The Dissertation Committee for Alexandra Rebecca Mey certifies that this is the approved version of the following dissertation:

Heme Utilization in *Vibrio cholerae* and Analysis of Domains Involved in the Specificity of TonB for TonB-dependent Receptors

Committee:

Shelley M. Payne, Supervisor

Charles F. Earhart

Ian J. Molineux

Kathleen Postle

Jon D. Robertus

Heme Utilization in *Vibrio cholerae* and Analysis of Domains Involved in the Specificity of TonB for TonB-dependent Receptors

by

Alexandra Rebecca Mey, B. A.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May, 2002

Dedication

To my husband and my family

Acknowledgements

This work was funded by the Foundation for Research and by the Institute for Cellular and Molecular Biology at the University of Texas at Austin. Support was also provided by the University of Texas in the form of a Preemptive Fellowship for Graduate Studies in Molecular Biology and a Teaching Assistant position in the Department of Microbiology. I gratefully acknowledge the assistance of Eva Rab, an undergraduate who worked with me on the *V. cholerae* heme receptor project. I am also grateful to my sister Kristianna Mey, who, as a medical student, spent six weeks at the bench with me, teaching and being taught. I am greatly indebted to Douglas Henderson for laying the foundation for my studies of heme utilization in *V. cholerae.* I am also indebted to Deborah Wrona, who helped get the TonB project off the ground, and whose friendship I cherish. I want to recognize Melissa Mann and Mary Lozano for their invaluable assistance with the animal studies. My thanks to Alfredo Torres, Stefan Seliger, Stephanie Reeves, Yuki Gleason, Georgiana Purdy, Ana-Maria Valle, Scott Mogull, Mandy Oglesby, Megan Whitney, Bonnie Reus and Enrique Gonzales for giving me friendship and support, and for making the Payne lab an exceptionally positive

working environment. I would like to acknowledge Laura Runyen-Janecky, whose dedication to science and to excellence has been a true inspiration for me. I am greatly indebted to Liz Wyckoff for sharing her wealth of knowledge, and for giving me excellent guidance throughout. I want to recognize the guidance and many helpful suggestions from the members of my committee Charles Earhart, Ian Molineux, Kathleen Postle and Jon Robertus. I am immensely grateful to Shelley Payne, my supervisor, for the opportunity to learn and grow as a scientist in her lab. Her intelligence, dedication and concern for her students' progress make her a truly remarkable mentor. I want to acknowledge the love and support of my family, who enrich my life in so many ways. Finally, a very special thanks to my husband Jimmy Perez, whose love, support and tremendous faith in me enabled me to achieve success without compromising my happiness.

Heme Utilization in *Vibrio cholerae* and Analysis of Domains Involved in the Specificity of TonB for TonB-dependent Receptors

Publication No._____

Alexandra Rebecca Mey, Ph. D.

The University of Texas at Austin, 2002

Supervisor: Shelley Marshall Payne

Vibrio cholerae has multiple iron transport systems, one of which involves heme uptake through the outer membrane receptor HutA. This study demonstrates that *V. cholerae* encodes two additional TonB-dependent heme receptors, HutR and HasR. HutR has significant homology to HutA and to other bacterial outer membrane heme receptors, and the role of HutR in hemin utilization and its localization in the outer membrane were confirmed. The *hutR* gene was co-transcribed with the upstream gene *ptrB,* and expression from the *ptrB* promoter was negatively regulated by iron. HasR is most similar to the hemophore-utilizing heme receptors from *Pseudomonas aeruginosa* and *Serratia marcescens*. A mutant defective in all three heme receptors was unable to utilize hemin as an iron source. HutA and HutR functioned with either *V. cholerae* TonB1 or TonB2. In contrast, hemin uptake through HasR was TonB2-

dependent. Efficient utilization of hemoglobin as an iron source required HutA and TonB1. The triple heme receptor mutant exhibited no defect in its ability to compete with its Vib^- parental strain in an infant mouse model of infection, indicating that additional iron sources are present *in vivo*. *V. cholerae* utilized heme derived from marine invertebrate hemoglobins, suggesting that heme may be available to *V. cholerae* growing in the marine environment.

Although *E. coli* TonB and *V. cholerae* TonB1 exhibit different specificities for outer membrane receptors, these TonB proteins are similar enough that functional chimeras can be created between them. The activities of the chimeric TonB proteins demonstrated that the C-terminal one-third of TonB constitutes a functional domain responsible for receptor specificity. A Pro238Thr substitution in *V. cholerae* TonB1 resulted in the ability of TonB1 to recognize a wider range of receptors, indicating that very C-terminal end of TonB1 determines receptor specificity. Domain-switching experiments between *E. coli* ChuA and *V. cholerae* HutA showed that the TonB box heptapeptide at the N-terminus of these receptors does not contain specificity determinants. Instead, specificity was controlled by the residue immediately preceding the TonB box. Taken together, these data suggest that functional interactions take place between the C-terminus of TonB and the very N-terminal domain of TonB-dependent receptors.

Table of Contents

List of Tables

List of Figures

I. INTRODUCTION

A. Microbial iron acquisition strategies

1. IRON REQUIREMENTS AND AVAILABILITY

Most living organisms require iron for a variety of cellular processes including electron transport, DNA synthesis, and antioxidant defense (46, 180). Iron is one of the most abundant elements in nature; nevertheless, acquiring enough iron for growth poses a significant problem for bacterial pathogens. Iron forms insoluble complexes at physiological pH in the presence of oxygen, and thus the iron is not easily accessible to microorganisms living in the natural environment (16). Pathogens that colonize humans also encounter an iron-limiting environment. Although the human body contains 4-5 grams of iron, virtually all of this iron is sequestered within cells in the form of heme proteins and ferritin. The small amounts of extracellular iron are quickly bound by host iron-binding proteins such as transferrin (Tf) in serum and lactoferrin (Lf) in secretions. Upon tissue damage, heme proteins may be released from cells and are a potential source of iron for pathogens. To limit the amount of heme in the extracellular environment, free heme is bound by the circulating host serum proteins albumin and hemopexin, and hemoglobin is bound by haptoglobin (reviewed in (23, 138, 180). The ability to successfully compete with the host for iron is an important determinant of microbial pathogenesis (129).

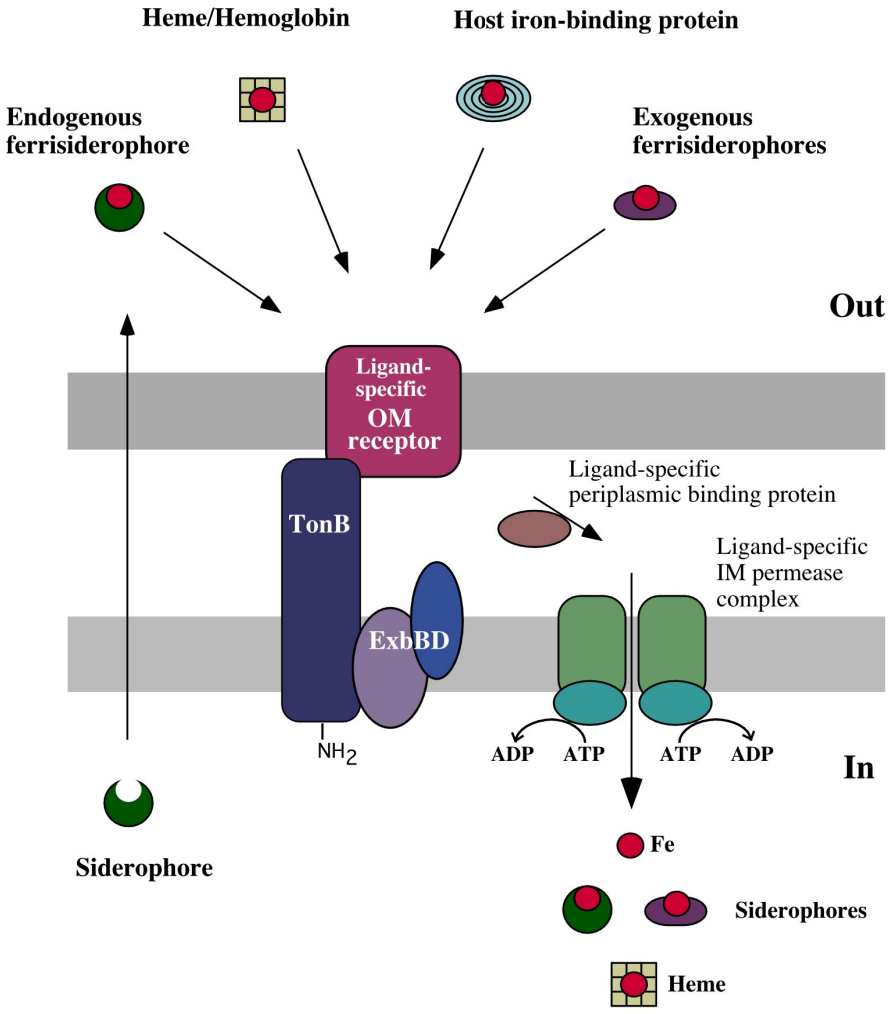
Another problem associated with iron uptake by bacterial cells stems from the potential of iron for catalyzing production of reactive hydroxyl radicals. Hydroxyl radicals are especially damaging to DNA. The uptake and storage of iron must therefore be tightly regulated and coordinated with the cell's needs, such that the requirements for iron are met but not exceeded (23).

2. OVERVIEW OF HIGH AFFINITY IRON TRANSPORT IN GRAM NEGATIVE BACTERIA

Most iron sources used by Gram negative pathogens are too large (> 600 Da) to pass through porins in the outer membrane, or too scarce to bioaccumulate in any significant amount by diffusion. Rather, iron complexes are transported across the outer membrane via high-affinity outer membrane receptors that are specific for their substrates (23, 65, 173). An overview of high affinity iron transport in Gram negative bacteria is shown in Figure 1. Following binding of an iron complex to its cognate receptors, the iron substrate is actively shunted through the transmembrane receptor pore into the periplasmic space. The TonB, ExbB and ExbD proteins play an essential role in the transport process by transducing energy produced at the cytoplasmic membrane to the receptors for iron complexes in the energy-deficient outer membrane (12, 14, 133). By interacting directly with ligand-bound receptors, energized TonB is thought to induce a conformational change in these receptors that facilitates internalization of the bound substrates (14, 133, 153). Once in the periplasm, iron complexes are shuttled to the cytoplasmic membrane by a substrate-specific periplasmic binding

Figure 1. High affinity transport of iron substrates across the Gram negative cell envelope.

An iron complex is bound by its cognate receptor in the outer membrane. Energy transduction by the TonB, ExbB, ExbD complex promotes the subsequent transport of the bound substrate into the periplasm. Once in the periplasm, the iron complex is shuttled to a substrate-specific inner membrane permease complex by a substrate-specific periplasmic binding protein (PBP). Binding of the substrate-loaded PBP to the inner membrane permease complex triggers ATP hydrolysis and facilitates transport of the substrate into the cytoplasm.


Heme/Hemoglobin
Host iron-binding protein
Endogenous ferrisiderophore
Exogenous ferrisiderophores
Out
Ligand-specific OM receptor
TonB
ExbBD
NH2
Ligand-specific periplasmic binding protein
Ligand-specific IM permease complex
ADP
ATP
ATP
ADP
In
Siderophore
Fe
Siderophores
Heme

protein (PBP). The substrate-bound PBP delivers its cargo to an integral inner membrane complex that is associated with ATPases at the cytoplasmic face of the membrane. The PBP-dependent transport systems (PBT) belong to the ABC (ATP binding cassette) superfamily of bacterial transporters (15, 138). Binding of the substrate-loaded PBP to the inner membrane complex triggers ATP hydrolysis and facilitates transport of the substrate into the cytoplasm. It is not currently known whether the PBP directly contacts the cytoplasmic ATPases, or whether a signal is transmitted through the integral membrane components of the transport complex to indicate docking of the substrate-bound PBP (16).

3. SPECIFIC HIGH AFFINITY IRON UPTAKE SYSTEMS IN GRAM NEGATIVE BACTERIA

a. Siderophore mediated iron acquisition

One of the most common strategies for bacterial iron acquisition is the synthesis and secretion of siderophores, low molecular weight (500-1000 Da) ferric iron chelating compounds with very high affinity for iron ($K_{aff} > 10^{30}$). In the extracellular environment, siderophores solubilize iron from insoluble complexes, and the iron-bound siderophores are subsequently taken up by the cell via specific cell surface receptors (42, 118, 138). Since siderophore-chelated iron is prevented from participating in oxy-radical production, siderophores also provide a solution to the toxicity problems associated with iron acquisition (23). The iron binding capacity of siderophores is considered strong enough to remove

iron from host iron-sequestering molecules such as Tf and Lf. Thus, these host compounds are potentially available as iron sources to siderophore-producing pathogens (138). Siderophores are synthesized and secreted under conditions of iron stress, and can be readily detected in the supernatant of cells grown in iron-limited media (118). Although chemically diverse, many of the siderophores produced by pathogenic bacteria can be classified as catechols, hydroxamates, or thiazolines (138). The prototype of a catechol siderophore is enterobactin, a cyclic compound made up of three molecules of 2,3-dihydroxybenzoylserine connected via ester linkages first identified in *Salmonella typhimurium* (131) and in *Escherichia coli* (122). Enterobactin is transported across the outer membrane of *E. coli* via the receptor FepA. An example of a hydroxamate siderophore is aerobactin, synthesized by certain *E. coli* and *Shigella* strains (130). Yersiniabactin, produced by *Yersinia* spp., contains two thiazoline rings as well as a phenolate group, all of which have the potential for coordinating iron (138). Many pathogens also use siderophores produced by other microorganisms, collectively termed xenosiderophores. The fungal hydroxamate siderophore ferrichrome, for example, is widely used by bacterial species. Ferrichrome transport across the *E. coli* outer membrane occurs via the receptor FhuA.

Following transport across the cell envelope, ferrisiderophores release their bound iron in the cytosol. Because siderophores bind Fe(II) much less tightly than Fe(III), reduction of the siderophore-bound Fe(III) has been proposed as a mechanism for liberating the iron from ferrisiderophores (42, 46, 138). Reductases capable of reducing ferrisiderophores *in vitro* have been identified in

many bacterial and fungal species (138). Following release of the iron, some siderophores are secreted once again into the extracellular environment, where they can participate in another round of iron scavenging. Aerobactin, for example, may be recycled up to three times before being degraded (46). Others, such as enterobactin, are degraded concomitantly with reduction of the bound iron. The enzyme responsible for enterobactin hydrolysis and iron release in the cytoplasm of *E. coli* is Fes, a ferrisiderophore reductase with esterase activity (17).

b. Utilization of host iron-binding proteins

The direct utilization of the host iron binding glycoproteins Tf and Lf as sources of iron has been best described in members of the Neisseriaceae and Pasteurellaceae families (63). The ability to use Tf and Lf directly may be particularly important in these non-siderophore producing organisms. It was demonstrated genetically that there are separate cell surface receptors for Tf and Lf in *Neisseria gonorrhoeae* (9), and the presence of genes encoding specific receptors for these substrates has been documented for most of the bacterial species that use either or both of these glycoproteins directly (126). The Tf and Lf cell surface receptors in the families Neisseriaceae and Pasteurellaceae are each composed of two proteins, both of which are capable of binding the substrate: Tf or Lf binding protein A (TbpA/LbpA), and Tf or Lf binding protein B (TbpB/LbpB). The TbpA/LbpA proteins are integral outer membrane proteins that resemble receptors for siderophores and other iron substrates. The

TbpB/LbpB proteins are lipoproteins that are peripherally associated with the outer membrane via an N-terminal lipid moiety (reviewed in (63). There is a high level of variation in LbpB among members of the same species, consistent with exposure of this protein at the cell surface where variation may aid in evading the host immune response. A recent model of the meningococcal Tf receptor proposes that TbpB provides the initial binding site for Tf, and that this binding ensures the proper orientation and proximity of Tf to a closely associated TbpA dimer. Subsequent binding of iron-loaded Tf by TbpA may result in a distortion of the Tf molecule, promoting release of the Tf-bound iron into the TbpA pore (10).

c. Utilization of heme as an iron source

Heme is by far the most abundant source of iron in the human body, and many pathogens have evolved mechanisms to take advantage of this reservoir (reviewed in Wandersman and Stojiljkovic, 2000 (175) and Genco and Dixon, 2001 (58)). Pathogens that multiply intracellularly can use heme directly; however, gaining access to intracellular heme stores is not trivial for extracellular pathogens. To facilitate the release of intracellular nutrients, many extracellular pathogens produce hemolysins/cytolysins that damage eukaryotic cells, causing intracellular heme proteins to leak into the extracellular milieu. *V. cholerae* El Tor strains produce the cytotoxic hemolysin Hly under conditions of iron deprivation, consistent with a role for Hly in iron acquisition (158). The *Pseudomonas aeruginosa* exotoxin A, which is cytotoxic toward a wide range of

eukaryotic cells, (8) and the *Serratia marcescens* ShlA hemolysin (132) are similarly under the control of iron.

Host heme-containing proteins are generally thought to be too large be taken up intact by the bacterial cell. One mechanism for liberating heme sequestered within hemoglobin and hemoglobin-haptoglobin, or bound to a serum protein carrier such as hemopexin or albumin, is the synthesis and secretion of proteases that degrade host heme proteins extracellularly. *V. vulnificus* synthesizes an exocellular protease that is required for the utilization of hemoglobin, hemoglobin-haptoglobin, and heme-hemopexin as sources of iron (121). The lysine-specific cysteine protease Kgp produced by *Porphyromonas gingivalis* binds to and degrades hemoglobin, and also exhibits proteolytic activity towards haptoglobin and hemopexin (101, 155). In addition, Kgp is a heme-binding protein, indicating that this protein is able to bind heme as it is released from the protein carrier. The pathogenic *E. coli* strain EB1 similarly produces a heme-binding hemoglobin protease, Hbp (128).

Several pathogens, including *S. marcescens* (96), *Haemophilus influenzae* (38) and *Pseudomonas spp*. (76, 100) synthesize and secrete hemophores, small extracellular proteins capable of binding heme in the extracellular environment. Hemophores are functionally similar to siderophores: after being secreted from the bacterial cell into the extracellular medium, they bind their substrate and deliver it to specific receptors at the bacterial cell surface. The best-characterized hemophore to date is the *S. marcescens* HasA protein, which is involved in capturing heme from hemoglobin (96). Secretion of the 19 kDa HasA proteins

depends upon a secretion signal within the C-terminal part of the molecule, and occurs via an ABC export system composed of HasD, HasE, and HasF (97). The mechanism by which HasA obtains heme from hemoglobin is not clear. HasA does not form a stable complex with hemoglobin, and if there is a direct transfer of the heme molecule from the hemoprotein to the hemophore, it may involve only a transient complex (98). HasA has a higher affinity for heme than hemoglobin does (98), and it is possible HasA acquires its heme simply by efficiently binding up any heme that dissociates from hemoglobin. *has*-like genes have been identified in *P. aeruginosa* (100, 125) and in *Pseudomonas fluorescens* (76, 99) and are required for efficient utilization of hemoglobin as an iron source in these organisms. Hemophore systems that are functionally similar, but genetically distinct from the HasA systems have also been identified. HxuA, an extracellular heme-binding protein which is both soluble and cell-surface associated, is involved in obtaining heme from heme-hemopexin in *H. influenzae* (38, 39). The *P. gingivalis* secreted protein Kgp, which degrades hemoglobin and binds the liberated heme, has been shown to interact with a cell surface receptor HmuR, and may function in the delivery of heme to this receptor (155).

The ability to bind heme, hemoglobin, and other host heme-containing proteins directly via cell surface receptors has been documented for a number of pathogenic species (58, 175). These receptors share many sequence features, suggesting that they may use similar mechanisms for binding and/or transport of heme. In particular, two amino acid sequence motifs, FRAP and NPNL, appear to be conserved among a large number of heme and hemoglobin receptors (11, 175).

Several histidine residues required for *Y. enterocolitica* HemR receptor function are conserved in other heme receptors as well (11). Ultimately, whether the heme carrier is a bacterial hemophore, or a host-derived heme protein, only the heme moiety is transported into the cell.

