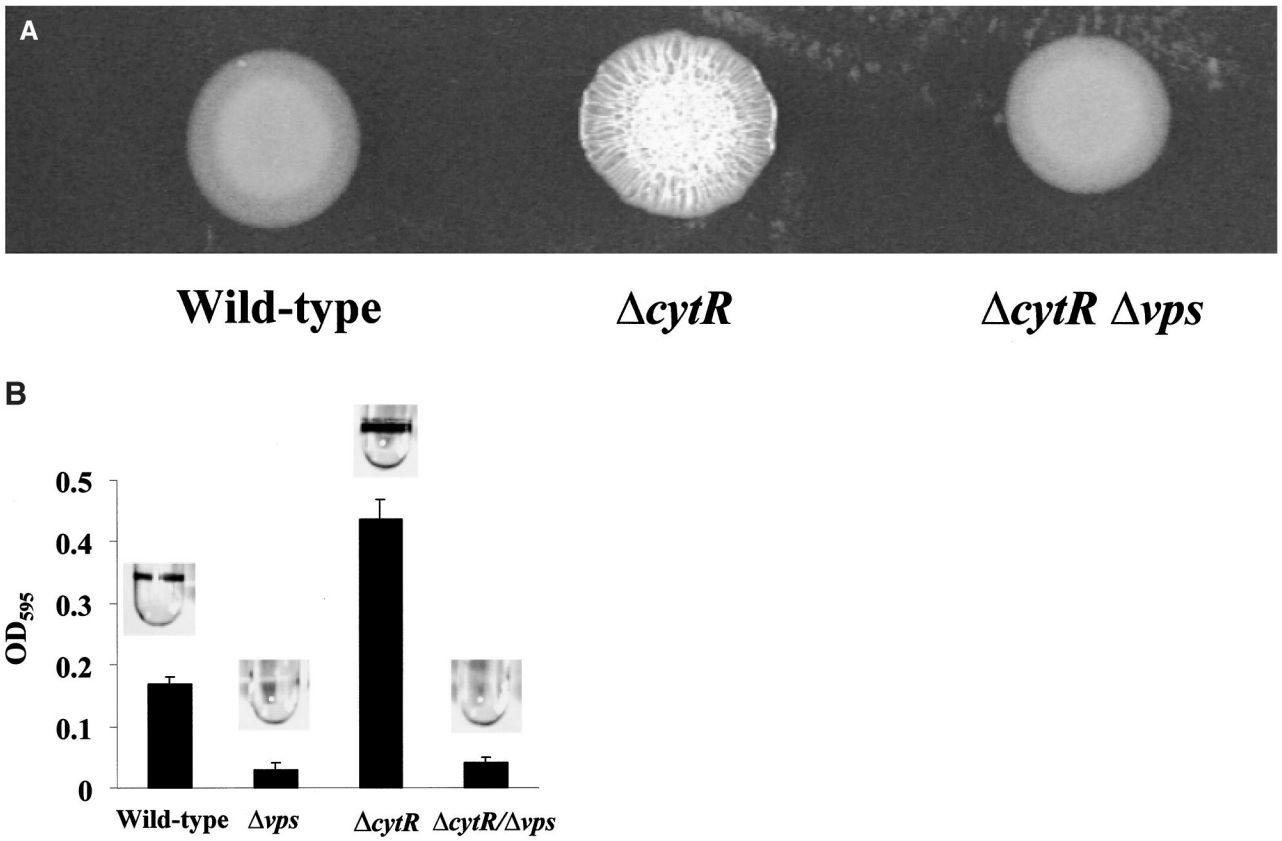

Figure 2.
Comparison of wild-type *V. cholerae* and Δ*cytR* mutant colony morphology and biofilm development.
A. Colony morphology of wild-type *V. cholerae* (MO10), a Δ*cytR* mutant (PW324) and a Δ*cytR*Δ*vps* double mutant (PW329).
B. Biofilm accumulation by wild-type *V. cholerae* (MO10), a Δ*vps* mutant (PW328), a Δ*cytR* mutant (PW324) and a Δ*cytR*Δ*vps* double mutant (pw329). Biofilms stained with crystal violet are shown above, and quantification of crystal violet staining is shown below.

