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The *Vibrio cholerae* VctPDGC System Transports Catechol Siderophores and a Siderophore-Free Iron Ligand

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Abstract

Vibrio cholerae, the causative agent of cholera, has an absolute requirement for iron. It transports the catechol siderophores vibriobactin, which it synthesizes and secretes, and enterobactin. These siderophores are transported across the inner membrane by one of two periplasmic binding protein-dependent ABC transporters, VctPDGC or ViuPDGC. We show here that one of these inner membrane transport systems, VctPDGC, also promotes iron acquisition in the absence of siderophores. Plasmids carrying the *vctPDGC* genes stimulated growth in both rich and minimal media of a *Shigella flexneri* mutant that produces no siderophores. *vctPDGC* also stimulated the growth of an *E. coli* enterobactin biosynthetic mutant in low iron medium, and this effect did not require *feoB*, *tonB* or *aroB*. A tyrosine to phenylalanine substitution in the periplasmic binding protein VctP did not alter enterobactin transport, but eliminated growth stimulation in the absence of a siderophore. These data suggest that the VctPDGC system has the capacity to transport both catechol siderophores and a siderophore-free iron ligand. We also show that VctPDGC is the previously unidentified siderophore-independent iron transporter in *V. cholerae*, and this appears to complete the list of iron transport systems in *V. cholerae*.

Iron is an essential element for nearly all forms of life, but its bioavailability is limited by its insolubility at neutral pH in the presence of oxygen. Pathogenic bacteria face additional challenges, since the mammalian host sequesters iron within cells or bound to proteins such as transferrin or lactoferrin. A mechanism for iron acquisition used by a wide variety of microorganisms is the synthesis and export of siderophores, small molecules that bind iron in the external environment with extremely high affinity. Ferri-siderophores are transported back into the cell via ligand-specific receptors, and iron is removed from the siderophore in the cytoplasm. In gram negative bacteria, the energy required for transport through the outer membrane receptor is transduced from the inner membrane by the TonB-ExbBD complex (Postle & Larsen, 2007). Siderophores are then transported across the inner membrane by a periplasmic binding protein-dependent ATP binding cassette (ABC) transport system (Braun *et al.*, 1998). Bacteria also have systems that transport iron independently of siderophores, including those for the transport of heme, of iron bound to host proteins such as transferrin and lactoferrin, and of free ferric and ferrous iron.

Vibrio cholerae, the causative agent of cholera, has an absolute requirement for iron and must obtain this element both within the human host and in its various environmental niches. A long term goal of our lab has been to identify the iron sources available to *V. cholerae* in each of these environments and to characterize the genes used to transport these iron sources. Multiple systems for the transport of iron have been identified in *V. cholerae* (see (Wyckoff *et al.*, 2007) for a review).

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V. cholerae synthesizes and exports the catechol siderophore vibriobactin (Griffiths *et al.*, 1984, Wyckoff *et al.*, 2007). Ferri-vibriobactin is transported across the outer membrane by its receptor ViuA (Butterton *et al.*, 1992) and across the inner membrane by one of two periplasmic binding protein-dependent ABC transporters, ViuPDGC or VctPDGC (Wyckoff *et al.*, 1999, Mey *et al.*, 2002). These ABC transporters consist of a monomeric periplasmic binding protein which delivers the ligand to its cognate permease complex in the inner membrane. These complexes consist of two distinct integral membrane permease proteins and two identical ATPase proteins located on the cytoplasmic face of the complex, which provide energy for transport through the simultaneous hydrolysis of ATP.

V. cholerae also transports siderophores that it does not synthesize, termed xenosiderophores, including the catechol siderophore enterobactin (Mey *et al.*, 2002, Griffiths *et al.*, 1984) and the hydroxamate ferrichrome (Rogers *et al.*, 2000). There are also iron transport systems that function independently of siderophores. *V. cholerae* uses heme as a source of iron (Stoebner & Payne, 1988) and has three heme receptors (Henderson & Payne, 1994a, Mey & Payne, 2001). The ferric iron transporter FbpABC is a periplasmic binding protein-dependent ABC transporter (Wyckoff *et al.*, 2006), while FeoABC transports ferrous iron (Wyckoff *et al.*, 2006).

In previous work, a *V. cholerae* mutant defective in the synthesis of vibriobactin, as well as in the Fbp and Feo systems was constructed. This *vibB*, *fbpA*, *feoB* mutant had increased sensitivity to iron chelators, but still formed colonies on LB agar (Wyckoff *et al.*, 2006). The heme and xenosiderophore transport systems were intact in this strain, but it is expected that the quantity of their ligands in LB agar is insufficient to support growth. Thus we reasoned that growth in LB medium must be due to the presence of at least one unidentified iron transport system in this mutant strain. No candidate genes for this iron transporter were found in analysis of the *V. cholerae* genome sequence (Heidelberg *et al.*, 2000), as no homologues of *sitABCD* (Kehres *et al.*, 2002, Zhou *et al.*, 1999), *mntH* (Makui *et al.*, 2000), *corA* (Hantke, 1997), *zupT* (Grass *et al.*, 2005), or *efeU* (Grosses *et al.*, 2006) are present.

In this work we show that iron is transported in the *vibB*, *fbpA*, *feoB* mutant strain by the VctPDGC system, which was previously identified as a periplasmic binding protein-dependent ABC transporter of catechol siderophores. We show that the VctPDGC system transports two discrete classes of ligands, catechol siderophores and an iron ligand that is siderophore independent.

RESULTS

Identification of a *V. cholerae* system that stimulates growth in low iron

A genetic screen was used to identify active iron transport systems in the *V. cholerae* *vibB*, *fbpA*, *feoB* mutant strain CFV1. In this screen, a library constructed using CFV1 genomic DNA was transferred to *Shigella flexneri* strain SM193w (Runyen-Janecky *et al.*, 2003, Wyckoff *et al.*, 2006), which is highly defective in iron transport. All of the clones that stimulated growth of SM193w contained a region of the *V. cholerae* small chromosome that included the *vctPDGC* operon. VctPDGC was previously characterized as a periplasmic binding protein-dependent ABC transporter for transit of the catechol siderophores vibriobactin and enterobactin across the inner membrane (Mey *et al.*, 2002). A clone from the lab collection containing only the *vctPDGC* region, pCAT120 (Mey *et al.*, 2002), was introduced into SM193w, and it also supported growth of SM193w on unsupplemented LB agar (Fig. 1), confirming that sequences from this region were sufficient to stimulate growth of SM193w.