Receptors involved in heme acquisition can be divided into several categories, depending on their substrate specificities. A large number of receptors, including *V. cholerae* HutA (68, 69), *Shigella dysenteriae* ShuA (112, 113), *E. coli* 0157:H7 ChuA (168), *V. vulnificus* HupA (106), *P. aeruginosa* PhuR (125), *Yersinina pestis* HmuR (167), *H. influenzae* HxuC (37), and *Plesiomonas shigelloides* HugA (71) have been shown genetically and/or biochemically to be involved in the transport of free heme. Although many of these receptors are also required for utilization of heme-containing proteins such as hemoglobin as iron sources, no direct binding to heme-protein substrates has been demonstrated for this group of receptors. For example, *V. cholerae* uses both heme and hemoglobin as iron sources, and the *V. cholerae* receptor HutA confers both heme and hemoglobin utilization in *E. coli* (68, 69). However, hemoglobin binding assays have failed to show binding of hemoglobin to the surface of *V. cholerae* (S. M. Payne, unpublished results), suggesting that liberation of heme in preparation for uptake occurs independently of the cell surface receptor. Similarly, the *H. influenzae* outer membrane receptor HxuC was shown to be involved in utilization of hemoglobin, but no binding to hemoglobin was detected (37). The *E. coli* 0157:H7 heme receptor ChuA is necessary for growth using either heme or hemoglobin as iron sources (168). However, based on its similarity to the

S. dysenteriae heme receptor ShuA (100% similarity; 99% identity) it is unlikely that ChuA is a hemoglobin receptor, since *S. dysenteriae* does not use hemoglobin as an iron source. Thus, hemoglobin utilization in *E. coli* 0157:H7 may involve a mechanism not found in *S. dysenteriae* that facilitates the release of heme from hemoglobin prior to heme binding by ChuA.

The second category of receptors includes those that have been shown biochemically to bind hemoglobin and/or hemoglobin-haptoglobin directly. Most of these receptors are capable of binding free heme as well, suggesting that an initial protein contact is made between the receptor and the substrate, followed by release of the heme into a secondary binding site on the receptor. *Neisseria meningitidis* expresses two distinct hemoglobin receptors: HpuAB, a two-component hemoglobin/hemoglobin-haptoglobin receptor similar to the Tf and Lf receptors (102, 104), and HmbR, which is specific for hemoglobin (159, 160). Both of these receptors are phase-variable (103, 141). Phase-variation may play a role in evasion of the host immune response during an infection, and may enable a given strain to infect the same host more than once. Hemoglobin-utilizing *N. gonorrhoeae* strains express only the HpuAB receptor (32). The gene encoding *hmbR* appears to be defective in the *N. gonorrhoeae* strains tested (160). *H. influenzae* produces three hemoglobin- and hemoglobin-haptoglobin-binding proteins, HgpA, HgpB and HgpC, all of which undergo phase-variation (36, 115). A mutant defective in all three receptors was unable to use hemoglobin-haptoglobin, and no longer bound hemoglobin to the cell surface (115). However, growth stimulation with hemoglobin was still observed, and this growth has been

attributed to the heme receptor HxuC (37). Finally, the *Y. enterocolitica* receptor HemR and the *P. gingivalis* receptor HmuR both participate in hemoglobin utilization, and have been shown to bind hemoglobin directly (11, 152).

The heme-hemopexin receptors constitute the third category of receptors. Direct binding of heme-hemopexin to bacterial cells has only been described in *H. influenzae*. *H. influenzae* HxuA binds both heme and heme-hemopexin extracellularly, and is found both in the extracellular medium and in association with the cell surface. Thus, HxuA may function both as a hemophore and as a cell surface receptor for heme-hemopexin. In addition to HxuA, many *H. influenzae* strains produce a 57 kDa cell surface exposed protein that facilitates binding of heme-hemopexin to the bacterial cell independently of HxuA. This protein has been proposed to function as an outer membrane receptor for heme-hemopexin complexes (179).

The final set of receptors includes those specific for hemophores. In *S. marcescens*, heme-bound HasA delivers its heme to the outer membrane receptor HasR, and although HasR is capable of transporting free heme in the absence of HasA, heme uptake is 100-fold more efficient in the presence of the hemophore (60). HasR binds both heme-bound and unbound HasA, suggesting that there are direct protein-protein contacts involved in binding of the hemophore to its receptor (95). A HasR type hemophore receptor has also been identified in *P. aeruginosa*, and is required for hemophore-dependent heme uptake in this organism (125). The *P. gingivalis* outer membrane receptor HmuR has been

classified as a hemoglobin receptor, but may also function like a hemophore receptor by binding to the extracellular hemophore-like protein Kgp (127).

Once heme enters the cytosol, it may be incorporated directly into heme-containing proteins, or it may undergo oxidative cleavage by a heme oxygenase to release the heme iron (58). A heme oxygenase from a bacterial pathogen was first characterized in the Gram positive pathogen *Corynebacterium diphtheriae*. *C. diphtheriae* HmuO exhibits heme oxygenase activity *in vitro* (33) and is required for utilization of heme as an iron source (147). Heme oxygenases have now been identified in several Gram negative pathogens as well, including in *Neisseria* spp. (186, 187) and *P. aeruginosa* (139).

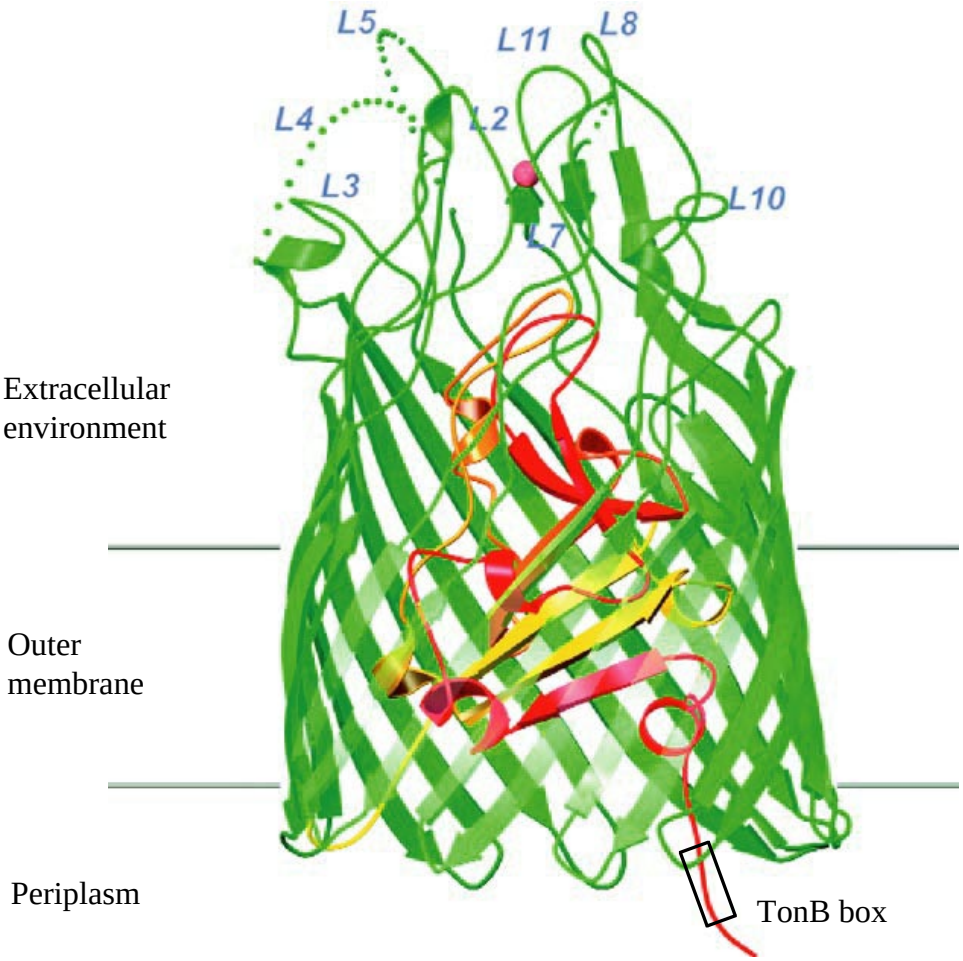
B. Mechanisms of TonB-dependent transport across the outer membrane

1. TonB-dependent outer membrane receptors

TonB-dependent receptors from many different organisms have been characterized and exhibit significant overall sequence conservation. Based on structural information available for two TonB-dependent receptors from *E. coli*, FepA (18) and FhuA (52, 108), it is predicted that all TonB-dependent receptors have a similar basic design: a 22-stranded transmembrane β-barrel porin structure with a unique N-terminal domain that fits tightly into the barrel like a plug from the periplasmic side (Fig. 2). The transmembrane β-strands are connected by short turns at the periplasmic face, and by long loops at the external face of the

Figure 2. Ribbon diagram of the *E. coli* enterobactin receptor FepA.

The diagram shows the β-barrel structure of FepA in green, and the N-terminal plug domain in red. A section of the β-barrel has been made transparent to show the location of the plug domain within the β-barrel pore. The long extracellular loops are labeled. The red sphere shows the putative position of the iron in ferrienterobactin. The location of the TonB box is indicated. Reprinted from Buchanan *et al.*, 1999, with permission from the authors and from *Nat. Struct. Biol.*


L5
L11
L8
L4
L2
L3
L10
L7
Extracellular environment
Outer membrane
Periplasm
TonB box

barrel. These loops extend into the extracellular environment and are likely to be involved in the initial binding of the substrate (28, 119). Following its initial adsorption, the substrate appears to bind with high affinity further into the channel (28, 108). The structure of ligand-bound FhuA shows that FhuA binds ferrichrome via residues in both the barrel and the N-terminal plug domain (108). Interestingly, a FhuA variant missing the plug domain (FhuAΔ5-160) still bound ferrichrome, suggesting that the plug domain residues are dispensable for binding (13). Further, a hybrid composed of the FhuA β-barrel and the FepA N-terminal plug bound ferrichrome, but not enterobactin, indicating that residues in the β-barrel confer ligand specificity, and that ligand-specific contacts between the N-terminal domain and the substrate are not absolutely required for substrate binding (148). In all binding studies, however, the plugless and hybrid receptor variants had significantly lower binding capacities than the wild type receptors (148), suggesting that the plug domain may be important for efficient binding under conditions of limited substrate availability.

The mechanism of substrate transport and the involvement of TonB in this process cannot be inferred from the structural information alone. In order for a substrate to pass through the channel, the plug domain must either come out of the barrel entirely, or must undergo some reorganization within the barrel to allow passage of the substrate. The plug domain is held in the β-barrel by a large network of hydrogen bonds and salt bridges, and ousting of the plug in order to facilitate passage of a substrate does not seem energetically feasible. It is also not clear how any reorganization of the plug domain within the barrel would create a

space large enough to accommodate the substrate. One model proposes that the plug domain undergoes an entropically favorable unfolding, which, with help from TonB, would offset the energy costs involved in disrupting surface interactions within the barrel (172). The unfolded plug domain might then come out of the barrel entirely, which would help explain the TonB-dependent passage of much larger molecules than siderophores, such as colicins and siderophore antibiotics, through the siderophore receptors. This model fails, however, to account for the large input of energy that would be required for refolding and insertion of the plug domain into the barrel once more. Interestingly, transport of ferrichrome through a plugless FhuA variant required TonB, indicating that removal of the plug domain is not sufficient for substrate internalization, and that interactions between TonB and the barrel also participate in the passage of the ligand through the receptor pore (13).

2. STRUCTURE OF TONB AND ORGANIZATION OF TONB FUNCTIONAL DOMAINS

E. coli TonB (Fig. 3) is a 26 kDa protein that is anchored in the cytoplasmic membrane via its uncleaved N-terminal signal sequence (134). The association of TonB with the inner membrane requires the ExbB and ExbD proteins (94), both of which interact directly with TonB (73, 90). This interaction is necessary for energy transduction by TonB (1, 79). While ExbB extends into the cytoplasm (47, 82), ExbD primarily occupies the periplasm (81), and together they may from a heterohexameric complex directly involved in energizing

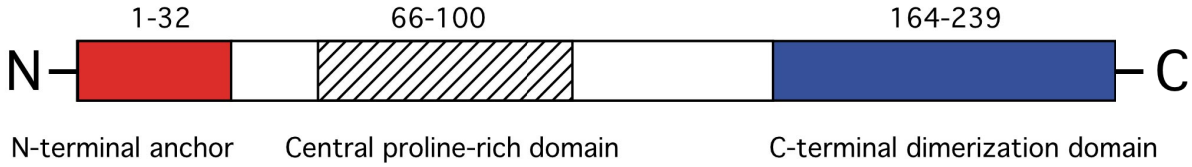

1-32
66-100
164-239
N
C
N-terminal anchor
Central proline-rich domain
C-terminal dimerization domain

Figure 3. Organization of *E. coli* TonB functional domains.

The N-terminal anchor domain (amino acid residues 1-32) is shown in red. The central proline-rich domain (amino acid residues 66-100) is hatched. The C-terminal dimerization domain (amino acid residues 164-239) is shown in blue.

TonB (73). The interaction between TonB and ExbB is well characterized and involves a set of conserved residues on one face of the α-helical transmembrane N-terminal anchor domain of TonB (88-90). These residues form a H*XXX*S motif that is required for the coupling of TonB to the electrochemical gradient of the inner membrane (88). The precise nature of the interaction between TonB and ExbD has not been defined (73).

From the cytoplasmic membrane, TonB extends into the periplasm (144) and may span the periplasmic space in order to make direct contacts with components of the outer membrane (153). The central proline-rich domain of TonB may be critical for the ability of TonB to bridge the periplasmic space by allowing TonB to adopt a rigid extended conformation (91). Deletion of this region only affects TonB activity under conditions of high osmolarity, conditions which may cause a widening of the periplasmic space (91, 149).

Membrane fractionation experiments have demonstrated that TonB localizes with both the inner and outer membranes, suggesting intimate associations with both membranes (94). Several lines of evidence support the hypothesis that TonB associates with the outer membrane via its C-terminal domain. A TonB variant that lacks the C-terminal 65 residues no longer fractionated with the outer membrane, whereas a variant lacking the N-terminal anchor domain was not impaired in outer membrane association (94). Loss of the final 48 residues rendered TonB inactive and prevented *in vivo* cross-linking to FepA, suggesting that residues within this region participate in the interaction with outer membrane receptors (87). Finally, a soluble TonB fragment consisting

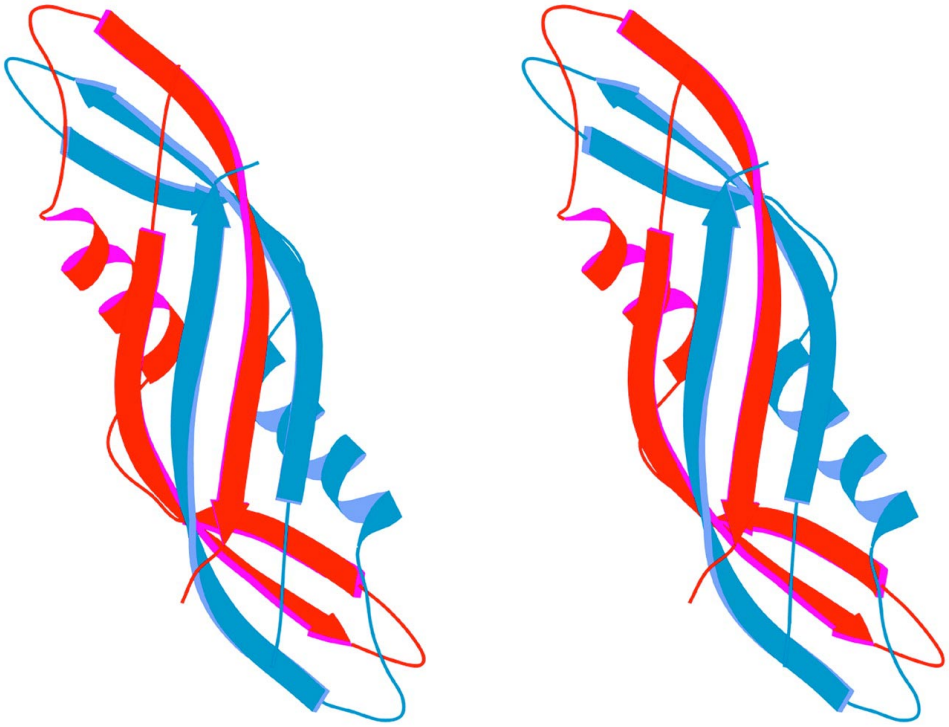
of the C-terminal half of TonB was shown to inhibit all TonB-dependent transport when synthesized *in vivo*, presumably by out-competing wild type TonB for binding to outer membrane receptors (75). Taken together, these data strongly implicate a region within the C-terminus of TonB in the association with outer membrane receptors and in energy transduction to these receptors. The recently solved crystal structure of the C-terminus of *E. coli* TonB showed that the region comprising the final 75 amino acids dimerizes to form a tightly intertwined cylinder-shaped structure (Fig. 4) (31). This structural feature of TonB may be responsible for productive interactions with outer membrane receptors.

3. INTERACTIONS BETWEEN LIGAND-BOUND RECEPTORS AND TONB

TonB interacts preferentially with ligand-bound receptors (114), and insights into how TonB recognizes the ligand-bound state have been gained from comparisons of liganded and unliganded FhuA. When FhuA binds its substrate, there are very few changes in the β-barrel. In contrast, a periplasmically exposed region of the plug domain undergoes dramatic conformational shifts, resulting in the unwinding of helix one (H1) and the displacement of this segment to the opposite wall of the barrel (108). It is conceivable that TonB discriminates between the bound and unbound states by sensing these changes at the periplasmic face of the receptor. Because TonB interacts with several different types of outer membrane receptors involved in high-affinity iron transport, it is expected that these receptors share a common binding motif for TonB. Indeed,

Figure 4. Stereo ribbon diagram of the C-terminal domain dimer of *E. coli* TonB.

The diagram depicts two TonB C-terminal domain monomers, A and B, intertwined to form a dimer. Monomer A is shown in red and monomer B is shown in blue. The arrows represent β-strands, and the cylinders represent α-helices. Reprinted from Chang *et al*., 2001, with permission from the authors and from *J. Biol. Chem*.



many TonB-dependent receptors share a short stretch of amino acid homology near the amino terminus termed the TonB box (80, 133, 136). In the FhuA structure, this heptapeptide domain was not resolved; however, the proximity of the TonB box to the periplasmically exposed region that shifts extensively upon ligand binding suggests that the TonB box could be involved in the signaling event (108). In support of this hypothesis, structural analyses recently demonstrated that the TonB box of the vitamin B_{12} receptor BtuB undergoes significant conformational changes upon substrate binding to the receptor (109). In FepA, the TonB box is ideally positioned for physical contact with TonB at the periplasmic face of the receptor, but it is not clear from the structure how this conformation relates to substrate binding (18). Both genetic and biochemical studies have previously implicated the TonB box in a direct interaction with TonB. Mutations in the BtuB TonB box that abolish TonB-dependent transport can be partially suppressed by mutations at residue Q_{160} in TonB (7). Site-directed disulfide cross-linking of cysteine residues engineered into TonB and BtuB later demonstrated that the region of TonB containing the suppressor mutations may be in close proximity to the BtuB TonB box (25). A soluble pentapeptide corresponding to the first five residues of the TonB box from the receptor FhuE inhibited transport through a variety of receptors, including FhuA and FepA, indicating that this peptide interfered with the ability of TonB to bind to these receptors (171). Finally, a mutation within the TonB box of FepA that eliminated enterobactin transport also abolished formaldehyde cross-linking of TonB to FepA (87). The data cited above are consistent with a model in which

TonB directly contacts the TonB box region of the receptor. However, it seems clear from studies showing TonB stimulation of ferrichrome transport through a plugless FhuA variant that TonB makes contacts with residues in the transmembrane β-barrel as well (13).