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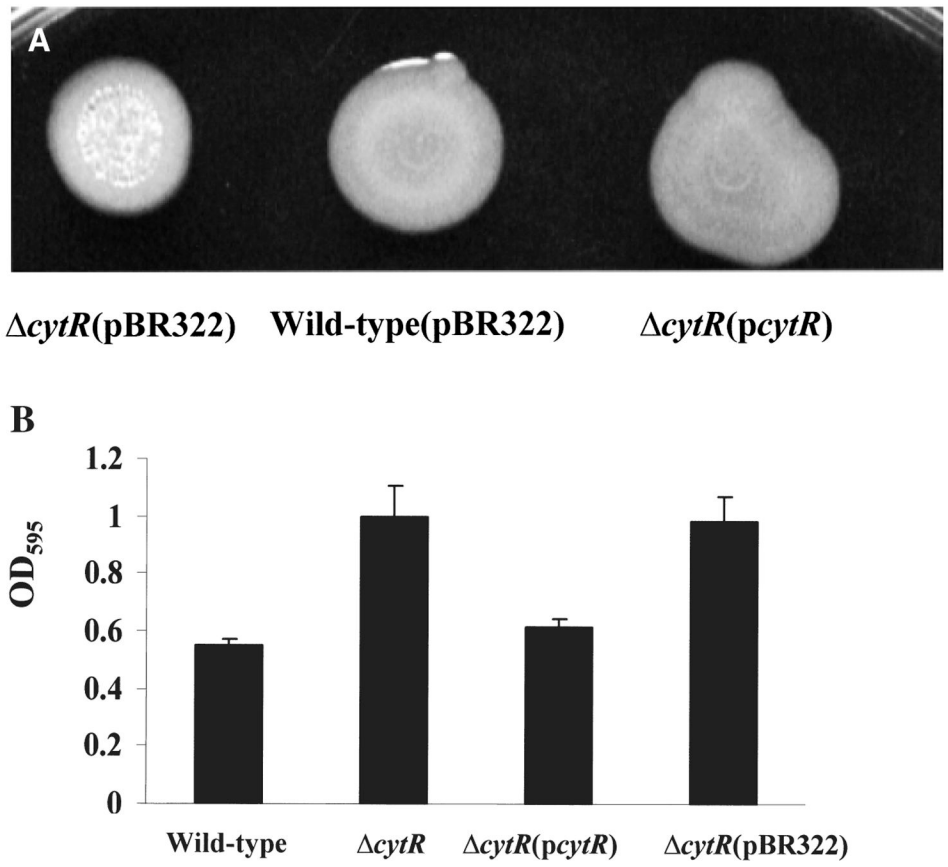

Figure 3.

Complementation of the rugose colony morphology and increased biofilm accumulation phenotype of a *V. cholerae* Δ*cytR* mutant by the *V. cholerae cytR* gene provided *in trans*.

A. Colony morphology of the *V. cholerae* Δ*cytR* mutant (PW324) transformed with the cloning vector pBR322, wild-type *V. cholerae* transformed with the cloning vector pBR322 and a *V. cholerae* Δ*cytR* mutant (PW324) transformed with the pBR322-based plasmid pAJH3, denoted p*cytR*, carrying the promoter and coding sequence of *V. cholerae cytR*.

B. Quantification of biofilm accumulation by wild-type *V. cholerae* (MO10), a *V. cholerae* Δ*cytR* mutant (PW324), a *V. cholerae* Δ*cytR* mutant transformed with the plasmid pAJH3, denoted p*cytR*, and a *V. cholerae* Δ*cytR* mutant transformed with the cloning vector pBR322.