The *vctPDGC* genes are located in an apparent operon on *V. cholerae* chromosome 2 (Heidelberg et al., 2000, Mey et al., 2002) (Fig. 2). The proteins have strong sequence similarity with other periplasmic binding protein-dependent ABC transporters with siderophore ligands, and from these data it is predicted that VctP is the periplasmic binding protein, VctD and VctG are in integral inner membrane permease proteins and VctC is the ATPase. Downstream of the operon is VCA0231, an uncharacterized ORF with similarity to AraC type transcriptional regulatory proteins, and VctA, a TonB-dependent outer membrane receptor for enterobactin (Mey et al., 2002).

Although pCat120 does not have any intact ORFs other than *vctPDGC*, it does contain several hundred nucleotides on each side of the operon (Fig. 2), and it was important to determine whether the *vctPDGC* operon was responsible for stimulating the growth of SM193w. Additional clones were constructed to determine which sequences on the plasmid are needed for growth stimulation. pCat125 was constructed using a PCR primer immediately upstream of the putative *vctPDGC* operon promoter. This plasmid stimulated growth of SM193w, indicating that sequences upstream of the putative *vct* promoter are not required (Fig. 2). In contrast, pCat123, which has a truncation that deletes most of *vctC*, did not stimulate growth of SM193w. pCat137 has an in-frame deletion that removes most of *vctP* and did not support growth of SM193w, but growth stimulation was restored when *vctP* was supplied in trans on a compatible plasmid (Fig. 2). Although these data do not directly demonstrate a role for VctD and VctG, we believe that taken together, they provide strong evidence that the *vctPDGC* ABC transporter genes are necessary and sufficient for promoting growth of SM193w on unsupplemented LB agar.

***vctPDGC* complements an *E. coli* *fepB* mutation**—VctPDGC was originally described as one of two largely redundant ABC transport systems in *V. cholerae* that transported either of the catechol siderophores vibriobactin or enterobactin across the inner membrane. In this work, the role of VctPDGC as a siderophore transporter was further characterized by transforming the *vctPDGC* genes into an *E. coli* strain carrying a mutation in *fepB*, which encodes the periplasmic binding protein component of the ABC transporter for enterobactin (Elkins & Earhart, 1989). The *vctPDGC* genes complemented the *fepB* mutation (Table 1), providing additional evidence that VctPDGC transports enterobactin. As expected, the *E. coli* *fepBDGC* genes complemented the *fepB* mutation. The zone of growth surrounding the enterobactin source when the *fepB* mutant carried the *vctPDGC* genes was similar in size to that observed when the mutant carried *E. coli* *fepBDGC* (Table 1). The plasmid pCat133, carrying only *vctP* did not complement the *fepB* mutation, indicating that Vct system periplasmic binding protein was not sufficient, and that it most likely lacks the ability to deliver the enterobactin ligand to the FepDGC permease in the inner membrane (data not shown). The hydroxamate siderophore ferrichrome, which is transported across the inner membrane independently of the FepBDGC system, stimulated growth of all strains in this study (Table 1).

It is of note that genes for the *V. cholerae* ferric iron transporter FbpABC did not complement the *fepB* mutation (Table 1), and the *fepB* mutant used in this study has an intact operon for the ferrous iron transporter Feo. Thus under the conditions used in this assay, the level of free iron in the periplasm is insufficient to allow either a ferric or a ferrous iron transporter to stimulate growth.

VctPDGC does not require a siderophore to stimulate the growth SM193w—It is not obvious how a siderophore transport system would stimulate growth of *S. flexneri* strain SM193w on LB agar, since SM193w is not expected produce a functional siderophore. There is a Tn5 insertion within the *iucD* gene for the biosynthesis of the hydroxamate siderophore aerobactin (Daskaleros & Payne, 1987, Runyen-Janecky et al.,

2003), and, as with most *S. flexneri* strains, an enterobactin locus is present, but multiple deletions and point mutations prevent enterobactin production (Schmitt & Payne, 1991, Wei *et al.*, 2003). SM193w uses enterobactin as a source of iron, but with reduced efficiency compared with *E. coli*. Although *fec* system genes for the transport of ferric citrate have been reported for at least one *S. flexneri* strain (Luck *et al.*, 2001), *fec* genes are not present in other characterized *S. flexneri* strains (Payne & Mey, 2010), including SM193w (data not shown).

To test whether an uncharacterized iron binding compound was secreted by the *S. flexneri* strains used in this study, cultures were spotted onto Chrome Azurol S (CAS) agar (Schwyn & Neilands, 1987). While an orange halo, indicative of siderophore secretion, formed around the wildtype strain, no halo formed around the aerobactin biosynthetic (*iucD*) mutant (Fig. 3). For completeness, SM193w was also tested, and no halo was formed. These data confirm that aerobactin is the only high affinity iron-binding compound secreted by these strains, which are all derived from *S. flexneri* strain SA100 (Payne *et al.*, 1983). The possibility remained, however, that SM193w might secrete another, lower-affinity iron-binding compound, and the VctPDGC system might allow this “cryptic siderophore” to be transported more efficiently into the cell. To test for this, either wildtype *S. flexneri* or a strain carrying the *iucD* mutation, were spotted onto agar seeded with SM193w with or without the *vctPDGC* genes. As expected, the wildtype cells secreted aerobactin which stimulated the growth of both *S. flexneri* strains. In contrast, the strain defective in aerobactin synthesis did not promote the growth of either strain (data not shown). Thus if a siderophore-like compound, other than aerobactin, is secreted by *S. flexneri*, we were unable to detect it in these assays.