C. Transcriptional regulation of gene expression by iron and Fur

A common feature of genes that participate in iron acquisition is that they are negatively regulated at the transcriptional level by iron. This regulation allows the bacterial cell to carefully coordinate iron uptake with the cell's iron requirements. Negative regulation by iron occurs via the repressor protein Fur. Fur is a 17 kDa iron-binding protein that, when complexed with Fe^{2+}, blocks transcription by binding to a conserved operator sequence termed the Fur box within the promoter region of iron-regulated genes. In the absence of iron, Fur cannot bind to the Fur box, and repression is relieved. The consensus Fur box in *E. coli* is a 19 base pair palindrome formed by two, nine base pair (bp) inverted repeats, separated by one bp: (GATAATGAT)A(ATCATTATC). Alternatively, this sequence can be defined as a series of 6 bp repeats. The Fur box consensus sequence, when engineered into a non-iron-regulated promoter, was shown to be sufficient for Fur-mediated repression of the promoter in the presence of iron (26). The palindromic nature of the Fur box consensus sequence, combined with the observation that Fur exists as a dimer *in vitro* (3), initially suggested a dimer-palindrome mechanism of binding similar to that employed by other bacterial

repressor-DNA complexes, such as the DNA-bound *lac* repressor. However, DNA footprinting experiments showed that Fur contacts the DNA on all sides of the helix in a symmetrical fashion (44), and electron microscopy images demonstrated Fur multimers wrapped around the DNA helix (92). These data are consistent with more than two Fur-DNA contacts, and recent studies using natural and synthetic Fur binding sites indicate that Fur may bind to a series of at least three direct or inverted GATAAT repeats throughout the Fur box region in *E. coli* (48).

D. Iron uptake systems and virulence in the host

The importance of iron for growth and virulence of bacterial pathogens *in vivo* has been suggested by epidemiological studies showing a direct correlation between disease progression and iron levels in the host. Infection by *Mycobacterium tuberculosis* elicits a host iron sequestration response, resulting in anemia in tuberculosis patients. Treatment of *M. tuberculosis* infection-induced anemia with dietary iron supplements was shown to promote the development of active tuberculosis and increase the risk of death from tuberculosis (45). Iron loading conditions, including hemochromatosis and alcoholism, are associated with a higher incidence of systemic infection with pathogens such as *V. vulnificus* and *Yersinia pseudotuberculosis*, which do not normally cause systemic infections (177). In experimental animal models, administering an iron source along with the bacterial inoculum greatly increases the infectivity and/or virulence of

V. vulnificus (181), *N. gonorrhoeae* and *N. meningitidis* (57), mycobacteria of the *Mycobacterium avium* complex (45), and many enteric pathogens (84, 130).

The importance of bacterial iron transport systems for *in vivo* growth and pathogenesis has been demonstrated directly by analysis of iron acquisition mutants. A *S. dysenteriae tonB* mutant was shown to be deficient in intracellular multiplication in cultured cells, and was avirulent in a guinea pig keratoconjunctivitis model of infection (140). A *Bordetella pertussis tonB* mutant did not multiply *in vivo* in the mouse respiratory infection model (135). In a mouse model of urinary tract infection by the uropathogenic *E. coli* strain CFT073, TonB was shown to be required for colonization of the bladder and kidneys (169). Other examples of pathogens that require a functional *tonB* gene for full virulence in an animal model include *P. aeruginosa* (163), *H. influenzae* (78), and *V. cholerae* (70, 149).

While studies using *tonB* mutants serve to illustrate the relevance of high affinity iron uptake in the host, they do not address the importance of individual iron transport systems. In some cases, it is evident that the *tonB* requirement correlates directly with the necessity for acquiring a specific iron substrate, not only within the host, but under all conditions. Such is the case for heme auxotrophic organisms such as *H. influenzae* (78), *H. ducreyi* (2, 156) and *P. gingigvalis* (59, 154), which require exogenous heme for growth. In other cases it has been necessary to create defined mutations in individual iron transport systems in order to assess their role *in vivo*. Human volunteers challenged with a *N. gonorrhoeae* strain defective in the transferrin receptor complex TbpAB did

not develop urethritis, demonstrating that transferrin uptake is critical for establishing infection in the human host (40). In *N. meningitidis*, the ability to acquire iron from hemoglobin via the receptor HmbR is essential for virulence, and has been proposed to be important for the intravascular stage of meningococcal bacteremia (159). Two iron transport systems, Ybt and Yfe, are required for *Y. pestis* virulence. The Ybt system, dedicated to yersiniabactin synthesis and uptake, is required for infection via the subcutaneous route. Thus, the Ybt system may be critical for iron acquisition immediately upon transmission of *Y. pestis* to the mammalian host from an infected flea (5). The Yfe system, consisting of a periplasmic binding protein-dependent ABC transporter complex, appears to be important for establishing a systemic infection (6). In *Helicobacter pylori*, the TonB-independent Feo iron transport system is required for virulence in a mouse model, suggesting that ferrous iron may be the most important source of iron for this pathogen *in vivo* (174).

In most cases, pathogens probably rely on multiple systems for iron acquisition *in vivo*, and the loss of just one of these systems may not have a measurable effect on virulence. In a mouse model of urinary tract infection, *E. coli* CFT073 mutants defective in either enterobactin or aerobactin siderophore synthesis were not impaired in colonization of the kidneys; however, a mutant defective in both siderophore systems exhibited a measurable decrease in colonization, suggesting that siderophore-mediated iron acquisition is important for infection of the kidney (169). It is likely that other iron transport systems contribute to colonization as well, since an *E. coli* CFT073 *tonB* mutant had an

even greater deficiency in colonization than that observed for the double siderophore synthesis mutant (169). An *S. dysenteriae* strain defective in both heme uptake and enterobactin-mediated iron acquisition did not appear to be starved for iron when growing in cultured cells, suggesting that this pathogen has alternative means for gaining access to host iron in the intracellular environment (140).

E. Life cycle of *V. cholerae*

The diarrheal disease cholera continues to be a major public health concern, and epidemics of this disease occur worldwide. The etiologic agent of cholera is the bacterium *Vibrio cholerae,* a Gram negative, motile, curved rod with a single polar flagellum. Transmission of *V. cholerae* to humans is via the fecal-oral route and occurs primarily via contaminated water supplies. Within the host, *V. cholerae* adapts to the intestinal environment and colonizes efficiently before being shed in great numbers into the environment. Outside the human host *V. cholerae* can persist in both freshwater and marine aquatic environments, and the presence of environmental reservoirs for this organism greatly increases the potential for epidemic outbreaks (reviewed in (41, 51, 83, 146)). To colonize the human host, *V. cholerae* must first survive the acid barrier of the stomach. The high infectious dose of *V. cholerae* is directly related to the sensitivity of this organism to stomach acids (83). Bacteria that survive transit through the stomach use their flagellum to penetrate the mucin layer of the small intestine to reach the

underlying intestinal epithelium (54). Colonization of epithelial cells is mediated by the toxin co-regulated pilus (TCP) (85, 166), and *V. cholerae* mutants deficient in TCP production do not cause disease in human volunteers (72, 162). Infection with *V. cholerae* is associated with massive fluid and electrolyte loss, potentially leading to collapse of the circulatory system and death if left untreated. The virulence factor responsible for the profuse diarrhea is cholera toxin (CT), which causes the activities of intestinal cell ion transporters to be altered, resulting in a net efflux of ions and water into the lumen of the gut (146).

Coordinate regulation of virulence gene expression within the human host is achieved through the ToxR/S, TcpP/H, and ToxT regulatory networks. ToxR and TcpP are membrane-localized transcription factors which, when associated with their respective partners ToxS and TcpH, directly regulate the expression of CT and ToxT in response to environmental stimuli. ToxT, in turn, activates transcription of the TCP and CT structural genes (41). Maximal expression of the CT genes *in vivo* occurs after, and is dependent upon, expression of TCP, suggesting that there is a correlation between colonization of the intestinal epithelium and efficient CT production (93). By influencing the ability of *V. cholerae* to successfully colonize the host, nutrient acquisition *in vivo* may also play a critical role in CT production, and thus in the pathogenesis of this organism.

F. Iron uptake systems in *V. cholerae*

1. *V. CHOLERAE* TONB SYSTEMS

V. cholerae employs a variety of high-affinity iron transport systems, all of which require a functional TonB, ExbB, ExbD complex (70, 124). Two sets of *tonB, exbB, exbD* genes have been identified in *V. cholerae.* The *tonB1, exbB1,* and *exbD1* genes are located on the smaller (1.07 Mb) replicon, whereas the *tonB2, exbB2,* and *exbD2* genes are carried by the larger (2.96 Mb) replicon (67, 170). The two *V. cholerae* TonB systems exhibit specific as well as overlapping functions (149), indicating that these systems are not completely redundant. Further, it was inferred from studies of the function of the *V. cholerae tonB* genes in *E. coli* that the two TonB systems are inherently different. While the *tonB2* system genes can complement an *E. coli tonB* mutant (124), the TonB1 complex cannot substitute for the native *E. coli* TonB complex (68, 124). The TonB1 complex is, however, able to mediate transport through a *V. cholerae* receptor in *E. coli* (68), suggesting that *V. cholerae* TonB1 may be highly specific for *V. cholerae* receptors. The presence of two TonB systems has been reported in other Gram negative pathogens as well, including *P. aeruginosa* (185), and *Vibrio parahaemolyticus* and *Vibrio alginolyticus* (123).

2. SIDEROPHORE-MEDIATED IRON ACQUISITION

Under iron-limiting conditions, *V. cholerae* secretes the siderophore vibriobactin, a catechol type iron chelator (64). Vibriobactin is composed of three

2,3-dihydroxybenzoic acid residues linked to a norspermidine backbone. Two of the dihydroxybenzoyl residues are connected to the backbone via oxazoline rings, while the third is joined directly to norspermidine (64). The vibriobactin biosynthetic genes are located in two separate loci, both of which also contain genes involved in the uptake of iron-bound vibriobactin by the cell (20, 21, 182-184). Transport of vibriobactin across the outer membrane occurs via the receptor ViuA (22, 157). Disruption of the *viuA* gene results in a complete defect in vibriobactin utilization, indicating that ViuA is the only receptor for vibriobactin in *V. cholerae* (157). Either of the two *V. cholerae* TonB systems mediate vibriobactin transport, indicating that ViuA is not specific for either TonB (124). *V. cholerae* can also use the *E. coli* catechol siderophore enterobactin (149, 184). There are two TonB-dependent receptors, IrgA and VctA, involved in enterobactin transport across the outer membrane (Mey *et al.*, manuscript in preparation). In contrast to vibriobactin uptake, enterobactin transport specifically requires the TonB2 complex (149). An ABC transporter system involved in shuttling both vibriobactin and enterobactin across the cytoplasmic membrane was recently described in *V. cholerae*, and there is evidence for at least one more inner membrane system dedicated to catechol transport in this organism (184). *V. cholerae* uses many xenosiderophores besides enterobactin. *V. cholerae* can use ferrichrome as an iron source (64); transport of this substrate occurs via the FhuA receptor (143) and either of the two TonB systems (124). *V. cholerae* also uses schizokinen (produced by *Bacillus spp.*, and by *Anabaena*

variabilis) as an iron source (149). Schizokinen uptake is TonB1-dependent, but a specific receptor for this substrate has not yet been identified (149).

3. UTILIZATION OF HEME AND HEMOGLOBIN

V. cholerae can use both heme and hemoglobin as sources of iron (69, 124, 158). Binding and transport of heme is facilitated by HutA, an iron-regulated outer membrane protein with many of the conserved features of TonB-dependent heme receptors (68). These include an N-terminal TonB box domain, a FRAP/NPNL motif (11), and at least one of the conserved histidine residues implicated in heme receptor function (11). Heme transport in *V. cholerae* is absolutely dependent upon the presence of a functional TonB complex (70, 124). Both *V. cholerae* TonB systems are capable of mediating heme transport; however, we recently demonstrated a preferential role for TonB1 in the utilization of heme and hemoglobin as iron sources (149). TonB1, the longer of the two TonBs, is required for transport of heme under high osmolarity conditions. TonB1 is also required for efficient utilization of hemoglobin as an iron source (149). In the same operon as *tonB1, exbB1, and exbD1* are genes for the heme-specific transporters HutB (periplasmic binding protein), and HutC and HutD (cytoplasmic membrane ABC transporter complex) (124), further implicating the *tonB1* locus in heme utilization.

4. REGULATION OF *V. CHOLERAE* GENE EXPRESSION BY IRON AND FUR

Many genes involved in iron uptake in *V. cholerae* are negatively regulated by iron and Fur. These include the vibriobactin synthesis genes (21, 158), the catechol transport genes (21, 184), and the genes encoding the heme receptor HutA (68, 164), the vibriobactin receptor ViuA (22, 107), the ferrichrome receptor FhuA (143), and the enterobactin receptors IrgA (61) and VctA (Mey *et al*., manuscript in preparation). Although a role in iron acquisition was not definitively established for the El Tor hemolysin gene *hlyA*, this gene is also regulated by Fur (158). *V. cholerae* Fur shares significant amino acid identity with *E. coli* Fur, and can complement an *E. coli* Fur mutant (105). The *E. coli* Fur protein can also substitute for *V. cholerae* Fur (158). As expected, Fur-binding sequences within the promoter regions of iron-regulated genes in *V. cholerae* have significant homology to the *E. coli* consensus Fur box (19, 21, 22, 61, 68, 183, 184). Fur-mediated repression of gene expression is not the only form of iron regulation in *V. cholerae*. Two-dimensional PAGE of iron-regulated proteins from wild type and *fur* mutant *V. cholerae* strains have demonstrated both positive and negative regulation by iron independent of Fur, suggesting that there are other iron responsive regulatory pathways in this organism (107).

G. Iron-regulated genes and virulence in *V. cholerae*

Several studies have addressed the importance of iron transport systems for *V. cholerae* virulence. *V. cholerae* strains defective in both TonB systems

were significantly impaired in the ability to colonize infant mice and cause disease, indicating that loss of all high affinity uptake systems compromises *in vivo* growth (70, 149). However, it has been more difficult to assess the contribution of individual iron transport systems to growth and disease production *in vivo*. Mutants defective in the synthesis or transport of vibriobactin were able to multiply and elicit a diarrheal response in infant mice, suggesting that vibriobactin-mediated iron acquisition is not required for virulence of *V. cholerae* in the mammalian host (151). A mutant defective in the outer membrane heme receptor HutA was not significantly compromised in its ability to compete with the parental strain in a rabbit ileal loop model of infection (164), indicating that other transport systems may compensate for the loss of HutA-mediated heme transport *in vivo*.

Regulation by iron and Fur may also serve to ensure increased expression of a virulence gene within the iron-limiting environment of the host (129). A role in virulence for the iron-regulated Hly hemolysin was suggested by a study showing that a strain deficient in hemolysin production exhibited a 100-fold increase in the LD_{50} in infant mice (178). *V. cholerae* 01 clinical isolates from Northern Brazil (Amazonia variants) were recently found to possess a cytotoxic vacuolating activity linked to the Hly hemolysin (34). The Hly cytotoxin may be intimately involved in the pathogenesis of the Amazonia variants, since these strains do not produce any of the other known *V. cholerae* toxins (35). The iron-regulated enterobactin receptor IrgA was proposed to be an important virulence factor in *V. cholerae.* The LD_{50} of a *V. cholerae irgA*::Tn*phoA* mutant was 100-

fold higher than that of the parental strain in an infant mouse model of *V. cholerae* infection (62). However, this study did not demonstrate that the loss of virulence could be complemented by introduction of the wild type *irgA* gene. A different *irgA* mutation in the same *V. cholerae* strain background had no effect on colonization of infant mice or disease production in these animals, although the mutation abolished IrgA-mediated enterobactin transport (Mey *et al*., manuscript in preparation). Thus, it is possible that the *irgA*::Tn*phoA* strain carries additional mutations that result in attenuation of virulence in the infant mouse model.

H. Purpose of this dissertation

The first goal of this study was to characterize further the heme utilization systems in *V. cholerae* by identifying and characterizing any additional receptors for heme expressed by this organism. Identification of all the *V. cholerae* heme receptors allowed for more definitive studies of heme receptor characteristics such as heme substrate specificity and TonB preference, and made it possible to analyze the role of heme uptake for growth of *V. cholerae in vivo*.

The second goal of this study was to analyze the regions involved in the productive interaction between TonB and TonB-dependent receptors. These analyses were extended to include an investigation of the specificity exhibited by *V. cholerae* TonB1 for a subset of *V. cholerae* TonB-dependent receptors.

II. MATERIALS AND METHODS

A. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are described in Table 1.

B. Media and growth conditions

Bacterial strains were routinely grown at 37°C in Luria (L) broth, in Bacto® (Difco Laboratories, Detroit, MI) brain heart infusion (BHI) broth, or on L agar, and maintained at –80°C in tryptic soy broth (TSB, Beckton Dickinson Microbiology Systems, Beckton Dickinson and Company, Sparks, MD) plus 20% glycerol. L broth was prepared by adding 10 g Bacto® tryptone, 5 g Bacto® yeast extract, and 10 g NaCl per liter ddH_2O. TSB and Bacto® BHI media were prepared according to the manufacturer's instructions. L agar was prepared by addition of 15 g Bacto® agar per liter of L broth. Iron-replete medium was prepared by the addition of 40 μM ferrous sulfate ($FeSO_4$), and iron-depleted medium was prepared by the addition of ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDA), deferrated by the method of Rogers (142). Bovine hemin was obtained from Sigma Chemical Company (St. Louis, MO). Hemoglobin was prepared fresh from whole ovine blood as follows: red blood cells were pelleted from 1 ml of whole blood at 5000 × *g*, and washed repeatedly with PBS to reduce contamination with free heme. The washed red cells were

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
Strains		
V. cholerae		
Lou 15	*V. cholerae* El Tor strain	(150)
CA401	*V. cholerae* Classical strain	(55)
CA401S	CA401, Str^R	(149)
CA40130	Vibriobactin synthesis mutant of CA401	(64)
CA40130N	CA40130, Nal^R	This study
DHH11	CA40130, Nal^R; defective in both TonB systems	(124)
ARM213	DHH11 *hutR::kan*	This study
ARM215	DHH11 *hutA::cam*	This study
ARM219	DHH11 *hutA::kan, hutR::cam*	This study
ARM303	CA40130N *ptrB::cam*	This study
ARM313	CA41030N *hutR::cam*	This study
ARM315	CA40130N *hutA::kan*	This study
ARM316	CA40130N *hutA::kan, irgA::cam*	This study
ARM516	CA40130N *irgA::cam, vctA::kan*	This study
ARM903	CA40130N *hutA::kan, ptrB::cam*	This study
ARM915	CA40130N *hutA::kan, hutR::cam*	This study
ARM930	CA40130N *hutA::kan, hutR::cam, vhrA::tmp*	This study
ARM932	CA40130N *hutA::kan, hutR::cam,* $hasR_{L9P}$	This study

AMV527	CA40130N *tonB1::kan*	A. M. Valle
E. coli		
DH5α	cloning strain	(66)
1017	HB101 *entF::*Tn*5*	(43)
SY327 λ*pir*	λ*pir recA56*, Nal^R; host strain for pGP704 Derivatives	(111)
SM10 λ*pir*	λ*pir recA*, Kan^R, mobilizing strain for pGP704 derivatives	(111)
W3110	F^- IN(*rrnD-rrnE)1*	(74)
KP1032	W3110 *tonB::kan*	(90)
KP1032N	KP1032, Nal^R	(124)
ARM100	KP1032N *entF::cam*	This study
ARM101	KP1032N *fepA::cam*	This study
ARM107	KP1032N *entF::cam*, *fepA*D11Y	This study
Plasmids		
pACYC184	medium-copy cloning vector, Cm^R, Tc^R	(30)
pHM5	suicide vector pGP704 carrying *sacB*; Cb^R, Suc^S	(145)
pQF50	promoterless *lacZ* reporter plasmid; Cb^R	(50)
pWSK29	low-copy cloning vector; Cb^R	(176)
pWKS30	low-copy cloning vector; Cb^R	(176)
pWKS30ΔX	pWKS30 with the *Xba*I site disrupted	This study
pAGO-cat1	pHM5 carrying *vctA::kan*	Mey *et al*., manuscript in preparation
pAMC22	pChuA encoding the HutA TonB box in place of the ChuA TonB box	This study
pAMH20	pACYC184 carrying *hutA*	This study

pAMH21	pAMH20 encoding the FepA TonB box in place of the HutA TonB box	This study
pAMH22	pAMH20 encoding the ChuA TonB box in place of the HutA TonB box	This study
pAML20	pQF50 with the *hutA* promoter cloned upstream of *lacZ*	This study
pAML21	pQF50 with the *ptrB* promoter cloned upstream of *lacZ*	This study
pAMR12	pWKS30 carrying *hutR*	This study
pAMR14	pACYC184 carrying *hutR*	This study
pAMS1	pHM5 carrying *hutA::kan*	This study
pAMS2	pHM5 carrying *hutR::cam*	This study
pAMS3	pHM5 carrying *ptrB::cam*	This study
pAMS4	pHM5 carrying *hasR::tmp*	This study
pAMS5	pHM5 carrying *irgA::cam*	This study
pAMS6	pHM5 carrying *vhrA::tmp*	This study
pAMS7	pHM5 carrying *entF::cam*	This study
pAMS8	pHM5 carrying *fepA::cam*	This study
pAMT12[1]	pvTonB1*X* carrying additionally v*exbBD1*	This study
pAMT111[a]	pWKS30ΔX carrying e*tonBX*, and v*exbBD1*, *hutBCD*	This study
pAMT112	pAMT12 carrying additionally v*hutBCD*	This study
pAMT113	pAMT112 encoding eTonB$X_{1\text{-}119}$/ vTonB1$X_{122\text{-}245}$ in place of vTonB1*X*	This study
pAMT114	pAMT111 encoding vTonB1$X_{1\text{-}121}$/ eTonB$X_{120\text{-}239}$ in place of eTonB*X*	This study
pAMT121	pAMT111 with an *Xho*I site introduced into e*tonBX* at codon 172 (e*tonBXXh*)	This study