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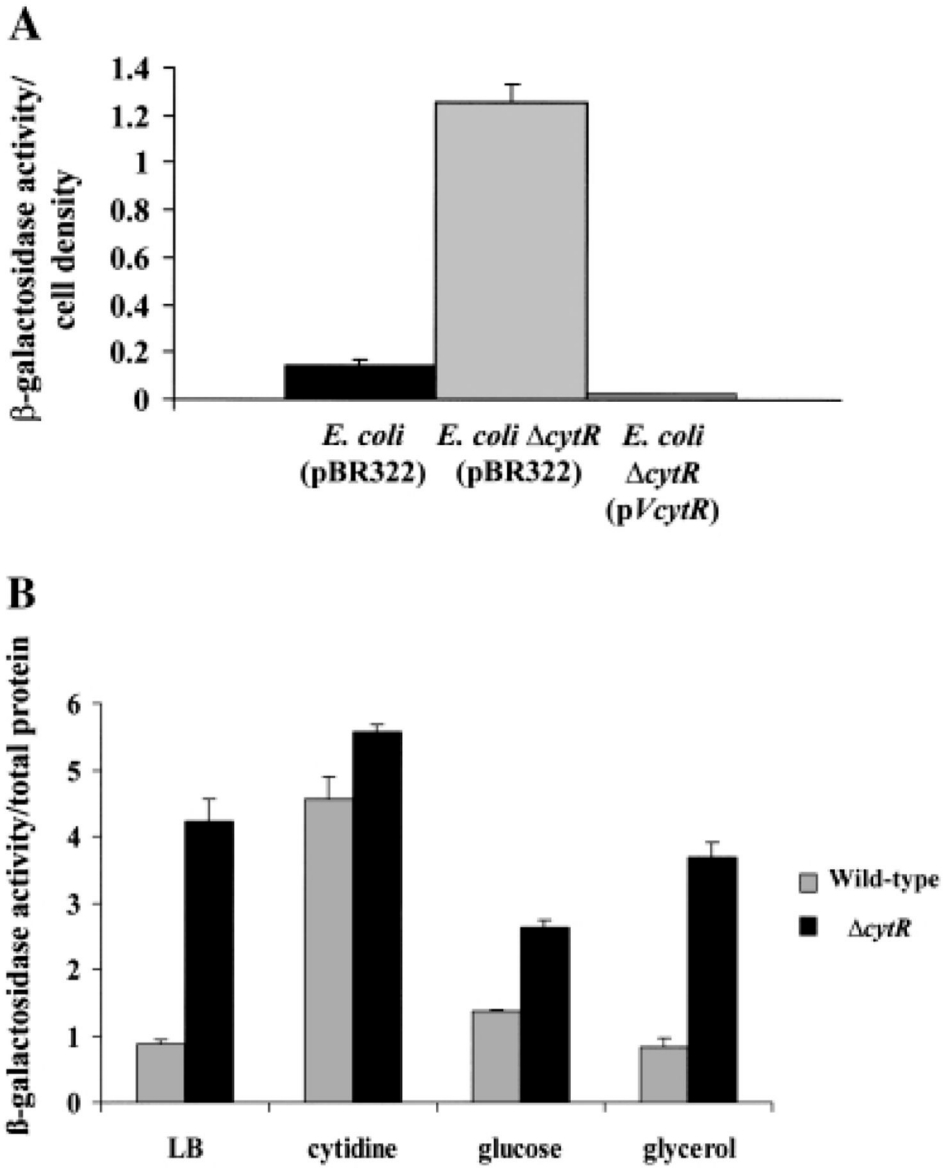

Figure 4.

Functional similarity of *E. coli* CytR and *V. cholerae* CytR.

A. Normalized β-galactosidase activity of an *E. coli* strain containing a chromosomal fusion of *lacZ* to the *E. coli udp* promoter (SS6005) transformed with pBR322, an *E. coli* Δ*cytR* mutant (SS6018) transformed with pBR322 and the same *E. coli* Δ*cytR* mutant transformed with the plasmid pAJH3, denoted p*VcytR*, carrying the promoter and coding sequence of *V. cholerae cytR*.

B. Normalized β-galactosidase activity of wild-type *V. cholerae* (PW387) and *V. cholerae* Δ*cytR* mutant (PW386) cells containing a chromosomal fusion of *lacZ* to the *V. cholerae udp* promoter grown in LB broth, LB broth supplemented with 0.3% cytidine, LB broth supplemented with 0.3% glucose and LB broth supplemented with 0.3% glycerol.