If VctPDGC increases the efficiency of transport of a catechol siderophore-like ligand, then other catechol siderophore transporters might also stimulate growth of SM193w. As shown in Figure 4, neither genes for the *E. coli* ABC transporter for enterobactin (*fepBDGC*) nor genes for the second *V. cholerae* ABC transporter of vibriobactin and enterobactin (*viuPDGC*) stimulated growth of SM193w on unsupplemented LB medium. This is additional evidence that a catechol siderophore is not available during growth of SM193w on LB agar. Further this indicates that the VctPDGC system is unique, in that its ligand specificity is different from that of the FepBDGC and ViuPDGC systems. Genes for the ferric iron transporter FbpABC and the ferrous iron transporter FeoABC were included as positive controls (Fig. 4).

Another possibility is that the complex medium LB may contain a siderophore-like compound that is transported more efficiently in the presence of the VctPDGC system. To investigate this, SM193w carrying genes for the indicated iron transport systems were spread at low cell density on either minimal T medium agar or LB agar. After 24 hours, the diameter of ten, well separated colonies was measured (Fig. 5). The VctPDGC system stimulated growth of SM193w on minimal agar, whereas the strain carrying only the plasmid vector failed to form colonies on this medium. For purposes of comparison, the colony sizes for SM193w carrying genes for either the ferric transporter FbpABC or the ferrous transporter FeoABC were determined in the same assay. As seen in Figure 5, the colonies formed when the *vctPDGC* genes were present were nearly identical in size to those formed when the *feoABC* genes were supplied and somewhat smaller than those formed by cells carrying *fbpABC*. These data show that none of the complex components of LB medium are required for VctPDGC to transport its ligand. All four strains used in this experiment formed larger colonies on LB than on minimal agar, but the pattern is otherwise the same as that seen on minimal agar (Fig. 5). This also quantifies the growth stimulation of SM193w by VctPDGC, which is shown qualitatively in Figures 1 and 4.

VctPDGC stimulates growth of *E. coli* iron transport mutants in the absence of *feoB*, *tonB* and *aroB*—The experiments described above were all performed with the *S. flexneri* strain SM193w. To determine whether siderophore-independent iron acquisition by VctPDGC was specific to *S. flexneri*, we tested its ability to stimulate growth of *E. coli* mutants defective in the synthesis of the endogenous siderophore enterobactin. The first mutant tested was an *entF*, *feoB* mutant, and, as shown in Figure 6A, the *vctPDGC* genes stimulated growth of the mutant in a colony size assay. The size of the colonies formed when the strain carried *vctPDGC* was similar to that of cells carrying genes for the known iron transport systems Fbp and the Feo, which were included for comparison. All strains grew similarly when excess iron was added to the medium. These data show that iron-acquisition by the VctPDGC system is not limited to *S. flexneri*, but also functions in *E. coli* K12. VctPDGC activity in this assay required neither Feo function nor the synthesis of enterobactin.

In gram negative bacteria, the TonB-ExbBD complex transduces energy from the inner membrane to receptors on the cell surface that transport ferri-siderophores and other large iron complexes across the outer membrane. Consistent with this, the *E. coli* enterobactin receptor FepA requires the activity of the TonB-ExbBD complex to transport enterobactin into the periplasm (see (Moeck & Letellier, 2001, Noinaj *et al.*, 2010) for reviews). The *vctPDGC* genes were tested for their ability to stimulate growth on an *E. coli tonB* mutant in low iron. As seen in Figure 6B, the *vctPDGC* genes stimulated growth of the *E. coli tonB*, *entF* mutant. Thus VctPDGC can transport a ligand that can cross the outer membrane independently of TonB, most likely in a form that is small enough to pass through a porin.

In ferri-enterobactin, the iron is bound within the complex by interactions with the catechol moieties of the siderophore. Although we have presented evidence that catechol siderophores are not required for growth stimulation by VctPDGC in these assays, it was possible that VctPDGC transports iron in a complex with a catechol-related molecule, perhaps derived from a precursor in catechol biosynthesis. Catechols are synthesized from chorismate, a product of the shikimic acid biosynthetic pathway. AroB (3-dehydroquinate synthase) catalyzes one of the reactions in the pathway in which shikimate is synthesized from erythrose-4-phosphate. The VctPDGC system stimulated growth of the *aroB* mutant in low iron (Fig. 6C), indicating that no shikimic acid pathway intermediates synthesized by the cell are required for its function in this assay.

Activity in the *fepB* complementation and in the SM193w growth stimulation assays can be separated genetically—We have seen that the VctPDGC system has activity in assays for the transport of catechol siderophores and also in assays for iron acquisition in the absence of siderophores. In principle, it could do this either by having a single ligand that allows iron acquisition under both assay conditions or by having two different ligands. If there are two different ligands, it may be possible to isolate point mutations that transport one ligand, but not the other. To identify amino acid residues that may contact one of the ligands, we aligned the amino acid sequence of the putative periplasmic binding protein VctP with the sequence of two related periplasmic binding proteins for which crystal structures are available. These are the enterobactin transporter CeuE of *Campylobacter jejuni* (Richardson & Park, 1995, Muller *et al.*, 2006) and the petrobactin transport protein YclQ of *Bacillus subtilis* (Kunst *et al.*, 1997, Zawadzka *et al.*, 2009) (Fig 7). In ligand binding studies, purified *B. subtilis* YclQ bound its siderophore petrobactin, but it also bound free ferric iron (Zawadzka *et al.*, 2009). The authors speculate that tyrosine-275 may be involved in ferric iron binding. This tyrosine is conserved in VctP (tyrosine 310, numbering from the annotated start of the *vctP* open reading frame), and this tyrosine was changed to phenylalanine (Y310F) using site-directed mutagenesis. A plasmid containing *vctPDGC*(P-Y310F) complemented the *E. coli fepB* mutation (Table 1),

indicating that the VctP protein carrying this mutation is properly folded and functional in the transport of enterobactin. However, the *vctPDGC*(P-Y310F) plasmid did not stimulate growth of SM193w on LB agar (Fig. 4), showing that tyrosine-310 is required to transport for siderophore-independent iron transport. This genetic separation of VctP activity in the *fepB* complementation assay and SM193w growth stimulation of assays is strong evidence that different ligands are transported in the two assays.