[1] *V. cholerae* genes and gene products are preceded by the letter v, whereas *E. coli* genes and gene products are preceded by the letter e.

pAMT122	pAMT112 with a *Xho*I site introduced into v*tonB1X* at codon 178 (v*tonB1XXh*)	This study
pAMT123	pAMT122 encoding eTonB1*XXh*$_{1\text{-}172}$/ vTonB1*XXh*$_{179\text{-}245}$ in place of vTonB1*XXh*	This study
pAMT124	pAMT121 encoding vTonB*1XXh*$_{1\text{-}178}$/ eTonB*XXh*$_{173\text{-}239}$ in place of eTonB*XXh*	This study
pAMT131	pAMT111 with an *Mlu*I site introduced into e*tonBX* at codon 208 (e*tonBXM*)	This study
pAMT132	pAMT112 with an *Mlu*I site introduced into v*tonB1X* at codon 214 (v*tonB1XM*)	This study
pAMT133	pAMT132 encoding eTonB1*XM*$_{1\text{-}208}$/ vTonB1*XM*$_{215\text{-}245}$ in place of vTonB1*XM*	This study
pAMT134	pAMT131 encoding vTonB1*XM*$_{1\text{-}214}$/ eTonB*XM*$_{209\text{-}239}$ in place of eTonB*XM*	This study
pAMT173	pAMT112 encoding eTonB*X*$_{1\text{-}158}$/ vTonB1*X*$_{158\text{-}245}$ in place of vTonB1*X*	This study
pAMT183	pAMT112 encoding eTonB*X*$_{1\text{-}158}$/ vTonB1*X*$_{165\text{-}245}$ in place of vTonB1*X*	This study
pCHU101	pACYC184 carrying *chuA*; Cm^R	(168)
pChuA	pACYC184 carrying *chuA*; Tc^R	This study
pChuAT1F	pChuA encoding a T1F substitution in ChuA	This study
pEENTF::Cm	pWSc-1 (E. E. Wyckoff) carrying *entF::cam*	A. G. Torres
peTonB*X*	pWKS30ΔX carrying *E. coli tonB* with an *Xba*I site introduced at codon 119	This study
pMS101	pBR322 carrying *entD, fepA, fes, entF*	C. F. Earhart
pMTLfepA	pMTL24 (29) carrying *fepA*	This study
pFepA	pACYC184 carrying *fepA*	This study
pFepAD11Y	pFepA encoding a D11Y substitution in FepA	This study

pOUT11	pWSK29 *V. cholerae exbB2, exbD2, tonB2*	(124)
pvTonB1*X*	pWKS30ΔX carrying *V. cholerae tonB1* with an *Xba*I site introduced at codon 121	This study
pv1/eTonB*X*	pWKS30ΔX carrying the hybrid $vTonB1_{1-121}/eTonB_{120-239}$ gene	This study

lysed in an equal volume sterile water, and cellular debris was removed by centrifugation at 7000 × *g*. The supernatant containing hemoglobin was removed, and the concentration of the hemoglobin preparation was calculated by reading the absorbance of heme at 572 nm (A_{572}=5.5 at 1 mM heme). The marine invertebrate hemoglobin preparations were obtained from A. F. Riggs. The preparations were filter-sterilized, and the heme content was calculated as described above. Antibiotics were used at the following concentrations for *E. coli*: 250 μg of carbenicillin per ml, 250 μg of trimethoprim per ml, 50 μg of kanamycin per ml, 30 μg of chloramphenicol per ml, 40 μg of nalidixic acid per ml, and 12.5 μg of tetracycline per ml. For *V. cholerae*, one-third of these concentrations were used, except for nalidixic acid, chloramphenicol, and tetracycline, which were used at one-fourth the concentrations used for *E. coli* strains.

C. Reagents and Enzymes

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Company. Restrictions enzymes, T4 DNA ligase, and DNA polymerase (Klenow) were obtained from New England Biolabs (Beverly, MA). Shrimp alkaline phosphatase and Polynucleotide kinase were purchased from Boehringer Mannheim Corporation (Indianapolis, IN). *Pfu* DNA polymerase was obtained from Stratagene (La Jolla, CA) and *Taq* DNA polymerase was purchased

from Qiagen Inc. (Valencia, CA). All reagents and enzymes were used according to instructions supplied by the manufacturer.

D. Utilization of hemin and siderophores

The ability of *V. cholerae* and *E. coli* strains to use various iron sources was tested in halo assays, in colony size assays and in liquid culture growth assays as described previously (124, 149). In halo assays, the indicated strains were seeded at 10^6 cells per ml into molten L agar containing EDDA. Each iron source to be tested was spotted directly onto the solidified agar, except $FeSO_4$, which was soaked into a sterile filter paper disk on the surface of the solidified agar. Plates were incubated for 24 hrs at 37°C, then for 24 hrs at room temperature, and the diameter of each zone of growth was measured. Colony-size assays were performed by plating 50-100 cells on L agar supplemented with $FeSO_4$, with EDDA, or with EDDA plus hemin or hemoglobin. Colony sizes were measured after 24 hrs of growth at 37°C. In broth culture assays, overnight cultures were diluted to 10^5 cells per ml into L broth supplemented with $FeSO_4$, with EDDA, and with EDDA plus hemin or hemoglobin. The optical densities were measured after 18-24 hrs. The concentrations of EDDA and of the iron sources used in the assays are indicated in each table or figure. All assays were repeated at least 3 times. In order to standardize assay conditions for all strains, antibiotics were not used in the assays; however, hemin and other iron sources

provided a positive selective pressure for plasmid retention under low iron conditions.

E. Isolation of plasmid and cosmid DNA

Plasmid or cosmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen Inc.). For plasmid preparations, 3 ml bacterial culture was processed according to the manufacturer's instructions, and the plasmid DNA was eluted from the QIAGEN column using 50 μl sterile double distilled (dd) H_2O. For cosmid preparations, 5 ml bacterial culture was processed, and the cosmid DNA was eluted from the QIAGEN column using 100 μl sterile ddH_2O at 65°C. Plasmid and cosmid DNA preparations were stored at –20°C.

F. Transformation of bacterial strains

1. TRANSFORMATION OF CACL$_2$-COMPETENT *E. COLI* CELLS

To prepare $CaCl_2$-competent *E. coli* cells, an overnight culture was diluted 1:100 into 25 ml fresh L broth and grown to an OD_{595} of 0.4-0.6. The culture was heated at 50°C for 10 minutes to inactivate nucleases, and then chilled on ice for 10 min. The cells were pelleted by centrifugation at 6000 x *g* for 10 minutes at 4°C and resuspended in 10 ml ice-cold 100 mM $CaCl_2$. After a 25 minute incubation on ice, the cells were harvested by centrifugation at 3000 x *g* for 10 minutes at 4°C and resuspended in 2.0 ml 0.1 M $CaCl_2$ plus 15% glycerol. The

cell suspension was incubated at 4°C overnight and then divided into 0.2 ml aliquots for transformation. Any unused aliquots of competent cells were stored at –80°C. Competent cells were transformed by adding 100-200 ng plasmid DNA to 0.2 ml cells. After incubating for 45 minutes on ice, the cells were heat-shocked for 2 minutes at 42°C and chilled on ice for 2 minutes. The cells were added to 1.6 ml L broth for recovery at 37°C for one hour, and then plated on L agar supplemented with the appropriate antibiotics. For iron uptake-deficient strains, or for strains transformed with a plasmid carrying a potentially toxic iron-regulated gene, 40 μM $FeSO_4$ was added to the L agar plates as well.

2. ELECTROPORATION OF *V. CHOLERAE*

Electroporation of *V. cholerae* were carried out as described previously (124). Briefly, *V. cholerae* strains grown overnight in BHI were diluted 1:100 into fresh BHI and grown to an OD_{650} of 0.4-0.6. Cells were harvested by centrifugation at 6000 x *g* for 10 minutes at 4°C and then resuspended in 20 ml ice-cold electroporation buffer G (138 mM sucrose, 1 mM Hepes, pH 8). The cells were pelleted at 2000 x *g* for 15 minutes at 4°C and washed again with 20 ml G buffer. The cells were resuspended in a final volume of 1 ml G buffer, and 0.4 ml of cells were added to a pre-chilled 0.2 cm Gene Pulser® electroporation cuvette (Bio-Rad). For electroporation, 200-500 ng plasmid DNA was added to the cells just prior to pulsing at 2.4 kV, 25 μF, and 200 Ω, and the cells were transferred immediately to 1.6 ml BHI and incubated for one hour at 37°C. The

cells were then plated to selective media as described above for transformation of $CaCl_2$-competent *E. coli* strains.

3. CONJUGATION

One ml overnight cultures of the donor strain, the recipient strain, and the mobilizing strain (in the case of tri-parental conjugations) were washed in L broth and resuspended in 0.1 ml L broth. A mixture containing 25 μl of each strain was spotted onto an L agar plate and incubated for 6-8 hours at 37°C. The cells were scraped into 1 ml L broth, and transconjugants were obtained by plating the cell suspension on media selective for the recipient strain and for the transferred plasmid or cosmid.

G. Polymerase chain reaction (PCR)

The oligonucleotide primers for PCR were purchased from IDT Inc. (Coralville, IA). All primers used in this study are listed in Table 2. The primers for PCR-amplification of previously uncharacterized *V. cholerae* sequences were designed using the published DNA sequence for *V. cholerae* El Tor N16961(67). PCR was performed in a GeneAmp PCR system 2400 (Perkin-Elmer) as follows: 1 μl bacterial culture (template) was mixed with 250 μM each dNTP, 1 μM each primer, 2.5 U *Pfu* polymerase (Stratagene) or *Taq* polymerase (Qiagen), and the supplied polymerase reaction buffer (1X final concentrations), in a total volume of 100 μl. After a 5 min incubation at 94°C, the reactions underwent 30 cycles of

Table 2. Oligonucleotide primers used in this study.

original name	new name	primer sequence (5'-3')	primer location	primer features
V. cholerae tonB primers				
v482(+)	v1	TTCTCAAGGCAGTC GACATACAACAAGG GCTGCGC	upstream of v*tonB1*	contains a *Sal*I site (underlined)
v1129X(-)	v2	GCCATTTCAGCGGT TCTAGACTCTACCTT CTCCGG	internal v*tonB1*	introduces an *Xba*I site; results in the insertion of a Ser residue at amino acid position 120 in vTonB1
v1098X(+)	v3	CCGGAGAAGGTAGA GTCTAGAACCGCTG AAATGGC	internal v*tonB1*	introduces an *Xba*I site; results in the insertion of a Ser residue at amino acid position 120 in vTonB1
v1517(-)	v4	GCTGGTGTTGGAGT TGTTGTAACGGATC CATTAGCC	downstream of v*tonB1*	contains a *Bam*HI site
v1470(+)	v5	AACTGCAGGAGGGA ATAATGGAATCGTT ACAACAACTCC	upstream of v*exbB*	contains a *Pst*I site; contains a Shine-Dalgarno sequence upstream of *exb*B1 for efficient translation of v*exbBD1hutBCD*

v2596(-)	v6	CTTGGATCCATTAA GAGTGAGAAGG	downstream of v*exbD1*	contains a *Bam*HI site
v5393B(-)	v7	GTCAATGGATCCTT CACCTTCTGCC	downstream of v*hutD*	contains a *Bam*HI site
v1276Xho(+)	v8	GCGGGCTCGAGGGC ACTGTG	internal v*tonB1*	introduces a *Xho*I site; results in an I178L substitution in vTonB1*X*
v1295Xho(-)	v9	CACAGTGCCCTCGA GCCCGC	internal v*tonB1*	introduces a *Xho*I site; results in an I178L substitution in vTonB1*X*
v1386Mlu(+)	v10	GCTCGACGCGTTGA AACAGTG	internal v*tonB1*	introduces an *Mlu*I site; results in an E213D and an I215L substitution in vTonB1*X*
v1405Mlu(-)	v11	CACTGTTTCAACGC GTCGAGCG	internal v*tonB1*	introduces an *Mlu*I site; results in an E213D and an I215L substitution in vTonB1*X*
v1221(+)	v12	CCCGCTTTAGTGAG CGC	internal v*tonB1*	no changes in the v*tonB1* sequence
v1242(+)	v13	GTTCAACCACGTTA TCCACG	internal v*tonB1*	no changes in the v*tonB1* sequence
E. coli tonB primers				
e167(+)	e1	AGATCTGCAACGGA AAGTCGACGTCTTT GTTAAGGCC	upstream of e*tonB*	contains a *Sal*I site

e719X(-)	e2	GTATTTTCAAACGG TGATGCCGGTCTAG ACTCTACGG	internal e*tonB*	introduces an *Xba*I site; no changes in the amino acid sequence of eTonB
e685X(+)	e3	GTAGAGTCTAGACC GGCATCACCGTTTG AAAATACGG	internal e*tonB*	introduces an *Xba*I site; no changes in the amino acid sequence of eTonB
e1079(-)	e4	GTCGGATCCTTTTG ACCTGCAGCTTACT GAATTTCGG	downstream of e*tonB*	contains *Pst*I and *Bam*HI sites
e841Xho(+)	e5	GCATTGCGCCTCGA GGGGCAGG	internal e*tonB*	introduces a *Xho*I site; results in an I172L substitution in eTonB*X*
e862Xho(-)	e6	CCTGCCCCTCGAGG CGCAATGC	internal e*tonB*	introduces a *Xho*I site; results in an I172L substitution in eTonB*X*
e950Mlu(+)	e7	AGGTGAAAAACGCG TTGCGC	internal e*tonB*	introduces an *Mlu*I site; results in an M210L substitution in eTonB*X*
e978Mlu(-)	e8	CGCAACGCGTTTTT CACCTC	internal e*tonB*	introduces an *Mlu*I site; results in an M210L substitution in eTonB*X*
e810(-)	e9	ACGGCTTAATGCGC GTGG	internal e*tonB*	no changes in the e*tonB* sequence
V. cholerae receptor primers				
hutA –138(+)Nco	hutA1	TCTCTCCCATGGAT AGTTCACACCG	5’ end *hutA* promoter region	contains an *Nco*I site
hutA 80(-)Bm	hutA2	GCAGGATCCATGTT GAGTAAAACGC	3’ end *hutA* promoter region	contains a *Bam*HI site

hutA 779(-)	hutA3	ACTTGACTAGTAAA TTATTAGC	internal *hutA*	no changes in the *hutA* sequence
5'hutA/fepbox	hutA4	ACTATTGTCGTTACC GCCACTCGCTTGAA TACTCAAATAACTG	*hutA* TonB box region	replaces the TonB box of HutA with that of FepA
3'hutA/fepbox	hutA5	GGCGGTAACGACAA TAGTATCGAATGAG GCATAATCATCCG	*hutA* TonB box region	replaces the TonB box of HutA with that of FepA
5'hutA/chuAbox	hutA6	GAAACCATGACCGT TACGGCAACTCGCT TGAATACTC	*hutA* TonB box region	replaces the TonB box of HutA with that of ChuA
3'hutA/chuAbox	hutA7	TGCCGTAACGGTCA TGGTTTCGAATGAG GC	*hutA* TonB box region	replaces the TonB box of HutA with that of ChuA
hbr 2955(+)RI	hutR1	GTGAATTCGCGTTC TTTCTCACC	upstream of *hutR*	contains an *Eco*RI site
hrp 5003(-)RI	hutR2	TGTGAATTCCATTG GTAGTTGAGGC	internal *hutR*	contains an *Eco*RI site
hbr 5178(-)Sal	hutR3	TAAGGTCGACGTTG ATTTCATC	downstream of *hutR*	contains a *Sal*I site
hutR 5187(-)RI	hutR4	GGCTAGAATTCAGG TCAACG	downstream of *hutR*	contains an *Eco*RI site
hasR 643(+)	hasR1	CGAGGCTTTTGGCT AAACGG	upstream of *hasR*	
hasR 3859(-)	hasR2	TGGCGAATCAGCAC CAGC	downstream of *hasR*	

3'irgB(RI)	irgBA1	AGTGAATTCAGCTA AAGAACTGGTGG	downstream of *irgB*	contains an *Eco*RI site
3'irgA(RI)	irgBA2	GGGAATTCTAACCG ATACTCTAGGC	downstream of *irgA*	contains an *Eco*RI site
hrp 777(+)RI	ptrB1	CTGGAATTCGACAT CGACTAAGCC	upstream of *ptrB*	contains an *Eco*RI site
hbr 3076(-)Sal	ptrB2	CAGTGTCGACATGA GCGAACCC	downstream of *ptrB*	contains a *Sal*I site
hbr 794(+)Nco	ptrB3	CTAAGCCATGGTGT ACAACTTAGC	5' end *ptrB* promoter region	contains an *Nco*I site
hbr 992(-)Bm	ptrB4	TAACGGATCCAAAT CGGTCGC	3' end *ptrB* promoter region	contains a *Bam*HI site
E. coli receptor primers				
chuA(+)RV/H3	chuA1	AGAGATATCGAAGC TTGCAGAGC	upstream of *chuA*	contains an *Eco*RV site and a *Hin*dIII site
chuA 780(-)	chuA2	CGCGATGATCATAG CCACGC	internal *chuA*	no changes in the *chuA* sequence
5'chuA/hutbox	chuA3	ACTGATGAAGTAGT AGTATCTACAACGG GGAATGCC	*chuA* TonB box region	replaces the TonB box of ChuA with that of HutA
3'chuA/hutbox2	chuA4	TGTAGATACTACTA CTTCATCAGTAGCA AAAGCAAACG	*chuA* TonB box region	replaces the TonB box of ChuA with that of HutA

5'chuA.T1F	chuA5	GCTTTTGCTTTCGAA ACCATGACCG	*chuA* TonB box region	introduces a T1F mutation into ChuA
3'chuA.T1F	chuA6	CATGGTTTCGAAAG CAAAAGCAAACG	*chuA* TonB box region	introduces a T1F mutation into ChuA
fepA118(s)	fepA1	GCGTAATGAGCTCC GTGGAAGCG	upstream of *fepA*	contains a *Sac*I site
fepA 1697(-)	fepA2	TCATCGCCTAAACC TTGCG	internal *fepA*	no changes in the *fepA* sequence
5'fepA.D11Y	fepA3	TTCACATTACGATA CTATTGTCG	*fepA* TonB box region	introduces a D11Y mutation into FepA
3'fepA.D11Y	fepA4	ATAGTATCGTAATG TGAAACAGG	*fepA* TonB box region	introduces a D11Y mutation into FepA

94°C for 30 sec; 50°C for 30 sec; 72°C for 2 min per kb (*Pfu*) or 1 min per kb (*Taq*), followed by a final extension at 72°C for 5 min per kb (*Pfu*) or 3 min per kb (*Taq*).