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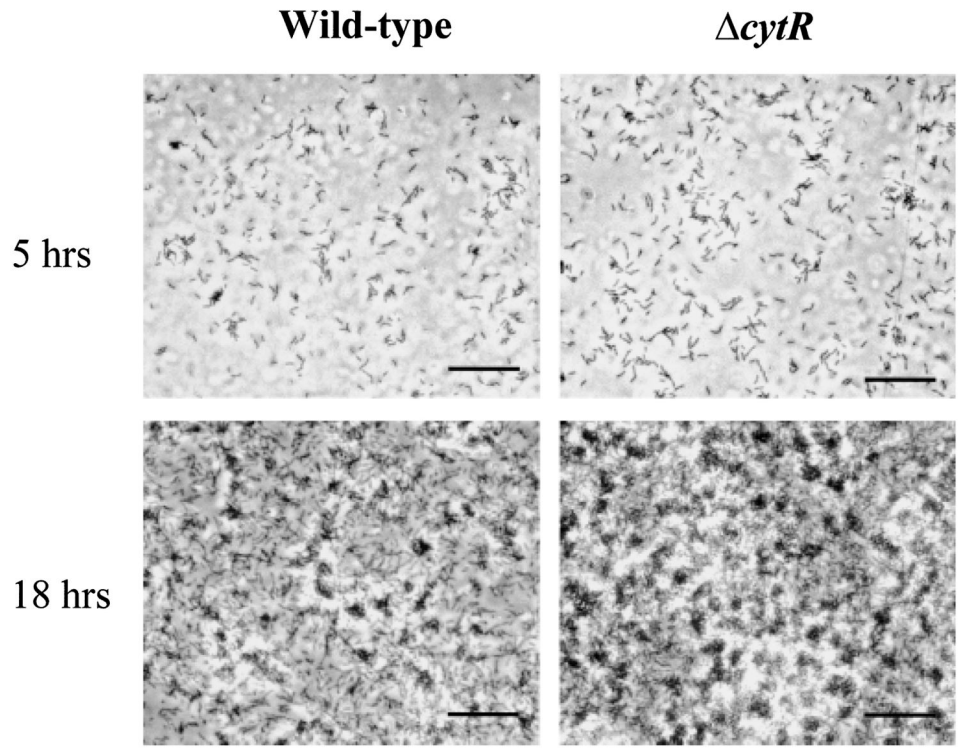

Figure 5.
Phase-contrast micrographs comparing batch biofilm development in LB broth by wild-type *V. cholerae* (MO10) and a *V. cholerae* Δ*cytR* mutant (PW324) after 5 h and 18 h of exposure to a polystyrene surface. Bar = 10 μm.

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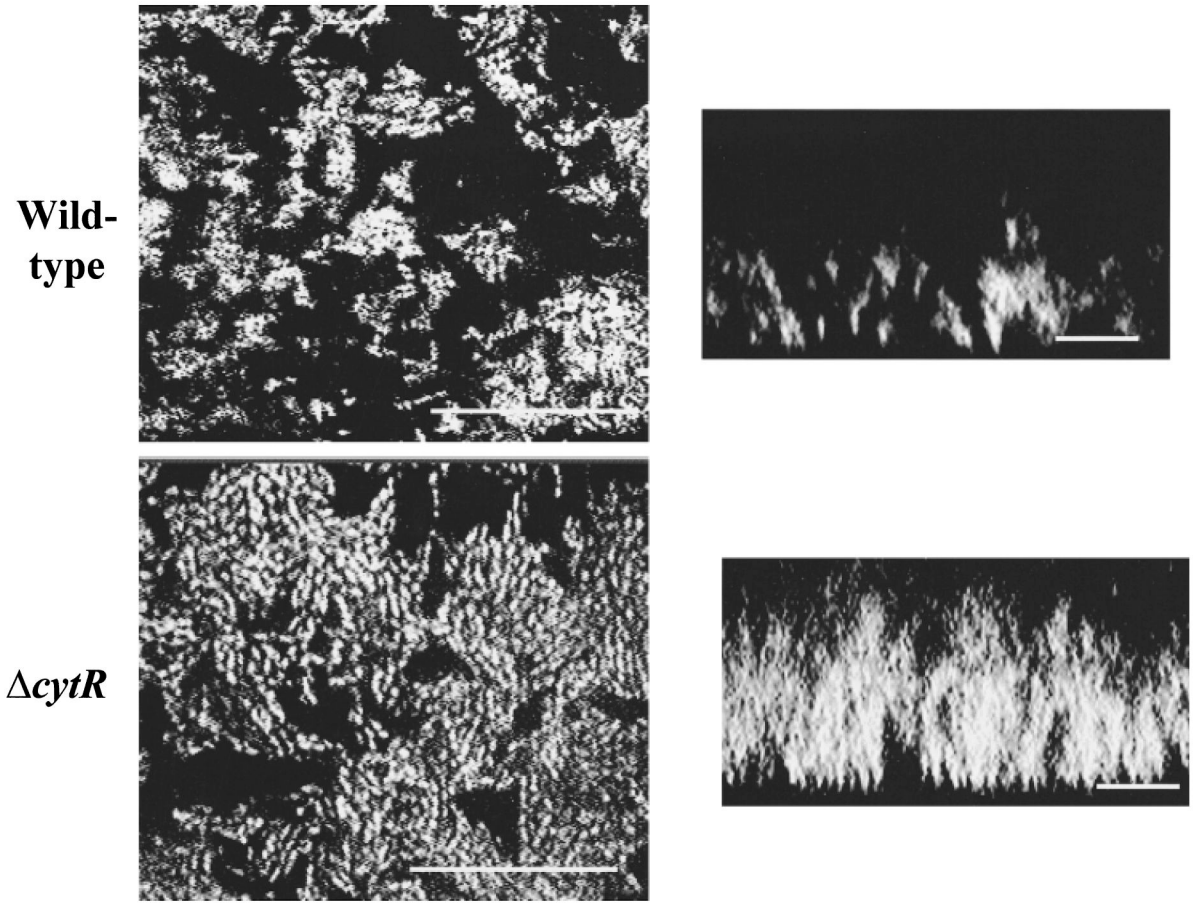