The CeuE protein was crystallized with the enterobactin mimic ferric mecam, and three arginine residues were found to interact with the oxygen atoms on the three catecholate moieties of the mecam (Muller et al., 2006). These arginines are conserved in both YclQ (Zawadzka et al., 2009) and in VctP (shaded in Fig. 6). Site-directed mutagenesis was used to change the most N-terminally located of these arginines to glutamine (R137Q). The cloned *vctPDGC*(P-R137Q) operon failed to transport enterobactin in the *fepB* complementation assay (Table 1) or to stimulate growth on SM193w on unsupplemented LB agar (Fig. 4). This suggests that this arginine is required for binding of both catechol and non-siderophore bound iron; however, it is also possible that this mutations makes the protein less stable.

VctPDGC is the functional iron transporter in the *V. cholerae vib, feo, fbp* mutant—As previously reported, a *V. cholerae* triple mutant defective in the synthesis of vibriobactin as well as in the Fbp and Feo systems grows on LB agar, albeit the colonies were smaller than those formed by the wildtype strain. This indicated that a reasonably efficient iron transport system is functional in this mutant. To test whether VctPDGC is the system that allows the *V. cholerae vib, feo, fbp* mutant strain CFV1 to grow on LB medium, a deletion within the *vct* locus was constructed in CFV1. To ensure growth of mutant strain candidates, allelic exchange was performed in the presence of either enterobactin or 20 μ M hemin. The resulting *vib, feo, fbp, vct* mutant strain EWV133 was tested for growth on LB agar that was not supplemented with an exogenous iron source (Fig. 8). EWE133 did not form colonies on LB agar, but the ability to grow was restored when the *vctPDGC* genes were supplied on a plasmid. This supports the conclusion that VctPDGC is the system responsible for the residual growth of strain CFV1, and it is likely to be the last remaining major uncharacterized iron transport system in *V. cholerae*.

Discussion

The VctPDGC system has characteristics of two distinct types of iron transport systems, those for the transport of catechol siderophores and those for transport of a siderophore-free iron ligand (Table 2). Evidence that the system transports catechol siderophores includes the observation that plasmids carrying this operon complemented an *E. coli fepB* mutation for the utilization of enterobactin (Table 2) as well as a *V. cholerae vctPD, viuP* double mutant for the transport of vibriobactin and enterobactin (Mey et al., 2002). Neither the cloned *fbpABC* genes nor the chromosomal *feoABC* operon stimulated growth of the *fepB* mutation. The failure of these transport systems to stimulate growth of the *fepB* mutation around an enterobactin source indicates that the *fepB* mutation is not suppressed by free ferric or ferrous iron transporters in this assay. Bioinformatics also supports the model that VctP transports a siderophore ligand. The VctPDGC proteins are most similar to ABC transporters for siderophores (Mey et al., 2002). Further, when the VctP amino acid sequence is aligned with that of the catechol transporters CeuE and YclQ, the arginine residues that contact the catechol in the CeuE crystal structure are conserved in VctP (Fig. 7).

However, unlike the FepBDGC and ViuPDGC siderophore transporters tested, the VctPDGC system also stimulated growth of *S. flexneri* and *E. coli* iron transport mutants

under conditions where siderophores are not present (Table 3). *S. flexneri* strain SM193w containing the *vctPDGC* genes did not secrete any detectable compounds with siderophore-like activity, and it could acquire iron during growth on minimal medium (Fig. 5). This suggests that if iron crosses the outer membrane as a complex, it must be bound to one of the ions present in the minimal medium or to a non-siderophore molecule secreted by the cell. This is consistent with the observation that VctPDGC stimulated iron acquisition in an *E. coli tonB* mutant (Fig. 6B), where its ligand would most likely need to cross the outer membrane in a form that is small enough to pass through a porin.

Based on these observations, we believe it is most likely that the VctPDGC system transports two discrete ligands. Purified YclQ bound both its siderophore ligand petrobactin and also free ferric iron (Zawadzka et al., 2009), and VctP may have similar binding properties. The observation that the tyrosine-310 to phenylalanine mutation prevented growth stimulation of SM193w without affecting its ability to transport enterobactin in the *fepB* complementation assay is consistent with the ferri-siderophore and the siderophore-free iron ligands being bound somewhat differently within the VctP ligand binding pocket. Interestingly, YclQ only bound free ferric iron when petrobactin was present in the assay. In contrast, our data indicate that siderophores are not required for iron transport by the Vct system. However, the YclQ binding studies were performed with highly purified protein, while VctP binds its ligand in the periplasm, a complex environment where an array of proteins, as well as ions and other small molecules, could facilitate iron solubility and/or binding to VctP. Although no siderophore is required for iron transport by the Vct system, our data do not exclude the possibility that iron is bound to VctP in a form that is complexed with an ion or other molecule that is present either in the medium or the periplasm. Since VctPDGC-mediated iron transport was observed in an *aroB* mutation, it is unlikely that any molecule promoting binding of iron to VctP, or binding VctP in a complex with the iron, is a catechol.

V. cholerae must acquire iron to grow both within the human small intestine and outside the host, where it is found primarily in aquatic environments, either in a free planktonic form or in association with organisms such as fish or crustaceans (Nelson et al., 2009, Reidl & Klose, 2002). *V. cholerae* has nearly 50 genes (greater than 1% of its genome) with an established role in iron acquisition. Some of these genes are for systems that transport siderophores or heme with high affinity. However, in some environments, such as moving water, these ligands may not be readily available, and free or loosely bound ferric or ferrous iron may be the primary source of iron. This variety of iron transport systems should allow optimal use of the iron sources available in any given environment. The ability of the VctPDGC system to transport two discrete ligands shows that a single system may possess unanticipated functional plasticity, potentially enhancing transport of catechol siderophores in environments where they are present while facilitating transport of siderophore-free iron when high affinity ligands are not available.