H. Plasmid construction

1. CONSTRUCTION OF PLASMIDS ENCODING HYBRID TONBS

Gene fusions between *E. coli tonB* and *V. cholerae tonB1* were created by engineering unique restriction sites into the cloned *E. coli* and *V. cholerae tonB1* genes by PCR, and exchanging equivalent *tonB* fragments. All clones derived from PCR fragments were verified by sequencing. The *Xba*I, *Xho*I, and *Mlu*I sites in *E. coli tonB* and in *V. cholerae tonB1* were introduced by overlap extension PCR using primers described in Table 2 (page 49). For example, to introduce the *Xba*I site into *E. coli tonB*, primer pairs e1/e2 and e3/e4 were used to create overlapping fragments containing an *Xba*I site. The overlapping PCR products were gel-purified and added in equimolar amounts as template for a second round of PCR in which the full-length *E.coli tonB* gene containing the *Xba*I site was created by polymerase extension of the overlapping PCR fragments followed by amplification of the resulting full-length e*tonBX* gene using primer pair e1/e4. An *Xba*I site was similarly introduced into *V. cholerae tonB1* using primers v1/v2 and v3/v4 to create the overlapping fragments, and primers v1/v4 to amplify the polymerase-extended final product containing v*tonB1X*. The e*tonB1X* and the v*tonB1X* PCR products were digested with *Sal*I and *Bam*HI and cloned into

pWKS30Δ*X* digested with *Sal*I and *Bam*HI to create peTonB*X* and pvTonB1*X*, respectively (Fig. 5A). The *Xba*I-*Bam*HI fragments of peTonB*X* and pvTonB1*X* were exchanged to create pe/v1TonB*X* and pv1/eTonB*X* (Fig. 5A).

Plasmids carrying native or hybrid *tonBX* genes in the context of the entire *V. cholerae tonB1* operon were constructed as follows: A fragment containing the 3' half of the v*tonB1X* gene and the v*exbBD1*, *hutBCD* genes was amplified by PCR using primers v3 and v7. The resulting PCR product was digested with *Xba*I and *Bam*HI and cloned into pvTonB1*X* or peTonB*X* digested with *Xba*I and *Bam*HI to create pAMT112 and pAMT113, respectively (Fig. 5B). To create pAMT111 and pAMT114, a fragment containing only the v*exbBD1* and *hutBCD* genes was amplified using primers v5 and v7. The PCR product was digested with *Pst*I and *Bam*HI and cloned into peTonBX or pv/eTonBX digested with *Pst*I and *Bam*HI, respectively (Fig. 5C).

To construct plasmid series pAMT101-4, the *Sal*I-*Bam*HI fragments from pAMT111-4 were cloned into pACYC184 digested with *Sal*I and *Bam*HI.

To introduce an *Xho*I or an *Mlu*I site into e*tonBX*, overlapping PCR products were created with primer sets e1/e6 and e5/v6 (*Xho*I) or with primer sets e1/e8 and e7/v6 (*Mlu*I) using pAMT101 as the template. The overlap extension products were amplified using primer set e1/v6. An *Xho*I or an *Mlu*I site was similarly introduced into v*tonB1X* using primer sets v1/v9 and v8/v6 (*Xho*I) or v1/v11 and v10/v6 (*Mlu*I) to create overlapping fragments from the pAMT102 template and primer set v1/v6 to amplify the overlap extension products. The final PCR products were digested with *Sal*I and *Kpn*I and cloned into pAMT101

Figure 5. Construction of plasmids carrying *E. coli* and *V. cholerae* hybrid *tonB* genes.

A. Construction of plasmids peTonB*X*, pvTonB1*X*, pe/v1TonB*X* and pv1/eTonB*X*.

B. Construction of plasmids pAMT112 and pAMT113

C. Construction of plasmids pAMT111 and pAMT114

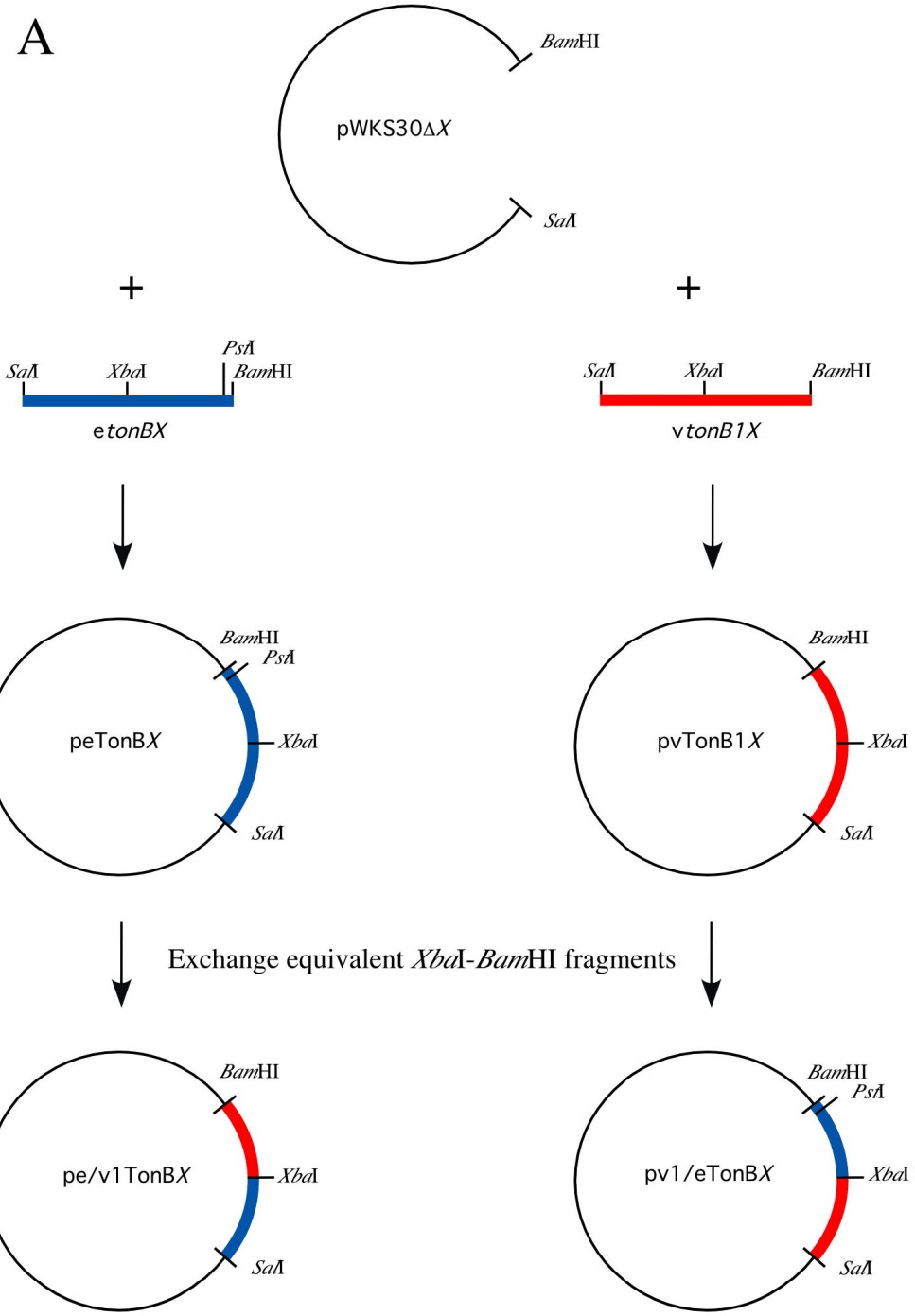
D. Construction of plasmids pAMT121 and pAMT131

E. Construction of plasmids pAMT122 and pAMT132

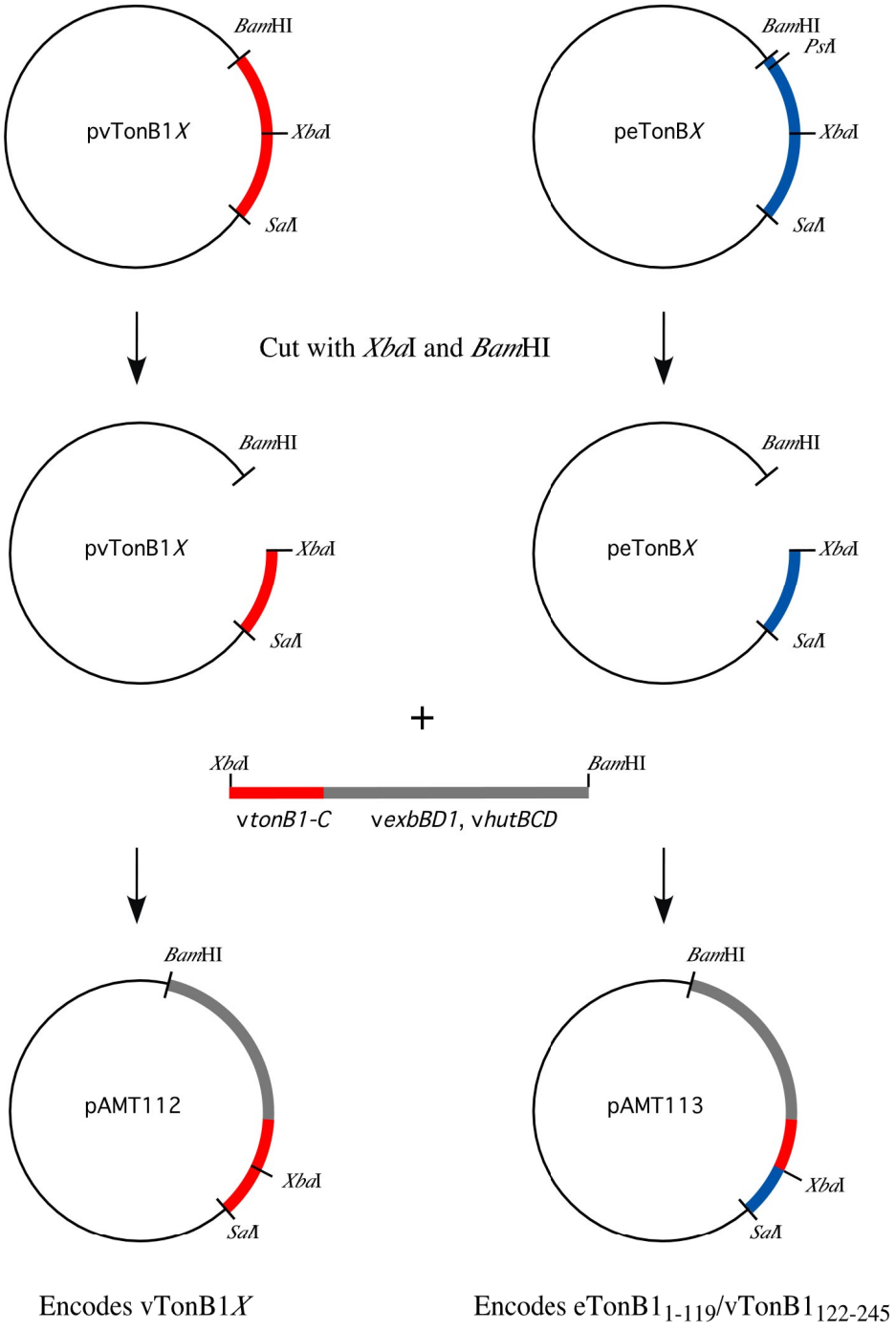
F. Construction of plasmids pAMT123 and pAMT124

G. Construction of plasmids pAMT133 and pAMT133

H. Construction of plasmids pAMT173 and pAMT183


A
BamHI
pWKS30ΔX
SalI
+
+
SalI
XbaI
PstI
BamHI
etonBX
SalI
XbaI
BamHI
vtonB1X
BamHI
PstI
peTonBX
XbaI
SalI
BamHI
pvTonB1X
XbaI
SalI
Exchange equivalent XbaI-BamHI fragments
BamHI
pe/v1TonBX
XbaI
SalI
BamHI
PstI
pv1/eTonBX
XbaI
SalI