Figure 6.
Confocal micrographs of 2-day-old wild-type *V. cholerae* (MO10) and *V. cholerae* Δ*cytR* mutant (PW324) biofilms. XY-sections through the biofilms are shown on the left (bar = 25 μm), and vertical sections through the biofilms are shown on the right (bar = 10 μm).

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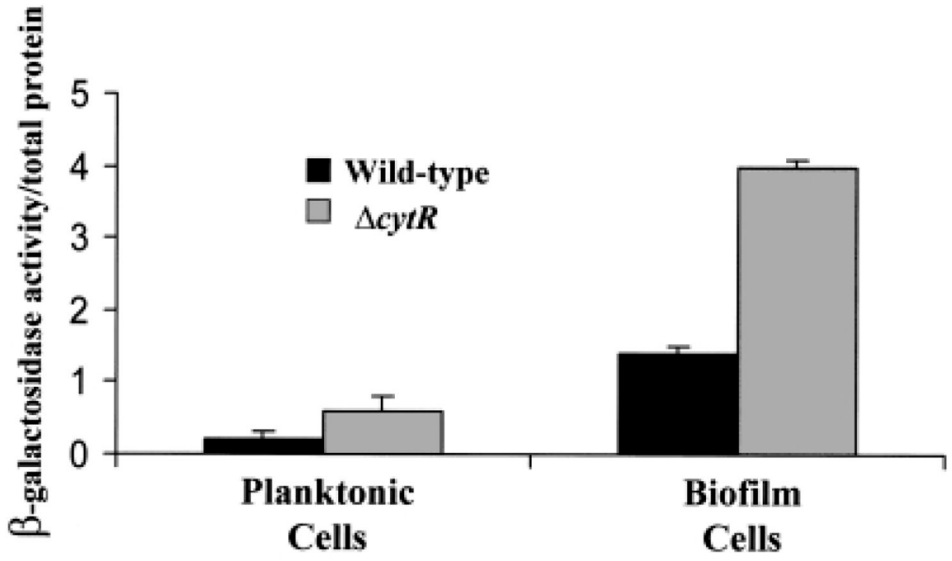

Figure 7.
Normalized β-galactosidase activity of biofilm-associated and planktonic wild-type *V. cholerae* (PW357) and *V. cholerae* Δ*cytR* mutant (PW358) cells carrying a chromosomal fusion of the *vpsL* promoter to *lacZ*. Black bars represent β-galactosidase measurements of wild-type *V. cholerae*, and grey bars represent β-galactosidase measurements of *V. cholerae* Δ*cytR* mutants.

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Table 1

Bacterial strains and plasmids.