The concept that an organism may optimize iron acquisition by both possessing multiple iron transport systems and by optimizing ligand use by one or more of those systems may be more widely applicable than currently appreciated. This is similar to our models for *V. cholerae* VciB, which potentiates iron transport by ferrous iron transporters, including Feo (Mey et al., 2008). If VciB functions as a ferric reductase, as hypothesized, it would allow ferrous iron transporters to function more optimally under oxidized conditions where free ferric iron is the predominant iron source (Mey et al., 2008). It has been known for some time that periplasmic binding protein-dependent ABC transporters may have some flexibility in their ligand specificity, as exemplified by the ability of the *E. coli* FhuDCD system to transport several distinct hydroxamate siderophores across the inner membrane (Braun et al., 1998). Recently transporters with more dissimilar ligands have been

identified, including the *E. coli* Dpp permease, which transports both dipeptides and heme (Letoffe *et al.*, 2006), and the HmuUV permease and ATPase of *Sinorhizobium meliloti* transports both heme and hydroxamate siderophores (Cuiv *et al.*, 2008).

Genetic analysis has been used to elucidate which iron transport systems are needed for growth of *V. cholerae* in animal models and under various in vitro conditions, with the goal of learning both about the function of each system and about the availability of various ligands within those environments (Henderson & Payne, 1994b, Mey & Payne, 2001, Seliger *et al.*, 2001, Tashima *et al.*, 1996, Sigel *et al.*, 1985). The interpretation of these experiments has been complicated by the large number of iron transport genes in *V. cholerae* and by the partial functional redundancy of the various systems. This situation was made more difficult by the apparent presence of at least one unidentified iron transport system, since iron acquisition by any mutant strain could potentially be carried out by the uncharacterized system. We show here that the activity attributed to the unidentified system is performed by VctPDGC. Although the VctPDGC system was identified as an inner membrane transporter of catechol siderophores, its role in siderophore-independent iron transport was not recognized. With this understanding, we can construct an essentially complete list of the iron transport systems present in *V. cholerae*, and this is summarized in Fig. 9. Previous work (Wyckoff *et al.*, 2006), together with the data in Figure 8, show that under the conditions tested Vib, Fbp, Feo and Vct are the only systems that can transport sufficient quantities of iron to support growth on unsupplemented LB medium. *V. cholerae* also has nine ligand-specific TonB-dependent receptors, and in most cases their ligands, either vibriobactin, enterobactin, heme, ferrichrome or B12, have been determined (Wyckoff *et al.*, 2007). The *vib*, *fbp*, *feo*, *vct* mutant has intact transport systems for all of the ligands transported by TonB-dependent receptors yet failed to grow on unsupplemented LB agar (Fig. 8). This shows directly that the quantity of heme or siderophore ligands in unsupplemented LB is insufficient to support growth of *V. cholerae*. This will allow functional analysis of the role of individual transport systems either by supplementing LB medium with a single ligand or by constructing strains that have only one of the four systems that allow growth on unsupplemented LB intact.

Experimental procedures

Bacterial strains, plasmids, media, and iron utilization assays

The strains and plasmids used in this study are listed in Table 3. The iron chelator ethylenediamine di(*ortho*-hydroxyphenylacetic acid) (EDDA) was deferrated by the method of Rogers (Rogers, 1973). The antibiotic concentrations used were carbenicillin, 250 µg/ml; kanamycin, 50 µg/ml; and chloramphenicol, 50 µg/ml (*E. coli*) or 5 µg/ml (*V. cholerae*). For the growth of strain SM193w, the medium was supplemented with sterile culture supernatant of *S. flexneri* strain SA101 as a source of the siderophore aerobactin as previously described (Runyen-Janecky & Payne, 2002). The minimal medium was Tris buffered T medium (Simon & Tessman, 1963) supplemented with 0.4% w/v glucose, 2 µg per ml nicotinic acid and FeSO₄ at a final concentration of 20 µM.

Colony size assays were performed by spreading diluted cultures at low density on LB or T minimal (Simon & Tessman, 1963) agar supplemented with FeSO₄ or EDDA where indicated and ampicillin for strains carrying plasmids. Colony diameter was measured after 24 hr incubation at 37°C. Only well-isolated colonies were measured. Bioassays for siderophore utilization were performed as previously described (Wyckoff *et al.*, 1997). Chrome Azurol S (CAS) agar, on which siderophore secreting bacteria produce an orange halo over the blue background, was used to detect siderophore production by *S. flexneri* strains (Schwyn & Neilands, 1987).

Sequence analysis

DNA sequencing was performed by the University of Texas Institute for Cellular and Molecular Biology DNA Core Facility using an Applied Biosystems Prism 377 DNA sequencer (Perkin-Elmer Corp.). Amino acid sequence alignments were performed using the ClustalW feature of the MacVector package (Olson, 1994). The BLAST program (Altschul *et al.*, 1990) was used to search the National Center for Biotechnology Information (NCBI) protein database. Some DNA sequence was obtained from The Institute for Genomic Research (TIGR) website (<http://cmr.tigr.org/tigr-cripts/CMR/GenomePage.cgi?database=gvc>) (Heidelberg *et al.*, 2000).