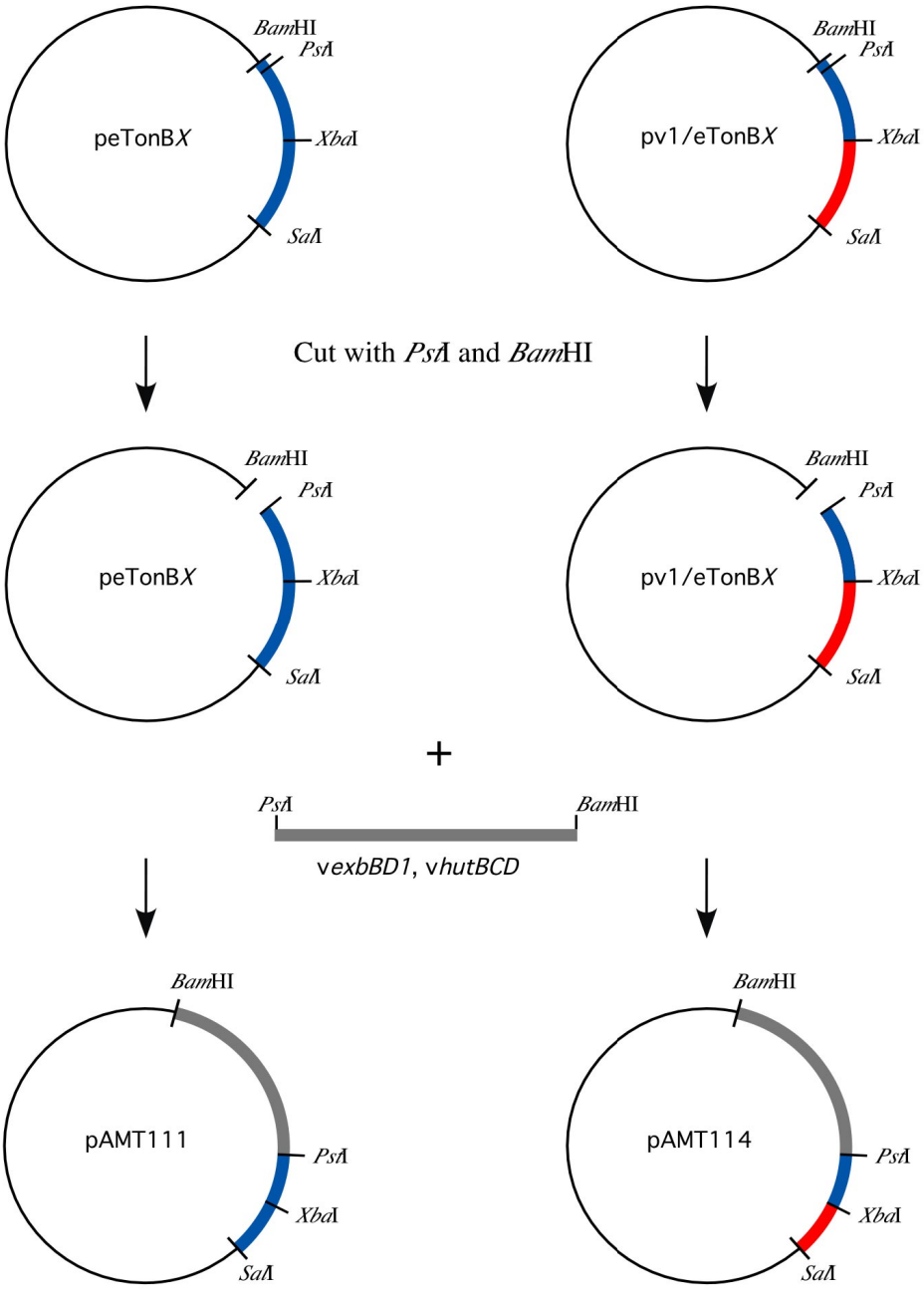
B



Encodes vTonB1*X*

Encodes eTonB1$_{1\text{-}119}$/vTonB1$_{122\text{-}245}$

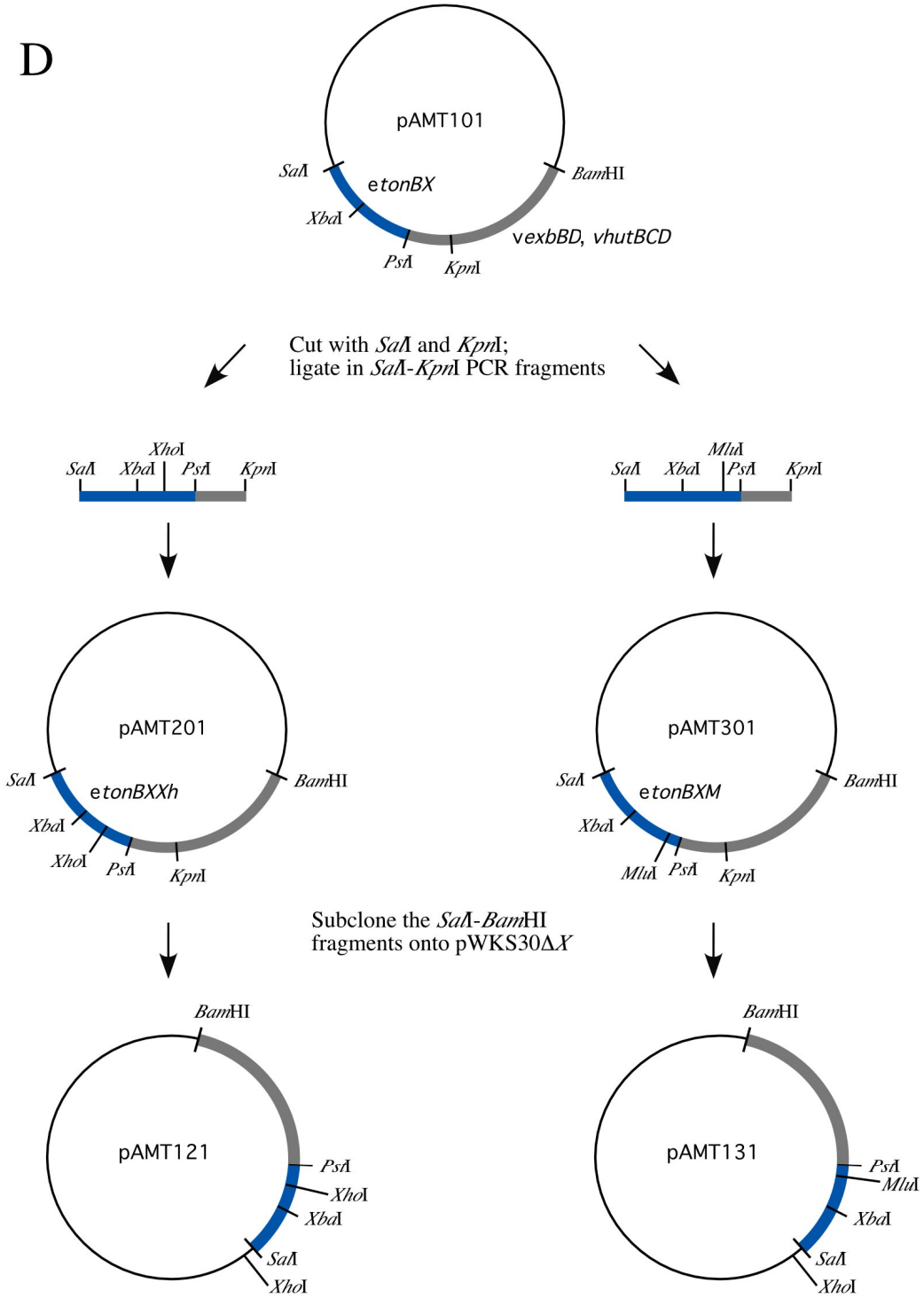
C



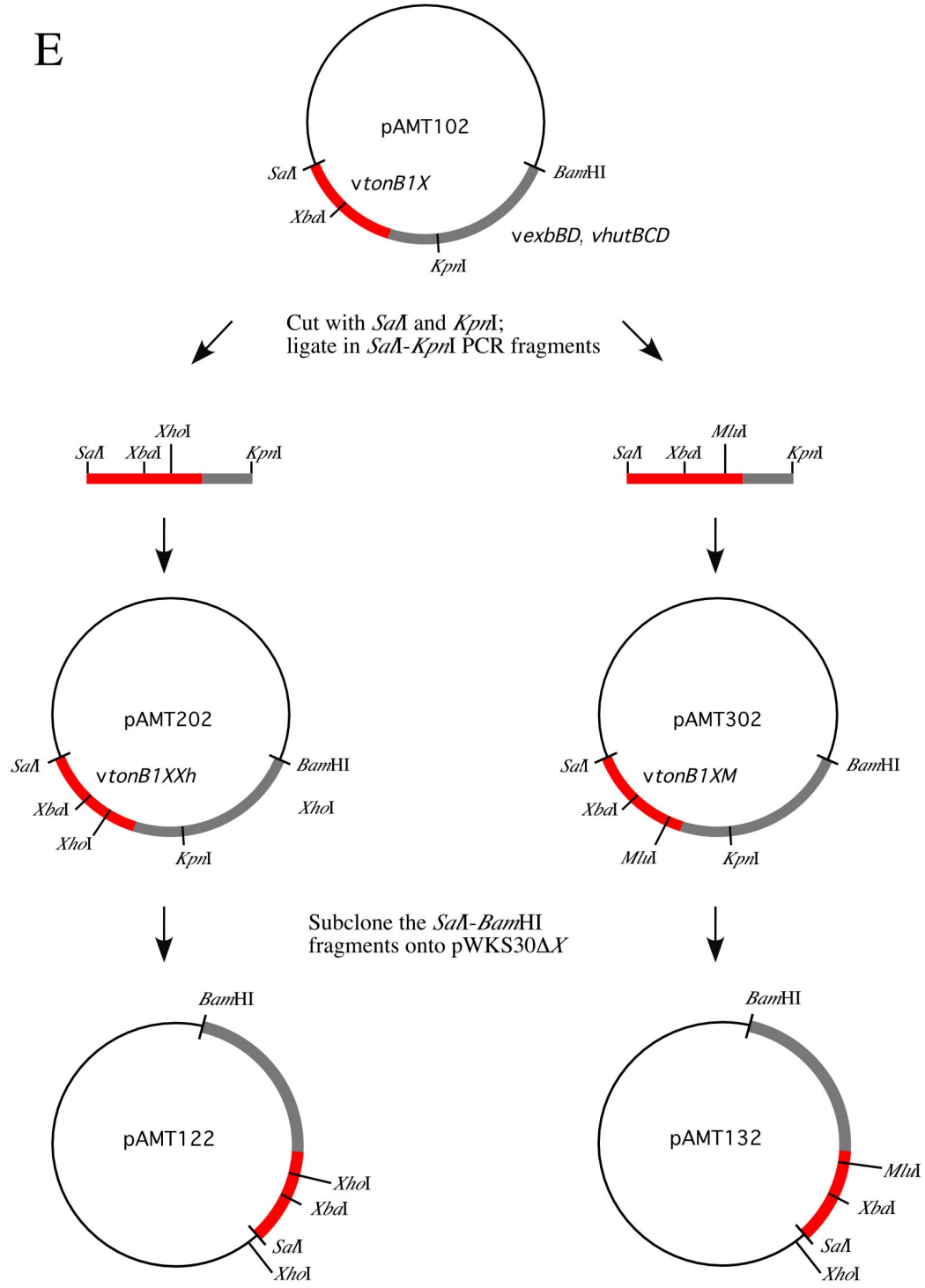
Encodes eTonB*X*

Encodes vTonB1$_{1\text{-}121}$/eTonB$_{120\text{-}239}$

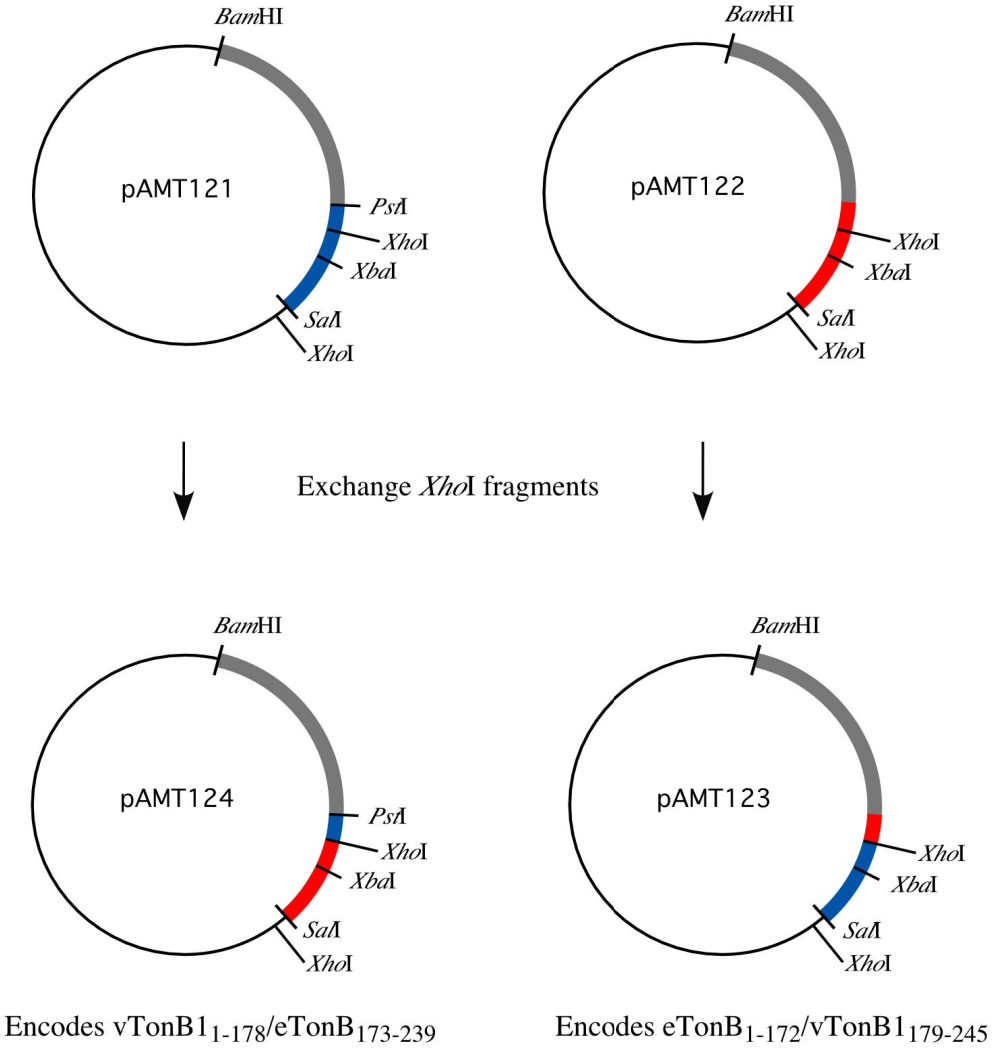
D


pAMT101
SalI
BamHI
etonBX
XbaI
vexbBD, vhutBCD
PstI
KpnI
Cut with SalI and KpnI;
ligate in SalI-KpnI PCR fragments
XhoI
SalI
XbaI
PstI
KpnI
MluI
SalI
XbaI
PstI
KpnI
pAMT201
SalI
BamHI
etonBXXh
XbaI
XhoI
PstI
KpnI
pAMT301
SalI
BamHI
etonBXM
XbaI
MluI
PstI
KpnI
Subclone the SalI-BamHI
fragments onto pWKS30ΔX
BamHI
pAMT121
PstI
XhoI
XbaI
SalI
XhoI
BamHI
pAMT131
PstI
MluI
XbaI
SalI
XhoI

E


pAMT102
SalI
vtonB1X
BamHI
XbaI
vexbBD, vhutBCD
KpnI
Cut with SalI and KpnI;
ligate in SalI-KpnI PCR fragments
XhoI
SalI
XbaI
KpnI
MluI
SalI
XbaI
KpnI
pAMT202
SalI
vtonB1XXh
BamHI
XbaI
XhoI
XhoI
KpnI
pAMT302
SalI
vtonB1XM
BamHI
XbaI
MluI
KpnI
Subclone the SalI-BamHI
fragments onto pWKS30ΔX
BamHI
pAMT122
XhoI
XbaI
SalI
XhoI
BamHI
pAMT132
MluI
XbaI
SalI
XhoI

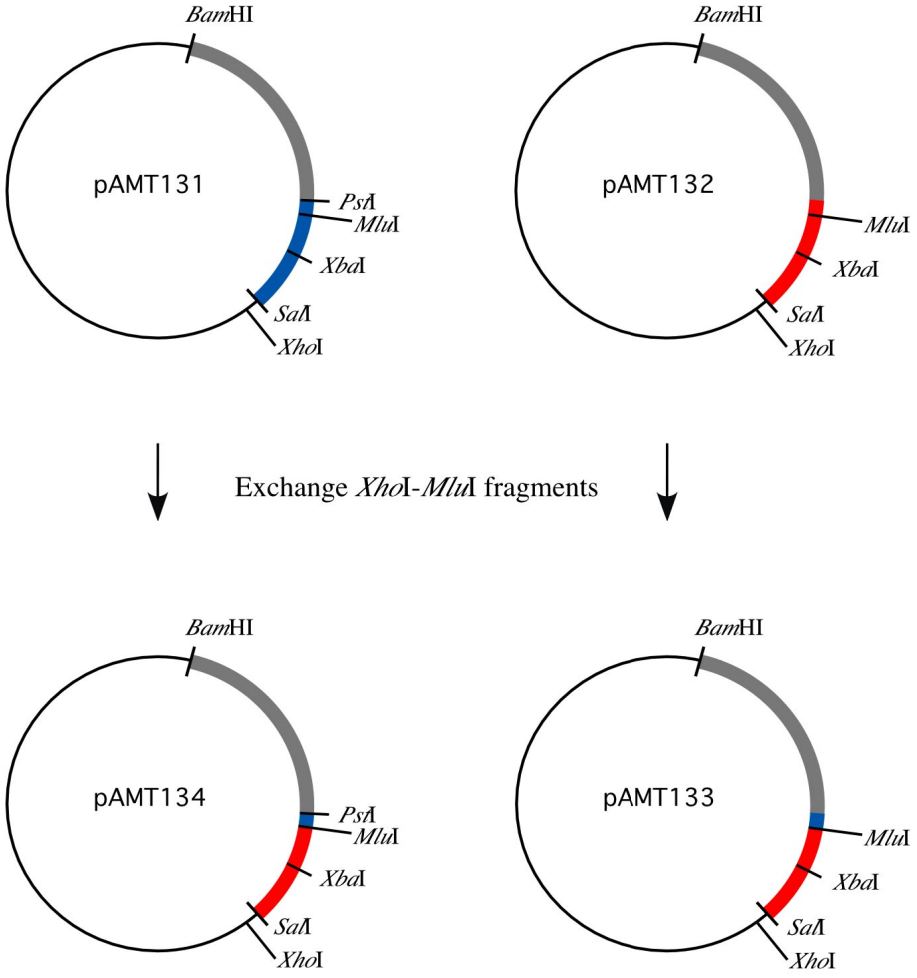
F



Encodes vTonB1$_{1\text{-}178}$/eTonB$_{173\text{-}239}$

Encodes eTonB$_{1\text{-}172}$/vTonB1$_{179\text{-}245}$

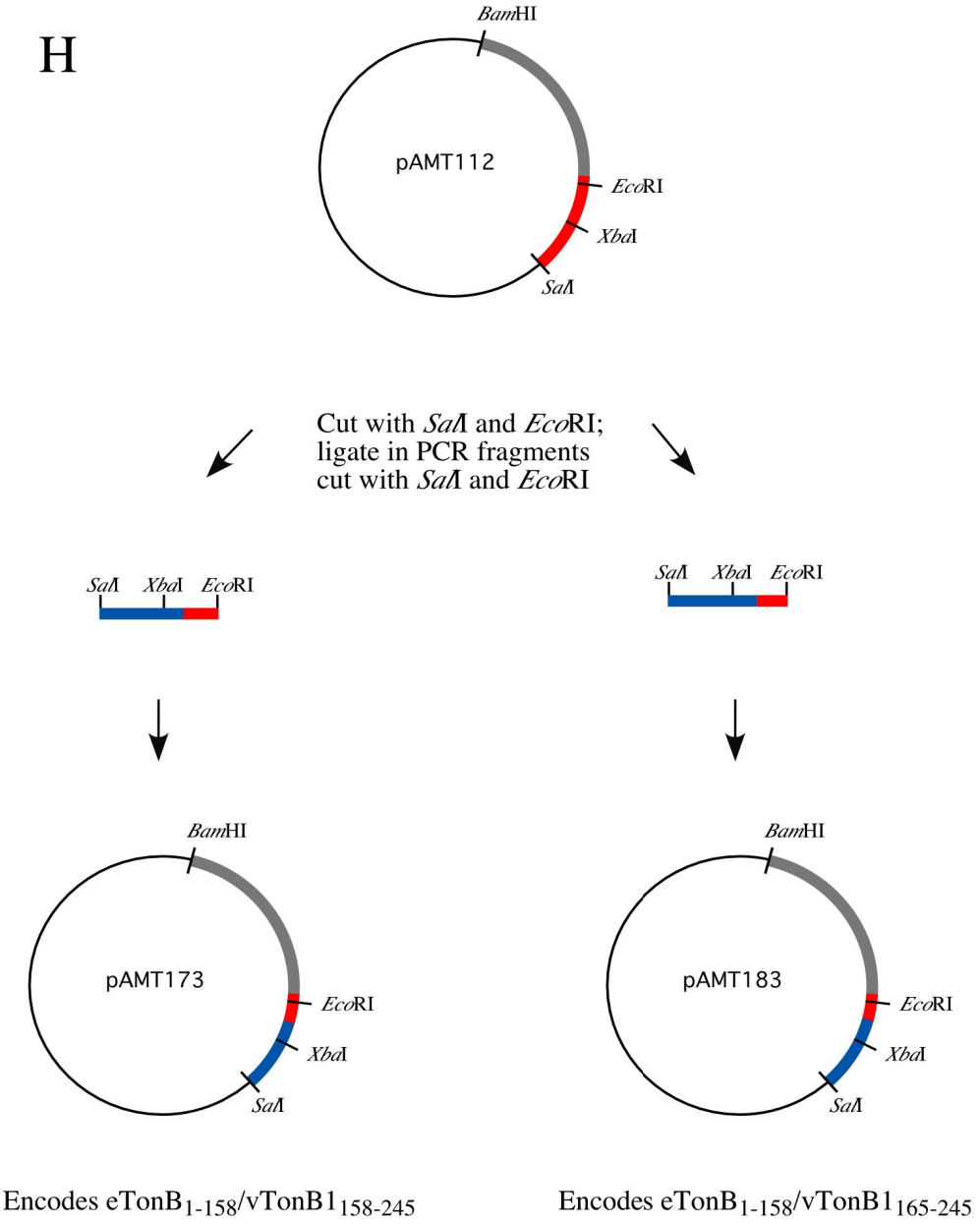
G



Encodes vTonB1$_{1\text{-}214}$/eTonB$_{209\text{-}239}$

Encodes eTonB$_{1\text{-}208}$/vTonB1$_{215\text{-}245}$

H



Encodes eTonB$_{1\text{-}158}$/vTonB1$_{158\text{-}245}$

Encodes eTonB$_{1\text{-}158}$/vTonB1$_{165\text{-}245}$

(e*tonB* products) or pAMT102 (v*tonB1* products) digested with *Sal*I and *Kpn*I to yield plasmids pAMT201 (e*tonBXX*), pAMT301 (e*tonBXM*), pAMT202 (v*tonB1XX*), and pAMT302 (v*tonB1XM*).

To create plasmid series pAMT121-4, the *Sal*I-*Bam*HI fragments from pAMT201 and from pAMT202 were first cloned into pWKS30Δ*X* digested with *Sal*I and *Bam*HI to create pAMT121 (Fig. 5D) and pAMT122 (Fig. 5E), respectively. The pWKS30Δ*X* vector backbones contain a *Xho*I site within the sequence upstream of the *Sal*I site used to clone in the *tonB* inserts. Hybrid *tonB* constructs were created by exchanging *Xho*I fragments between pAMT121 and pAMT122 to give rise to pAMT123 and pAMT124 (Fig. 5F).

To construct plasmid series pAMT131-4, the *Sal*I-*Bam*HI fragments from pAMT301 and from pAMT302 were cloned into pWKS30Δ*Xba*I digested with *Sal*I and *Bam*HI to create pAMT131 (Fig. 5D) and pAMT132 (Fig. 5E), respectively. Hybrid *tonB* constructs were created by exchanging *Xho*I-*Mlu*I fragments between pAMT131 and pAMT132 to give rise to pAMT133 and pAMT134 (Fig. 5G).

Hybrid *tonB* genes were also created by ligating together phosphorylated *E. coli* or *V. cholerae tonB* PCR fragments followed by PCR-amplification of the desired full-length *tonB* ligation product. The hybrid *tonB* genes contained in pAMT173 and pAMT183 were created by PCR amplification of a portion of the e*tonBX* gene using primers e1/e9, and amplification of a portion of the v*tonB1X* gene using primers v12/v6 or primers v13/v6. The gel-purified PCR products were 5' phosphorylated and ligated together, and the desired ligation products

were amplified directly from the ligation reactions using primers e1/v6. The amplified hybrid *tonB* ligation products were gel-purified and digested with *Sal*I and *Eco*RI and cloned into pAMT112 digested with *Sal*I and *Eco*RI to create pAMT173 and pAMT183 (Fig. 5H). All clones derived from PCR fragments were verified by sequencing.

2. CONSTRUCTION OF PLASMIDS ENCODING OUTER MEMBRANE RECEPTORS

To construct pAMH20, the ~3 kb *Hind*III-*Sal*I fragment containing the *hutA* gene was excised from pHUT3, blunted with Klenow, and cloned into the *Sca*I site of pACYC184. To exchange the TonB box of HutA with that of either FepA or ChuA, overlapping PCR fragments were created with primer sets P1/hutA5 and hutA4/hutA3 (FepA TonB box) or P1/hutA7 and hutA6/hutA3 (ChuA TonB box) using pAMH20 as template. Primer pair P1/hutA3 was used to amplify the mutant overlap extension products, and the final PCR products were digested with *Bsu*36I and *Spe*I and cloned into pAMH20 digested with *Bsu*36I and *Spe*I to create pAMH21 or pAMH23. The hybrid receptor genes were verified by sequencing.

To construct pAMR12, the *hutR* gene was amplified from CA40130N by PCR with *Pfu* polymerase using primers hutR1 and hutR3. The PCR fragment was sequenced to ensure that no mutations were introduced during the amplification. To allow expression of the promoterless *hutR* gene, the 2.2 kb PCR product was digested with *Eco*RI and cloned in the same orientation as *lacZ* into pWKS30 digested with *Eco*RI and *Sma*I to yield pAMR12. To construct

pAMR14, the *hutR* gene was PCR-amplified from CA40130N using *Pfu* polymerase and primers hutR1 and hutR4. The PCR product was digested with *Eco*RI and cloned in the orientation of the *cam* gene in pACYC184 to yield pAMR14.

To construct pAMR18, a fragment containing *irgA* and *irgB* was PCR-amplified using *Pfu* polymerase and primers irgBA1 and irgAB2. The 3.2 kb PCR product was digested with *Eco*RI and cloned into the *Eco*RI site of pACYC184. The *irgBA* insert was moved as an *Eco*RI fragment to pWKS30 to yield pCAT121.

To construct pChuA, the ~2.7 kb *Eco*RV fragment containing the *chuA* gene was excised from pCHU101 and cloned into pACYC184 digested with *Sca*I. To introduce the HutA TonB box or the T1F mutation into ChuA, primer sets chuA1/chuA4 and chuA3/chuA2 (HutA TonB box) or chuA1/chuA6 and chuA5/chuA2 (T1F) were used to amplify overlapping fragments from the pCHU101 template. The full-length overlap extension products were amplified using primers chuA1/chuA2, and the final mutant products were digested with *Hpa*I and *Kpn*I and ligated into pChuA digested with *Hpa*I and *Kpn*I to create pAMC23 or pChuAT1F. The mutations were verified by sequencing.

To construct pFepA, the ~2.5 kb *Ssp*I-*Stu*I fragment containing the *fep*A gene was excised frompMS101and cloned into pACYC184 cut with *Sca*I and *Pvu*II. The $FepA_{D11Y}$ mutation was introduced into FepA by creating overlapping mutant fragments using primer sets fepA1/fepA4 and fepA3/fepA2, and pFepA as template. After overlap extension, the full-length fragment containing the

mutation was amplified using primers fepA1/fepA2. The PCR product was digested with *Bsu*36I and *Eco*RI and cloned into pFepA digested with *Bsu*36I and *Eco*RI to create pFepAD11Y. The mutation was verified by sequencing.