Strains or plasmids	Genotype	Reference/source
E. coli		
β2155	*ThrB1004 pro thi strA hsdS lacZΔM15* (F' *lacZΔM15 lacI*^{q} *trajD36 proA*$^{+}$ *proB*$^{+}$) *ΔdapA*::*erm pir*.:RP4, Erm^{r}, Km^{r}	Dehio and Meyer (1997)
SM10λpir	*Thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pirR6K*; Km^{r}	Miller and Mekalanos (1988)
SS6005	F^{-} *thi leu rpsL* Δ(*argF-lac*)*U169 Φ*(*udp-lac*)*8* (λRS45)	Barbier and Short (1992)
SS6018	SS6005, *cytR*::Tn*10*dTet	Barbier and Short (1992)
V. cholerae		
MO10	1992 clinical isolate of *V. cholerae* O139 from India, Sm^{r}	Waldor and Mekalanos (1994)
PW324	MO10Δ*cytR*, Sm^{r}	This study
PW328	MO10Δ*vps*, Sm^{r}	This study
PW329	MO10Δ*cytR*Δ*vps*, Sm^{r}	This study
PW357	MO10*lacZ*::*vpsLp*→*lacZ*, Sm^{r}	This study
PW358	PW324*lacZ*::*vpsLp*→*lacZ*, Sm^{r}	This study
PW386	PW324*lacZ*::*udp1p*→*lacZ*, Sm^{r}	This study
PW387	MO10*lacZ*::*udp1p*→*lacZ*, Sm^{r}	This study
Plasmids		
pBSL180	*OriR6K mobRP4 lacI pTac tnp* mini-Tn*10Km;* Km^{r}, Ap^{r}	Alexeyev and Shokolenko (1995)
pWM91	*OriR6K OriT sacB lacZα*, Ap^{r}	Metcalf *et al.* (1996)
pAJH1	pWM91 carrying a fragment of *cytR* harbouring an internal, unmarked deletion	This study
pAJH5	pWM91 carrying a fragment of a *vps* operon harbouring an internal, unmarked deletion	This study
pAJH3	A pBR322 derivative containing the promoter and coding sequence of *cytR*; Tc^{r} Ap^{r}	This study
p6891MCS	pBR327 with 8 kbp *Sau*3A fragment of *V. cholerae lacZ* gene interrupted by a multiple cloning site	Butterton *et al.* (1995)
PAJH8	p6891MCS derivative with *udp1p*→*lacZ* inserted in the multiple cloning site	This study
pAJH2	p6891MCS derivative with *vpsLp*→*lacZ* inserted in the multiple cloning site	This study
pSMC2	pKEN carrying a bright mutant of *gfp* and 1.8 kb stabilizing fragment from pUC181.8; Ap^{r}	Bloemberg *et al.* (1997)

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Table 2

Primers used for PCR.

Primer	Location in chromosome I	Sequence
Construction of *V. cholerae vps* deletion		
P62	998815–998834	GGCTGGTCTATGTGG CTTGT
P63	999215–999195	TTACGAGCGGCCGCATCCCGAAGAATAATTGGAATG
P64	1005215–1005235	TGCGGCCGCTCGTAATGGTTGGTGATTGAACATCTG
P65	1005561–1005542	CAACGTCAGCAACATGGCTA
Construction of *V. cholerae cytR* deletion		
P38	2843448–2843465	CGGATATGGCTGCTGATG
P39	2844012–2843994	TTACGAGCGGCCGCACTTGAAAGGTTTGAACTCG
P40	2845064–2845081	TGCGGCCGCTCGTAACTCTATCGAGTGATGTTG
P41	2845571–2845554	GCTGCAATCCTTGGTTTG
Cloning of *V. cholerae cytR*		
P55	2845374–2845354	ATTGATCCGCAAGATTTGAGC
P56	2843961–2843978	TCTCAGCGCTGCGACAGG
Construction of *V. cholerae vpsLp→lacZ*		
P75	998382–998403	TAGTTCAATCGCGGCTTGTACCACA
P76	999402–999382	TAGACCCCTAGCAAGGCAACCGAA
Construction of *V. cholerae udp1p→lacZ*		
P95	1099912–1099931	TAGCGTCTGCTTAAA TTCCGCTC
P96	1100416–1100435	TAGCACCAAGGTGGA ATACGGTT