Plasmid construction

pWKSmob was constructed by subcloning the *SacI* fragment containing the origin of transfer fragment *oriT* from pSS125 (Suh *et al.*, 2004) into the *SacI* site of pWKS30. To construct pWMB1, the *oriT* region of pWKSmob was PCR amplified with Pfx DNA polymerase (Invitrogen) using the primers mob.for (5'-GGGGCCAGCCTCGCAGAGCAGG) and mob.rev (5'-GGGGAAGCGCTTTTCCGCTGCAT). The resulting 254 base pair fragment was inserted into the *BsaAI* site of pWKS30. pCAT126 was constructed by inserting the *NotI* fragment of pCAT116 (Mey *et al.*, 2002) into the *NotI* site of pCVD442N. pCAT125 was constructed by amplifying *V. cholerae* strain Lou15 DNA with the primers vct.14945 (CTCACACAGTTGCATTGCCAAG) and Liz181 (5'-CGTTATCTCAGCACCAAGAGGGAC) using Pfx DNA polymerase. The resulting fragment was cloned into the *SmaI* site of pWKS30. To construct pCat133, the *vctP* gene was amplified using the primers vct.14542 (5'-GAATCATCTTAGCCTCCGAGCC) and vct.16085.rev (5'-TTGCCCACGCCAACAAACAG), Pfx Platinum DNA polymerase (Invitrogen) and Lou15 DNA template. The resulting fragment was cloning into the *ScaI* site of pACYC184 (Chang & Cohen, 1978). A plasmid containing the *vct* operon with an in-frame deletion of *vctP* was constructed by splice overlap PCR. One of the initial reactions used the primers Liz 180 (5'-GCCAAACCATTGCGGAAATAGAAG) and vct.15054.sol.rev (CGATGTACCAATGAATACGAGATTTTCATATG) with Pfx polymerase and Lou15 DNA as template, while the other reaction used the primers vct.155881.sol.mod (5'-CTCGTATTTCATTGGTACATCGCTATCTCTGGC) and Liz181 using KOD Hotstart polymerase (Novagen) and Lou15 DNA template. The resulting PCR fragments were gel purified and used as template in a PCR reaction with Liz180 and Liz 181 primers and KOD Hotstart polymerase. The resulting fragment was cloned into the *SmaI* site of pWKS30 to create the plasmid pCat137. Plasmids to replace VctP tyrosine 310 with phenylalanine were constructed by a modification of the Quikchange method (Stratagene). The plasmid pCat120 was used as template with the primers vctp.Y310F.for (5'-CTCGATGTGAATGCTTGGTTTATCGCTATCTCTGGCGTGA) and vctp.Y310F.rev (TCACGCCAGAGATAGCGATAAACCAAGCATTACATCGAG) using KOD polymerase. The resulting fragment was digested with *DpnI* and used to transform *E. coli* strain DH5 α , and a plasmid from that transformation was named pCat135. The R137Q mutation in *vctP* was constructed using the same Quikchange protocol, except that the primers used were Vctp.R137Q.for (5'-CGACTTTTGGTGCCAATTACAAATTCTCTTCGGTGTACAAAGAT) and Vctp.R137Q.rev (5'-ATCTTTGTACACCGAAGAGAATTGTGAATTGGCACCAAAAAGTCG). The inserts from the plasmids pCat133, pCat134, pCat135, pCat136 and pCat137 were completely sequenced and found to have the expected mutation or deletion and no other changes.

Library construction and screening

Genomic DNA was isolated from CFV1 using the method of Marmur (Marmur, 1961) and partially digested with *Sau3AI*. The resulting fragments were ligated with *Bam*HI-digested pWMB1, and *E. coli* strain XL10-Gold was transformed. The plasmid library was transferred to *S. flexneri* strain SM193w by triparental mating, and serial dilutions of the final mating mix were spread on LB agar plates containing ampicillin and chloramphenicol, but no iron chelator or supplemental source of iron. Plasmid DNA was isolated from the resulting colonies and introduced into SM193w to confirm that the growth phenotype was conferred by the plasmid. All plasmids with the confirmed phenotype obtained in this screen contained the *vctPDGC* region.

Mutant construction

The *V. cholerae* mutant strain EWV133 (O395, *vibB*Δ, *fbpA::cam*, *feoB::tmp*, *vctPD::kan*) was constructed by introducing the plasmid pCAT126 into CFV1 by triparental mating. Allelic exchange was performed as previously described (Mey & Payne, 2001).

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Fig. 1.

Clones containing the *vctPDGC* genes stimulate growth of *S. flexneri* iron transport mutant SM193w. SM193w carrying either the plasmid vector (pWKS30), a clone derived from screening a plasmid library of CFV1 DNA, or a defined clone containing the *vctPDGC* genes (pCat120) were spread on LB agar containing ampicillin. The plate was incubated overnight at 37°C.

Fig. 2.

Genetic map of the *vct* region of *V. cholerae* chromosome 2. The arrows indicate the direction of transcription, and the locus designation from the TIGR database (Heidelberg et al., 2000) is used for unnamed ORFs. The approximate regions cloned on the indicated plasmids is shown below the map. The + indicates that a clone stimulated growth of SM193w on unsupplemented LB agar.

Fig. 3. Secretion of siderophores by *S. flexneri* strains. 5 μ l of the indicated cultures was spotted onto the surface of CAS agar (Schwyn & Neilands, 1987), and formation of an orange halo around the spot indicated the secretion of a siderophore. Strains used were Wildtype (SA101), Iuc (SA341) and SM193w/pCat120.

Fig. 4. SM193w growth stimulation by iron transport genes. SM193w containing plasmids carrying the indicated genes were streaked on unsupplemented LB agar. The plasmids used were vector (pWKS30), *fbpABC* (pFbp1), *feoABC* (pfeo101), *vctPDGC* (pCat120), *viuPDGC* (pVib147), *fepBDGC* (pCP111), *vctPDGC vctP-Y310F* (pCat135) and *vctPDGC vctP-R137Q* (pCat134).

Fig. 5.

The VctPDGC system stimulates growth of SM193w on both rich and minimal agar. SM193w carrying a plasmid with no insert (pWKS30), *fbpABC* (pFbp1), *feoABC* (pFeo101) or *vctPDGC* (pCat120) was grown overnight in LB medium containing ampicillin and aerobactin. These were diluted to low density and spread on minimal T agar or LB agar. The diameter of ten, well separated colonies was measured after 24 hours at 37°C. The “Δ” indicates that no colonies were formed. A “*” indicates $P < 0.01$ in the Student’s t test for strains with a plasmid carrying genes for the indicated transport system compared with the same strain carrying the plasmid vector.