3. CONSTRUCTION OF SUICIDE PLASMIDS FOR ALLELIC EXCHANGE IN *E. COLI*

To construct pAMS7, the ~3.8 kb *Sal*I-*Eco*RV fragment containing *entF::cam* was excised from pEENTF::Cm and cloned into pHM5 digested with *Sal*I and *Eco*RV.

To disrupt the *fepA* gene, a *cam* cassette was inserted as a *Kpn*I fragment into the *Kpn*I site within *fepA* in pMTLfepA. The *fepA::cam* insert was excised as a *Sal*I fragment and cloned into pHM5 digested with *Sal*I to create pAMS8.

4. CONSTRUCTION OF SUICIDE PLASMIDS FOR ALLELIC EXCHANGE IN *V. CHOLERAE*

To create an insertional mutation in *hutA*, the kanamycin resistance cassette from pUC4K (Pharmacia) was cloned as a *Pst*I fragment into the *Nsi*I site in the *hutA* gene on pHUT3. The disrupted *hutA* gene was subcloned as a *Hinc*II fragment into the *Eco*RV site of pHM5 to create pAMS1.

To disrupt *hutR*, a fragment containing *ptrB* and *hutR* was amplified by PCR with *Pfu* polymerase using primers ptrB1 and hutR2. The 4.2 kb PCR fragment was digested with *Eco*RI and cloned into the *Eco*RI site of pACYC184 to yield pAMR1. The *Eco*RV-*Xba*I fragment of pAMR1 containing *hutR* was

then subcloned into the *Eco*RV and *Xba*I-digested pHM5, and the chloramphenicol resistance cassette (*cam*) from PMTLcam (E. E. Wyckoff) was inserted into the *Nru*I site in *hutR* to create pAMS2.

To construct a polar insertion in *ptrB*, a 2.3 kb fragment containing *ptrB* was PCR-amplified with *Taq* polymerase using primers ptrB1 and ptrB2. The PCR product was digested with *Eco*RI and *Sal*I and cloned into pWKS30 digested with *Eco*RI and *Sal*I to yield pAMP1. The *ptrB* gene was disrupted by insertion of the *cam* cassette into the Klenow-blunted *Nhe*I site in *ptrB*. The *ptrB::cam* fragment was excised using *Sal*I and *Sma*I and inserted into pHM5 digested with *Sal*I and *Eco*RV to create pAMS3.

To create a mutation in *hasR*, a fragment containing *hasR* was PCR-amplified from the *V. cholerae* El Tor strain Lou15 using Taq polymerase and primers hasR1 and hasR2. The PCR fragment was digested with *Bam*HI and *Eco*RI, and cloned into *Bam*HI and *Eco*RI-digested pWKS30 to yield pAMR16. The *hasR* gene was disrupted by inserting a trimethoprim (*tmp*) cassette (E. E. Wyckoff) into the *Msc*I site. The *hasR::tmp* fragment was excised using *Xho*I and *Spe*I, and cloned into pHM5 cut with *Sal*I and *Xba*I to yield pAMS4. During the allelic exchange procedure it was discovered that exchanging just the 5' end of the *hasR* gene in ARM915 with the equivalent region from *hasR* in pAMS4 completely abolished hemin utilization in the resulting strain, ARM932. It was determined by sequence analysis that the *hasR* allele present in pAMS4 and in ARM932, but not in ARM915, contained a PCR-introduced mutation that causes

an L9P substitution within the putative signal sequence of HasR. This substitution most likely interferes with export of HasR to the outer membrane.

To inactivate *irgA*, the *cam* cassette was inserted as a *Sma*I fragment into the *Sma*I site of the *irgA* gene carried on pAMR18. The *irgA::cam* insert was moved as a Klenow-blunted *Cla*I fragment into pHM5 digested with EcoRV to yield pAMS5.

The construct pAMS6 carrying the disrupted *vhrA* gene, the ~2.3 kb *Hpa*I-*Sal*I fragment from pAMR30 was cloned into pHM5 digested with *Eco*RV and *Sal*I, and the *tmp* cassette was inserted as a *Sma*I fragment into the Klenow-blunted *Nco*I site within *vhrA*.

5. CONSTRUCTION OF PROMOTER FUSION PLASMIDS

The *hutA* promoter region was PCR-amplified from CA40130N with *Pfu* polymerase using primers hutA1 and hutA2; the *ptrB* promoter region was similarly amplified using primers ptrB3 and ptrB4. The PCR fragments were digested with *Nco*I and *Bam*HI and cloned into pQF50 digested with *Nco*I and *Bam*HI to create the *hutA-lacZ* (pAMP20) and the *ptrB-lacZ* (pAMP21) transcriptional fusions.

I. Construction of chromosomal mutations in *E. coli* and *V. cholerae* genes

Cloned *E. coli* or *V. cholerae* genes were disrupted by insertion of an antibiotic resistance cassette, and transferred to the λ*pir*-dependent suicide vector pHM5, which also contains the *sacB* gene conferring sensitivity to sucrose. The resulting suicide plasmids were transferred by mating from *E. coli* SM10λ*pir* into the *E. coli* or *V. cholerae* recipient strain, and transconjugants were selected as described above. After growth overnight, *E. coli* transconjugants were plated directly on selective media containing 5% (w/v) sucrose, whereas *V. cholerae* transconjugants were diluted 1:100 into L broth containing 5% (w/v) sucrose and grown for 3-4 hours, and then plated on selective media containing 10% (w/v) sucrose. Because *V. cholerae* strains carrying the *sacB* gene encoded by pHM5 are not lysed immediately when grown on sucrose, it was necessary to distinguish sucrose resistant *V. cholerae* colonies by colony morphology: sucrose-resistant colonies appeared clear and flat, whereas sucrose-sensitive colonies appeared opaque and globular and underwent lysis after approximately 24 hours. *E. coli* or *V. cholerae* colonies that were resistant to sucrose and to the antibiotic specified by the marked allele were screened for sensitivity to carbenicillin to verify loss of the suicide plasmid from the strain. The allelic exchange mutants were analyzed by PCR using primers annealing to chromosomal sequences outside of the region included in the mutagenic suicide construct.

J. Sequence analysis

DNA sequencing was performed by the University of Texas Institute for Cellular and Molecular Biology DNA Core Facility using an ABI Prism 377 DNA sequencer. Analysis of DNA sequences, including ORF analysis, restriction mapping and ClustalW alignments, was carried out using MacVector™ 7.0. Protein sequence alignments were also carried out using the CLUSTAL-W program from within MacVector™ 7.0. Signal peptide predictions for putative periplasmic, outer membrane or exported proteins were made using the SignalP program on the SignalP server at www.cbs.dtu.dk/services/SignalP. Secondary structure predictions for amino acid sequences based on similarity to solved crystal structures were carried out using the 3D-PSSM program on the 3D-PSSM Web Server V 2.6.0 at www.bmm.icnet.uk/~3dpssm. Protein sequence comparisons to the COG database (NCBI) were performed using the COGnitor search engine at www.ncbi.nlm.nih.gov/COG/xognitor.html. The BLASTX or BLASTP programs were used to search for homologous DNA or amino acid sequences in the Genbank database (NCBI, at www.ncbi.nlm.nih.gov/BLAST/) and the *V. cholerae* genome database (TIGR, at http://tigrblast.tigr.org/cmr-blast/index.cgi?database=GVC.seq).

K. β-galactosidase assays

Strains were grown to mid-log phase in iron-replete media containing 40 μM $FeSO_4$, or in iron-depleted media containing 300 μg per ml EDDA. The procedure described by Miller (110) was used to quantify β-galactosidase activity in the cells. Briefly, 1 ml bacterial cultures were pelleted and resuspended in 1 ml β-galactosidase assay buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 10 mM Mg $SO_4 \cdot 7H_2O$; pH adjusted to 7.0; 40 μl 1 M DTT added fresh per 100 ml assay buffer), and the OD_{660} was measured. The cells suspensions were diluted 1:10 into 1 ml of assay buffer in a 100 x 13 mm glass tube. To permeabilize the cells, 40 μl 0.1% SDS and 20 μl chloroform were added to the cells, and the tubes were vortexed for 5 seconds and then incubated for 10 minutes at room temperature. To start the assay, 0.2 ml ONPG was added to each tube, and the time lapsed until yellow color was clearly visible was recorded. The assay was stopped by adding 0.5 ml 1 M Na_2CO_3 per tube. The OD_{420} and OD_{550} of each reaction was measured, and the following formula was used to calculate β-galactosidase activity: Units = 1000 x $[OD_{420} - (1.75 \times OD_{550})]/t \times V \times OD_{660}$, where t = time (minutes), and V = volume (ml) of cell suspension added to each assay tube.

L. Cell fractionation

Overnight cultures were diluted 1:100 into 50 ml L broth containing 10 μg per ml EDDA to induce expression of iron-regulated genes. The diluted cultures were grown to an OD of 0.5 and harvested by centrifugation at 7000 x *g* for 10 minutes at 4°C. After resuspension in 3 ml Hepes buffer (10 mM, pH 7.4), the cells were frozen at –80°C. Complete™ EDTA-Free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) was added after thawing, and the cells were broken by passage twice through a FRENCH® pressure cell (SLM-AMINCO®, Thermo Spectronic, Rochester, NY) at 12,000 psi. Unbroken cells were pelleted at 8000 x *g* for 20 minutes at 4°C, and the broken cell extract was centrifuged for 45 minutes at 100,000 × *g* at 12°C to collect the total membrane fraction (77). The supernatant containing the cytosolic fraction was added to Laemmli solubilization buffer (86) and frozen at –20°C. The total membrane fraction was washed twice with 1 ml sterile ddH_2O using a blunt needle, and resuspended in a final volume of 150 μl sterile ddH_2O. The Sarkosyl (N-lauroylsarcosine)-insoluble outer membrane fraction was isolated by the method of Filip et al. (53). This method was used previously to obtain outer membrane preparations from *V. cholerae* with less than 5% inner membrane contamination (150). Sarkosyl (0.5% final concentration) was added to the total membrane suspension, and after incubating for 20 minutes at room temperature the Sarkosyl-treated membranes were centrifuged for 45 minutes at 100,000 × *g* at 12°C. The supernatant containing the inner membrane fraction was collected and frozen at

–20°C in Laemmli solubilization buffer (86). The pellet containing the Sarkosyl-insoluble outer membrane fraction was washed in 1 ml sterile ddH_2O containing 1% Sarkosyl, and centrifuged at 100,000 × *g* for 45 minutes at 12°C. The outer membrane fraction was resuspended in a final volume of 0.2 ml. Membrane fractions from equivalent numbers of cells (4 x 10^8) were resolved by SDS- 7.5% PAGE, and the proteins were visualized by Coomassie Brilliant Blue staining.

M. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins from membrane preparations were analyzed by SDS- 7.5% PAGE to resolve proteins in the 70-80 kDa range. The 7.5% resolving and 4% stacking gels were prepared using a 30% Acrylamide/Bis solution (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions, and the gels were cast using the Mini-PROTEAN® II gel casting system (Bio-Rad). Protein samples and low range SDS-PAGE standards (Bio-Rad) were boiled for 3 minutes in sample buffer and loaded onto the gels. Gels were electrophoresed in a Mini-PROTEAN® II cell (Bio-Rad) in SDS-PAGE running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS, pH 8.3) at 20 mA through the stacking gel and 40 mA through the resolving gel. To visualize proteins, the gels were Coomassie-stained (0.1% R-250 Brilliant Blue, 50% MeOH, 10% acetic acid) for 30 minutes and then destained in 7% acetic acid and 5% MeOH for 24 hours. Gels were

dried between 2 sheets of cellophane using an Easy Breeze Drying Frame from Hoefer Scientific Instruments (San Francisco, CA).

N. *In vivo* competition assays

In vivo competition assays were performed by a protocol modified from Taylor *et al*. (166). Five-day old pre-starved BALB/c mice were inoculated intragastrically using a 1cc tuberculin syringe with a 1 inch 23 gage needle fitted with 2 cm of intramedic PE-50 polyethylene tubing (inner diameter 0.58 mm; outer diameter 0.965 mm). Each inoculum consisted of 50 μl saline containing 0.5% sucrose, 0.02% Evan's Blue dye, and 5 x 10^5 cfu of each strain grown to mid-log phase under iron-limiting conditions. The mice were sacrificed after 24 hrs. Mice that had been properly inoculated exhibited dye accumulation in the large intestine and colon. The small intestine and the upper part of the large intestine were isolated and homogenized in saline using a Dounce homogenizer. Serial dilutions of the homogenate were plated on selective media to determine the viable counts for each strain. The output ratio of the competing strains was normalized to the input ratio to determine the competitive index.

III. RESULTS

A. Identification and characterization of two additional TonB-dependent heme receptors in *V. cholerae*

1. CHARACTERIZATION OF A *V. CHOLERAE* HUTA MUTANT

To further characterize the heme uptake system of *V. cholerae*, a mutation in the gene encoding the heme receptor HutA was generated in a vibriobactin (Vib)-synthesis mutant, CA40130N. The Vib$^-$ strain was chosen in order to eliminate background growth due to vibriobactin-mediated iron uptake in iron-limited media. The growth of the *hutA* mutant ARM315 was tested in a colony size assay as described previously [Occhino, 1998 #490]; this assay yields the most reproducible results when measuring subtle growth differences. The *hutA* mutant had only a slight defect in the ability to use hemin as the sole source of iron, compared to its Vib$^-$ parental strain. This defect was remedied by supplying *hutA* on a plasmid (Table 3). The *hutA* mutation had no effect on transport of other TonB-dependent substrates, including ferrichrome and vibriobactin (data not shown). These data suggest that there is a HutA-independent mechanism for transport of heme across the outer membrane in *V. cholerae*. Because our earlier studies showed that TonB-defective strains of *V. cholerae* do not use hemin as an iron source (70, 124), this additional heme uptake system in *V. cholerae* must involve one or more TonB-dependent receptors.

Table 3. HutA- and HutR-dependent hemin utilization in *V. cholerae.*

Strain	Relevant	Average colony size (mm) after 24 hrs on L agar containing:[a]		
		$FeSO_4$	EDDA	EDDA + hemin
CA40130N	parent	1.9	NG	1.3
ARM315[c]	$HutA^-$	1.8	NG	1.0[e]
ARM315/pAMH20	$HutA^+$	1.6	NG	1.3
ARM313	$HutR^-$	1.8	NG	1.3
ARM915[d]	$HutA^-$, $HutR^-$	1.9	NG	< 0.2[e]
ARM915/pAMR12	$HutA^-$, $HutR^+$	1.8	NG	1.4
ARM915/pAMH20	$HutA^+$, $HutR^-$	1.8	NG	1.2

a. The indicated strains were plated on L agar supplemented with 40 μM $FeSO_4$, with 100 μg EDDA per ml, or with EDDA and 5 μM hemin. Each number represents the mean diameter of 10 well-isolated colonies (> 5 mm apart). The standard deviation for each data set was less than 0.15 mm for growth on L agar + $FeSO_4$, and less than 0.1 mm for growth on iron-depleted media supplemented with hemin. NG = No growth.

b. All strains are derived from CA40130N and thus are Vib^-.

c. ARM315 carrying the vector pACYC184 had the same average colony size as ARM315.

d. ARM915 carrying the vectors pWKS30 or pACYC184 had the same average colony size as ARM915.

e. $P < 0.001$ (Student's *t* test) compared to CA40130N.

2. IDENTIFICATION OF A PUTATIVE SECOND RECEPTOR FOR HEME IN *V. CHOLERAE*

A BLAST search of the TIGR *V. cholerae* genome database using the HutA amino acid sequence identified several uncharacterized, putative TonB-dependent receptors. One of these, VCA0064, showed significant homology to *V. cholerae* HutA (68) (41% similarity; 25% identity) and to *V. vulnificus* HupA (106) (39% similarity; 22% identity), as well as to other heme receptors. We named this putative heme receptor HutR. HutR has a calculated mass of 80.5 kDa in the unprocessed form. Cleavage of the putative N-terminal signal sequence (120) would yield a 78.5 kDa mature protein with a calculated pI of 5.1.

The *hutR* gene is located on the smaller of the two *V. cholerae* replicons, and is the second open reading frame (ORF) in a predicted five-member operon that includes a type II protease gene, *ptrB*, as well as three hypothetical ORFs of unknown function (67) (Fig. 6a). A potential Fur box is present upstream of the putative transcriptional start site for this operon, indicating that these genes may be iron regulated (Fig. 6b). No promoters between *ptrB* and *hutR* were predicted from analysis of the DNA sequence. A potential Rho-independent transcription terminator is located downstream of the last ORF in the *hutR* operon.

Sequence analysis revealed that HutR contains many of the conserved features of TonB-dependent outer membrane receptors, including a TonB box similar to the *V. cholerae* HutA and *V. vulnificus* HupA TonB boxes (Fig. 6c). Significant homology to outer membrane receptors was also found at the C-terminus of HutR: HutR contains the terminal phenylalanine residue thought to

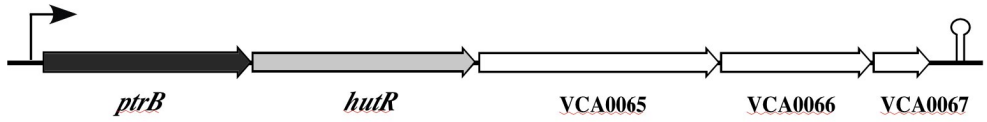
Figure 6. Sequence analysis of the *hutR* locus.

A. The thick shaded arrows delineate the open reading frames in the potential five-member operon containing *hutR*. The thin right-angle arrow shows the location of the putative promoter for this operon, and a potential transcriptional terminator is indicated by a stem-loop structure downstream of ORF VCA0067.

B. Analysis of the predicted promoter region for the *hutR* operon shows a putative Fur box sequence (boxed). The translational start site for *ptrB* is indicated by the arrow, and the N-terminal residues of PtrB are labeled using the single-letter code.

C. HutR, HutA and HupA were aligned using CLUSTAL-W. The numbers refer to amino acid position in the unprocessed protein. The arrowhead indicates the predicted N-terminal signal sequence cleavage site for HutR. Conserved residues are marked by an asterisk, and similar residues are denoted by a period. The TonB box is delineated by the horizontal bar. The FRAP and NPNL motifs are boxed, and the conserved histidine residue is shown in bold.

A



B

putative Fur box

gaatttttgctctaaaataacacaaatgataattgatcttatttagatttgtgttagag

ptrB →

tggcgaccgatttttatttgttatagttgtcacattttcg ATG AAA CTG CTC

M K L L

C

TonB box

```
V. cholerae HutR          1   MKLSPVSAAVLSVLAAGFAHAETEPSHYEEVVVTANR
V. cholerae HutA          1   MYKKSLLSSAIMLALVP--SAYADDYASFDEVVVSTTR
V. vulnificus HupA        1   MYNRTFLSASILFALAT--PVQAQDAGLFDEVVVSATR
                                  . .*....  *         .   ..****...*

V. cholerae HutR        474   GFRAPSYDKAYGASDHSFVPLTPFIIKPNNKLRAE
V. cholerae HutA        459   GFRAPDFQELYYSFGN---PAHGYVFKPNPNLEAE
V. vulnificus HupA      471   GFRAPSFNELYYTYDN---PGHGYTNRPNPNLESE
                              *****     * .      *   .  .**  * .*

V. cholerae HutR        700   EPGRYFTVHAKYVF
V. cholerae HutA        680   QAKRNFGITAKYEF
V. vulnificus HupA      699   QAERNYNISVKYEF
                                 * . .  ** *
```

be required for proper incorporation into the outer membrane (161), as well as the invariant arginine residue at position 11 from the C-terminus (Fig. 6c). HutR also contains motifs conserved among outer membrane receptors specific for heme and hemoglobin, including a complete FRAP box, a partial NPNL box, and one of the highly conserved histidine residues implicated in heme receptor function (Fig. 6c) (11).