Fig. 6.

Growth stimulation of *E. coli* iron transport mutants by the VctPDGC system did not require *feo tonB* or *aroB*. *E. coli* K12 mutants with the indicated genotype were transformed with the plasmid vector (pWKS30), carrying *fbpABC* (pFbp1), *feoABC* (pFeo101) or *vctPDGC* (pCat120). Cultures were grown overnight in LB medium, diluted and spread at low density. The diameter of ten colonies on each plate was measured after 24 hours incubation at 37°C. A “*” indicates $P < 0.01$ in the Student’s *t* test for strains with a plasmid carrying genes for the indicated transport system compared with the same strain carrying the plasmid vector.

A. The strain ARM114 (*entF, feoB*) was plated on LB agar containing 3 µg/ml EDDA or 20 µM FeSO₄.

B. The strain ARM113 (*entF, tonB*) was plated on LB agar containing 2 µg/ml EDDA or 20 µM FeSO₄.

C. The strain EWE1014 (*entF, aroB*) was plated on LB agar containing 5 µg/ml EDDA or on unsupplemented LB agar.

VctP	1	-MVLIIIVRTLMLRISIKMIPLAYLNHFRKEHMKSRHWAALGLLAFAAQAETVTIEHRL	59
YclQ	1	MKK-FALLFIALVTAVVISACGNQSTSSKGS DTKKEQITVKHQ---LDKNG-----	47
CeuE	1	MKKSLVFAFFAFFLSLILTACDSKSNENNASTAIKTSTTVKVIPISMSDEGDSFLVKDSL	60
			
VctP	60	GKTTLEQKPQRVVVIGVGALDAIDSFGIEPVAVSKFDG-TPDYLAKYKSDKYPSAGSLFE	118
YclQ	48	--TKVPKNPKKVVVDFGSLDTLTKLGLDDIVAGLPKQVLPKYLSKFKDDKYADVGLKE	105
CeuE	61	GENKIPKNPSKVILDLGILDTFHALRLNDKVAGVPAKNLPKYLQQFKDK--PSIGGVQQ	118
		. . . * .** . * ** * ** . . * . .	
VctP	119	PDFETIYTQKPD LIVIGPRASKSYDELSKIAPTIVFAAEADQGYWESTQQQWRNLGKVFA	178
YclQ	106	PDFDKVAELDPDLIIISARQSESYKEFSKIAPTIYLGVD TAK-YMESFKSDAETIGKIFD	164
CeuE	119	VDFEAINALKPDLIIISGRQSKFYEKL-KIAPTMFVGLDNAN-FLSSFENNVL SVAKLYG	176
		** . . . **** . * * * * . . .	
VctP	179	IEPAVEAKIEQVDAQFKSIMQYNQQHKSDAMLMSSGGNLTTFGANSRFS SVYKDFGFSE	238
YclQ	165	KEDKVKDELANIDHSIADVKKTAEKL NKNGLVIMANDGKISAFGPKSR YGLIHDVFGVAP	224
CeuE	177	LEKEASEKIADIKNEIEQA KSIVDE-DKKALIVLTNSNKISAFGPQSRFGI IHDVLGINA	235
		* ** ** . . *	
VctP	239	TVPVSKESSHGDLISFEYIREHNP KTLVVD RDKVVTKGETNIRQTFENDLVKATTAYKN	298
YclQ	225	ADQNIKASTHGQSVSYEYISK TNP DYLFVIDRGTAIGE-TSSTKQVVENDYVKNVNAVKN	283
CeuE	236	VDENVKVGTHGKSINSEFILEKNPDYLFVVD RNIIVGN-KERAQGILDNALVT KTNAATN	294
		* . ** . * . * ** * * . * *	
VctP	299	GHIAYLDVNAWYIAIS-GVKATEQM VADMKASVGMQ	333
YclQ	284	GHVIYLD SATWYLSGG-GLESMTQMIKEVKDGLEK-	317
CeuE	295	NKIIYLDPEYWY LASGNGLSLKTMILEVTNAIK--	328
		. . . *** ** . . * . . .	

Fig. 7.

Clustal W alignment of *V. cholerae* VctP with YclQ of *Bacillus subtilis* (Kunst et al., 1997) and CeuE of *Campylobacter jejuni* (Richardson & Park, 1995). The arginine residues that contact the mecA in the CeuE crystal structure (Muller et al., 2006), as well as the tyrosine residue of YclQ that was proposed to interact with ferric iron (Zawadzka et al., 2009), are highlighted in gray.

Fig. 8.

VctPDGC is a functional iron transporter in *V. cholerae*

Wildtype *V. cholerae* strain O395 carrying the vector pWKS30, CFV1 (O395

$\Delta vibB, fbp, cam, feo::tmp$) carrying pWKS30, EWV133 (O395 $\Delta vibB, fbp::cam, feo::tmp,$

$vct::kan$) carrying either the pWKS30 or the *vctPDGC* genes (pCat120) were spread on LB agar containing ampicillin. The plate was incubated overnight at 37°C.

Fig. 9. Summary of iron transport systems identified in *V. cholerae*. There are seven outer membrane receptors with identified iron-containing ligands: three for heme (Mey & Payne, 2001), two for enterobactin (Mey et al., 2002) and one each for vibriobactin (Butterton & Calderwood, 1994) and ferrichrome (Rogers et al., 2000). The two TonB systems that provide the energy for ligand transporters in the outer membrane have both unique and overlapping functions (Occhino *et al.*, 1998, Seliger et al., 2001). The TonB2 protein is shorter than TonB1 and requires the novel protein TtpC, in addition to ExbB2 and ExbD2, for its activity (Kuehl & Crosa, 2009, Stork *et al.*, 2007). Ligands that cross the outer membrane through TonB-dependent receptors are then transported across the inner membrane by periplasmic binding protein-dependent ABC transporters. Catechol siderophores cross the inner membrane through either the ViuPDGC or the VctPDGC systems (Mey et al., 2002), and we show here that the VctPDGC also transports a siderophore-free iron ligand. There are also the ferric iron transporter FbpABC and the ferric iron transporter FeoABC (Wyckoff et al., 2006), as well as VciB, which stimulates iron acquisition by ferrous iron transporters, possibly by functioning as a ferric reductase (Mey et al., 2008).

Table 1Complementation of *E. coli* *fepB* mutation

Indicator Strain (<i>E. coli</i> <i>fepB</i> / <i>entF</i> carrying the indicated plasmid genes)	Zone of stimulation (mm) with ^a :	
	DH5α (Ent ⁺)	Ferrichrome
Vector only	0	13
<i>vctPDGC</i>	14	15
<i>fepBDGC</i>	15	17
<i>vctPDGC</i> (<i>vctP</i> Y310F)	17	15
<i>vctPDGC</i> (<i>vctP</i> R137Q)	0	15
<i>fbpABC</i>	0	15

^aCultures of the indicator strains were seeded into molten LB agar containing 300 µg/ml EDDA. 5 µl *E. coli* strain DH5α (as a source of enterobactin) or 200 µM ferrichrome were spotted onto the solidified medium. The zone of growth was measured 18 hours after inoculation.

Table 2

Summary of activity of various systems

Iron transport system	<i>fepB</i> complementation	SM193w growth stimulation
Vector only	–	–
<i>vctPDGC</i>	+	+
<i>fepBDGC</i>	+	–
<i>fbpABC</i>	–	+
<i>feoABC</i>	^a –	+
<i>vctPDGC</i> (<i>vctP</i> R137Q)	–	–
<i>vctPDGC</i> (<i>vctP</i> Y310F)	+	–

^a *feoABC* present on the chromosome

Table 3

Bacterial strains and plasmids

Strain or Plasmid	Description	Reference or source
Strains		
<i>V. cholerae</i>		
O395	Classical biotype	(Mekalanos <i>et al.</i> , 1983)
CFV1	O395 $\Delta vibB$, $fbp::cam$, $feo::tmp$	(Wyckoff <i>et al.</i> , 2006)
EWV133	O395 $\Delta vibB$, $fbp::cam$, $feo::tmp$, $vct::kan$	This study
Lou15	El Tor biotype	(Sigel & Payne, 1982)
<i>E. coli</i>		
DH5 α	Cloning strain, ent ⁺	(Hanahan, 1983)
XL10-Gold	Cloning strain	Stratagene
1017	HB101 $ent::Tn5$	(Daskaleros <i>et al.</i> , 1991)
W3110N	W3110 Nal-resistant	(Mey <i>et al.</i> , 2008)
ARM110	W3110N $entF::cam$	(Mey <i>et al.</i> , 2008)
ARM113	W3110N $entF::cam$, $tonB::kan$	(Mey <i>et al.</i> , 2008)
ARM114	W3110N $entF::cam$, $feoB::kan$	(Mey <i>et al.</i> , 2008)
JW3352	BW25113 $aroB::kan$	(Baba <i>et al.</i> , 2006)
EWE1014	W3110N $entF::cam$, $aroB::kan$	This study
JW0584	BW25113 $fepB::kan$	(Baba <i>et al.</i> , 2006)
EWE1017	W3110N $entF::cam$ $fepB::kan$	This study
<i>S. flexneri</i>		
SM193	SM100 $iucD::Tn5$, $feoB::tmp$, $sitA::cam$	(Runyen-Janecky <i>et al.</i> , 2003)
SM193w	Nonvirulent derivative of SM193	(Wyckoff <i>et al.</i> , 2006)
SA101	Nonvirulent derivative of SA100	(Lawlor <i>et al.</i> , 1987)
SA241	SA100 $iucDTn5$, Nonvirulent	(Daskaleros & Payne, 1987)
Plasmids		
pBluescript SK ⁻	High copy cloning vector	Stratagene
pWKS30	Low copy cloning vector	(Wang & Kushner, 1991)
pGEM-Teasy	High copy cloning vector	Promega
pACYC184	Medium copy cloning vector	(Chang & Cohen, 1978)
pRK1013	Helper plasmid for triparental mating	(Figurski & Helinski, 1979)
pWMB1	pWKS30 with <i>oriT</i> inserted in BsaAI site	This study
pSS125	Source of <i>oriT</i>	(Suh <i>et al.</i> , 2004)
pUC4K	<i>kan</i> resistance gene	Pharmacia
pCVD442N	pCVD442 with a NotI adaptor inserted in the SacI site	(Wyckoff <i>et al.</i> , 2006)
pFbp1	pWKS30 carrying <i>V. cholerae fbpABC</i>	(Wyckoff <i>et al.</i> , 2006)
pFeo101	pWKS30 carrying <i>V. cholerae feoABC</i>	(Wyckoff <i>et al.</i> , 2006)
pCat116	pGEM-Teasy carrying <i>vctPDGC</i> region with <i>vctP</i> and <i>vctD</i> replaced by <i>kan</i>	(Mey <i>et al.</i> , 2002)
pCat120	pWKS30 carrying <i>V. cholerae vctPDGC</i>	(Mey <i>et al.</i> , 2002)

Strain or Plasmid	Description	Reference or source
pCat123	pCat120 with KpnI deletion that truncates <i>vctC</i>	This study
pCat125	pWKS30 carrying <i>vctPDGC</i> with minimal upstream sequence	This study
pCat126	NotI fragment of pCAT116 subcloned in pCVD442N	This study
pCat133	pACYC184 carrying the <i>vctP</i> gene	This study
pCat134	pWKS30 carrying <i>vctPDGC</i> , with VctP R137Q	This study
pCat135	pWKS30 carrying <i>vctPDGC</i> , with VctP Y310F	This study
pCat137	pWKS30 carrying the <i>vct</i> operon with an in-frame deletion in <i>vctP</i>	This study
pVib147	pWKS30 carrying <i>V. cholerae viuPDGC</i>	(Wyckoff et al., 1999)
pCP111	pACYC184 carrying <i>E. coli fepBDGC</i>	(Pierce & Earhart, 1986)