3. CHARACTERIZATION OF A *V. CHOLERAE* HUTA, HUTR MUTANT

In order to ascertain whether HutR functions as a heme receptor in *V. cholerae*, a *hutR* single mutant and a *hutA, hutR* double mutant were generated by allelic exchange in the Vib$^-$ background. In a colony size assay, the *hutR* single mutant ARM313 used hemin iron as efficiently as the parental strain CA40130N (Table 3); however, the *hutA, hutR* double mutant ARM915 was significantly impaired in hemin utilization compared to both the parental strain and to the *hutA* single mutant ARM315 (Table 3). These data suggest that both HutA and HutR participate in heme transport in *V. cholerae*. To verify that the transport defect observed for the *hutA, hutR* mutant is dependent upon loss of *hutR* expression, and not due in part to polar effects on downstream genes, ARM915 was transformed with pAMR12, which contains only the *hutR* gene. Expression of *hutR* from pAMR12 complemented the hemin transport deficiency of the *hutA, hutR* mutant ARM915 (Table 3); therefore, the phenotype of ARM915 cannot be attributed to the disruption of downstream genes. These data confirm that *hutR* encodes a functional heme receptor in *V. cholerae*. A plasmid carrying *hutA,*

pAMH20, also restored hemin transport to the *hutA, hutR* mutant (Table 3). In both cases, the complemented strains used hemin as efficiently as the parental strain, despite lacking one of the heme receptors. Thus, the presence of multiple copies of one receptor gene on a complementing plasmid appeared to compensate for the loss of the other receptor gene, showing that both receptors are capable of efficient hemin transport under the conditions tested.

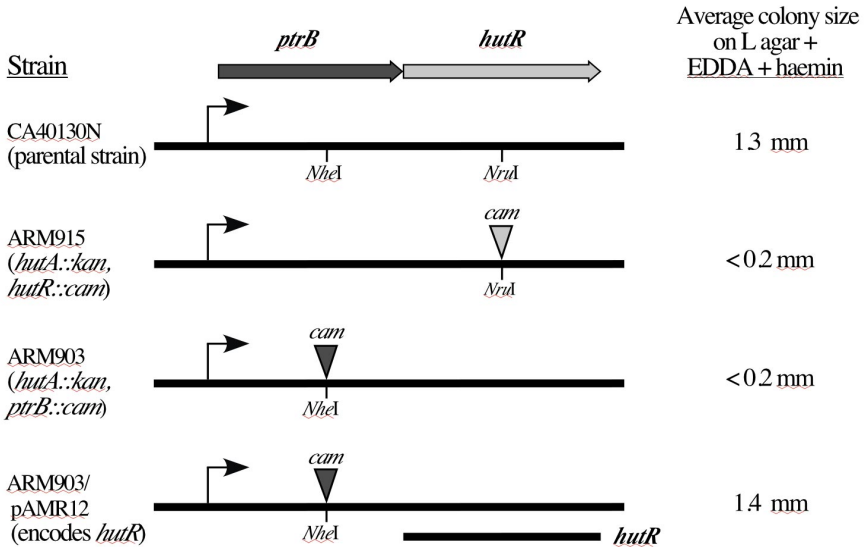
4. IDENTIFICATION OF AN IRON-REGULATED PROMOTER FOR *HUTR*

Sequence analysis predicted that the *hutR* and *ptrB* genes may be co-transcribed. To determine whether *hutR* expression is controlled by the promoter upstream of *ptrB*, or whether an internal promoter exists for *hutR*, an insertion mutation in *ptrB* was constructed in the *hutA::kan* background. The resulting strain ARM903 was significantly impaired in growth on media containing hemin as the sole iron source, and thus it behaved like the *hutA*, *hutR* double mutant ARM915 (Fig. 7). Plasmid-encoded HutR restored hemin utilization to the *hutA, ptrB* mutant, showing that the defect in hemin uptake was due to loss of *hutR* expression rather than to the disruption of *ptrB* (Fig. 7). These data suggest that *ptrB* and *hutR* are co-transcribed, and that PtrB is not required for utilization of hemin under the conditions tested.

The promoter region upstream of *ptrB* contains a potential Fur box, suggesting that the operon containing *ptrB* and *hutR* is iron-regulated. To analyze the regulation of these genes, the *ptrB* promoter was cloned upstream of the promoterless *lacZ* gene in pQF50 to create a transcriptional fusion. As a control,

Figure 7. The *hutR* and *ptrB* genes are co-transcribed.

The right-angled arrow indicates the location of the putative promoter for the *hutR* operon. A partial restriction map of the *hutR* region shows the sites of the polar *cam* cassette insertions. The ability to use hemin as an iron source was measured by plating the indicated strains on L agar containing 100 μg EDDA ml^{-1} and 5 μM hemin. Each number in the right hand column represents the mean diameter of 10 well-isolated colonies (> 5 mm apart) after 24 hrs. The standard deviation for each data set was less than 0.1 mm.


Strain
ptrB
hutR
Average colony size on L agar + EDDA + haemin
CA40130N (parental strain)
NheI
NruI
1.3 mm
ARM915 (hutA::kan, hutR::cam)
cam
NruI
<0.2 mm
ARM903 (hutA::kan, ptrB::cam)
cam
NheI
<0.2 mm
ARM903/ pAMR12 (encodes hutR)
cam
NheI
hutR
1.4 mm

a transcriptional fusion between the iron-regulated *hutA* promoter (69) and *lacZ* was created as well. *V. cholerae* strains carrying these constructs were grown to mid-log phase under high or low iron conditions, and the expression of *lacZ* was measured as β-galactosidase activity. Both the *hutA* and *ptrB* promoters were strongly induced in the absence of iron, and the level of induction was comparable for the two promoters (Fig. 8).

5. CELLULAR LOCALIZATION OF HUTR

The *hutR* gene encodes a potential N-terminal signal sequence, consistent with export of HutR to the outer membrane. To determine the subcellular localization of HutR, cellular fractions were prepared from iron-limited CA40130N (Vib^- parental strain; Fig. 9, lanes 1 and 5), ARM915 (*hutA*, *hutR* mutant; lanes 2 and 6), and ARM915 carrying either *hutR* (lanes 3 and 7) or *hutA* (lanes 4 and 8) on a plasmid. The 78.5 kDa mature HutR protein was present in the total membrane fraction of cells expressing *hutR* from a plasmid, but not in the cytosolic fraction (data not shown). Separation of the inner and outer membranes by Sarkosyl extraction showed that HutR is associated with the outer membrane (lane 7). Plasmid-encoded 75.0 kDa mature HutA was similarly detected in the total membrane and outer membrane fractions (lanes 4 and 8), consistent with earlier studies showing that HutA is present in the outer membrane (69). Interestingly, HutA, but not HutR, was visible in the outer membrane fraction isolated from the parental strain CA40130N (lane 5), indicating that the levels of HutA exceed the levels of HutR in the outer membrane of wild type cells in low iron conditions.

Figure 8. Regulation of *hutA-lacZ* (pAML20) and *ptrB-lacZ* (pAML21) transcriptional fusions by iron.

Strains were grown to mid-log phase in iron-replete media (+ $FeSO_4$) or in iron-depleted media (+ EDDA), and β-galactosidase activity was measured. The data represent the averages of three independent experiments. Standard deviations are indicated by the error bars.

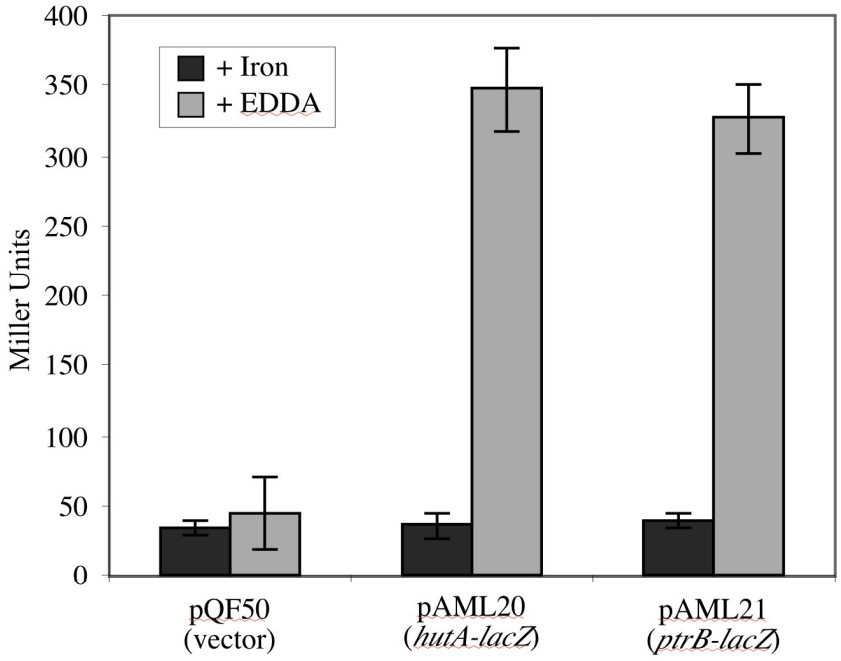
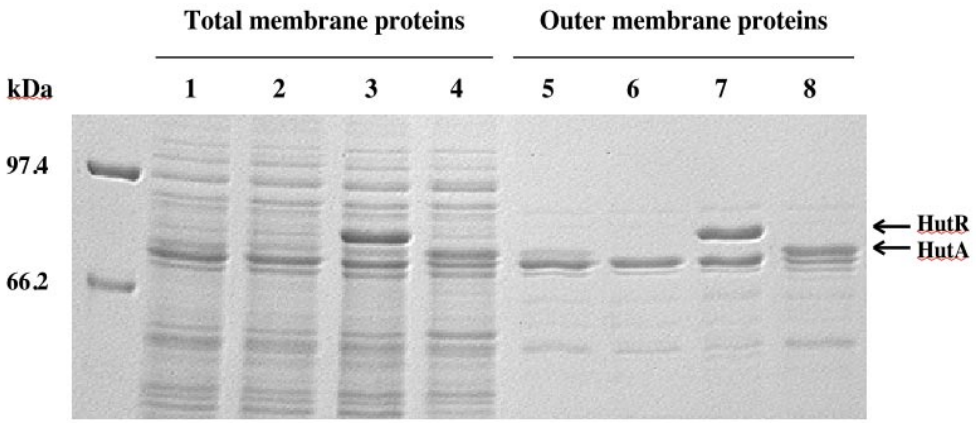

400
350
300
250
200
150
100
50
0
Miller Units
+ Iron
+ EDDA
pQF50
(vector)
pAML20
(hutA-lacZ)
pAML21
(ptrB-lacZ)

Figure 9. Cellular localization of HutR and HutA.

Total membrane and outer membrane fractions were isolated from *V. cholerae* CA40130N (Vib- parental strain; lanes 1, 5), ARM915 ($HutA^-/HutR^-$; lanes 2, 6), ARM915/pAMR12 (expressing plasmid-encoded HutR; lanes 3, 7), and ARM915/pAMH20 (expressing plasmid-encoded HutA; lanes 4, 8), grown in low iron conditions. Membrane fractions from equivalent numbers of cells (4×10^8) were resuspended in Laemmli buffer, and the proteins were resolved by SDS-7.5% PAGE and visualized by Coomassie Brilliant Blue staining.


Total membrane proteins
Outer membrane proteins
kDa
1
2
3
4
5
6
7
8
97.4
66.2
HutR
HutA

6. ANALYSIS OF HEMOGLOBIN UTILIZATION IN *V. CHOLERAE*

V. cholerae growing within the human host is not likely to encounter free heme, but rather host heme-containing proteins such as hemoglobin. We had observed earlier that the ability to use hemoglobin as an iron source specifically requires the TonB1 system (149). This TonB-dependence may reflect the preferential use of a particular receptor for utilization of hemoglobin. To address this issue, single mutants in *hutA* (ARM315) or *hutR* (ARM313) were compared with respect to growth in media containing hemin or hemoglobin as the sole source of iron (Table 4 and Fig. 10). The mutation in *hutR* had no effect on the ability to use either hemin or hemoglobin, suggesting that HutR does not have a specific function in hemin or hemoglobin utilization that cannot be carried out by HutA or some other protein under these conditions. The *hutA* mutant, however, was significantly impaired in growth using hemoglobin as the iron source. The defect observed for the *hutA* mutant was comparable to the defect observed for a *tonB1* mutant in the same background (AMV527), suggesting that the TonB1-HutA pair plays a critical role in the ability to access heme-iron from hemoglobin (Table 4 and Fig. 10).

Utilization of hemoglobin as an iron source likely requires the release of heme from hemoglobin prior to transport of heme into the cell. Indeed, CA40130N had a slower growth rate in media containing hemoglobin than in media containing free hemin (Fig. 10). These data suggest that either the affinity for hemoglobin is lower than for free heme, or that release of the heme moiety is the limiting step in the process of hemoglobin utilization. To test whether the

Table 4. Hemoglobin utilization by *V. cholerae.*

Strain	Relevant phenotype [b]	Average colony size (mm) after 24 hrs on L agar containing: [a]			
		$FeSO_4$	EDDA	EDDA + hemin	EDDA + hemoglobin
CA40130N	parent	1.8	NG	1.6	0.8
ARM303	$PtrB^-$, $HutR^-$	1.9	NG	1.7	0.8
ARM313	$HutR^-$	1.9	NG	1.6	0.8
ARM315	$HutA^-$	1.9	NG	1.3 [c]	<0.2 [d]
ARM915	$HutA^-$, $HutR^-$	1.9	NG	<0.2 [c]	NG
AMV527	$TonB1^-$	1.9	NG	0.9 [c]	<0.1 [d]

a. The indicated strains were plated on L agar supplemented with 40 μM $FeSO_4$, with 50 μg EDDA per ml (in this experiment it was necessary to reduce the EDDA concentration from 100 to 50 μg per ml for optimal growth using hemoglobin as the sole iron source), or with EDDA and 5 μM hemin or hemoglobin. Each number represents the mean diameter of 10 well-isolated colonies (> 5 mm apart). The standard deviation for each data set was less than 0.15 mm for growth on L agar + $FeSO_4$, and less than 0.1 mm for growth on iron-depleted media supplemented with hemin. NG = No growth.

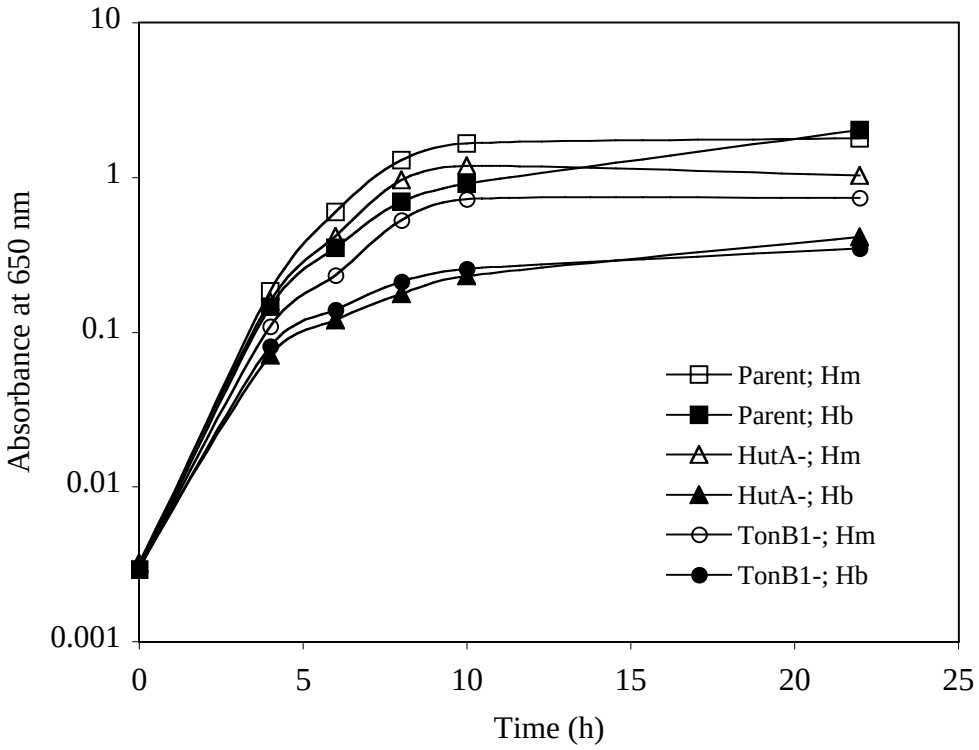
b. All strains are derived from CA40130N and thus are Vib^-.

c. $P < 0.001$ (Student's *t* test) compared to CA40130N on EDDA + hemin.

d. $P < 0.001$ (Student's *t* test) compared to CA40130N on EDDA + hemoglobin.

Figure 10. Role of HutA and TonB1 in utilization of hemin or hemoglobin as the sole iron source.

Strains were inoculated into L broth containing 75 μg EDDA per ml, and 2.5 μM hemin or hemoglobin. The optical density was measured at the indicated time points. Growth in iron-replete media was comparable for all the strains tested, and none of the strains grew in iron-depleted media (data not shown). (□) CA40130N (Vib^- parental strain), hemin; (■) CA40130N (Vib^- parental strain), hemoglobin; (Δ) ARM315 ($HutA^-$), hemin; (▲) ARM315 ($HutA^-$), hemoglobin; (○) AMV527 ($TonB1^-$), hemin; (●) AMV527 ($TonB1^-$), hemoglobin.


10
1
0.1
0.01
0.001
Absorbance at 650 nm
0
5
10
15
20
25
Time (h)
Parent; Hm
Parent; Hb
HutA-; Hm
HutA-; Hb
TonB1-; Hm
TonB1-; Hb

putative protease encoded by the *ptrB* gene upstream of *hutR* plays a role in heme acquisition from hemoglobin, a strain carrying an insertion in *ptrB*, ARM303, was tested for ability to grow on solid media containing hemoglobin as the sole source of iron (Table 4). The *ptrB* mutation did not adversely affect hemoglobin utilization, indicating that either PtrB is not involved in hemoglobin degradation, or *V. cholerae* encodes additional proteins that can facilitate the release of heme from hemoglobin prior to uptake by the cell. The lack of an effect of the *ptrB* mutation on hemin or hemoglobin utilization was confirmed in a growth curve experiment in liquid medium as well as in a halo assay (data not shown).

7. IDENTIFICATION OF A THIRD HEME RECEPTOR IN *V. CHOLERAE*

Although the *hutA, hutR* double mutant was significantly impaired in hemin utilization, producing only a pin-point colony in a colony size assay (Table 3, page 79), a small amount of growth around hemin was clearly detectable in halo assays (Table 5). We were interested in determining whether this residual growth might be due to a third heme transport mechanism in *V. cholerae.* A BLAST search of the TIGR *V. cholerae* protein database using HutA as the query sequence identified a third candidate heme receptor, VCA0625. We named this putative heme receptor HasR because of its overall similarity to the HasR hemophore receptors from *P. aeruginosa* (125) (32% similarity) and from *S. marcescens* (60) (31% similarity). A mutation in the *hasR* gene was created by allelic exchange in the *hutA, hutR* mutant to yield ARM932. Unlike the *hutA, hutR* double mutant ARM915, the triple mutant ARM932 was completely

Table 5. HasR-dependent hemin utilization in *V. cholerae.*

		Zone of growth (mm) after 48 hours[a]		
Strain	Relevant Phenotype[b]	$FeSO_4$	Hemin	Vibriobactin[c]
CA40130N	parent	23	15	32
ARM915	$HutA^-$, $HutR^-$	23	7	30
ARM932	$HutA^-$, $HutR^-$, $HasR^-$	25	NG	31

a. The indicated strains were seeded at 10^6 cells per ml into molten L agar containing 100 μg EDDA per ml. Iron sources were spotted onto the solidified agar as follows: 20 μl 10 mM $FeSO_4$; 5 μl CA401 overnight culture (vibriobactin); 5 μl 50 μM hemin. NG = No growth. Plates were incubated for 24 hrs at 37°C, then for 24 hrs at room temperature, and the diameter of each zone of growth was measured. The assays were repeated at least 3 times. Although the relative differences between strains were consistent between experiments, the absolute values for each strain varied due to subtle changes in the media and assay conditions, and thus only one representative experiment is shown.

b. All strains are derived from CA40130N and thus are Vib^-.

c. Vibriobactin utilization shows proper TonB function.

deficient in hemin utilization, and we conclude that HasR is the third, and likely final, heme receptor in *V. cholerae* (Table 5). HasR-mediated growth required a relatively high local concentration of hemin, such as that provided by spotting the hemin directly onto the surface in a halo assay. Thus, HasR may be a less efficient heme transporter than either HutA or HutR, or may be expressed at lower levels under the conditions used for the hemin utilization assays.

8. TonB SPECIFICITIES OF THE THREE *V. CHOLERAE* HEME RECEPTORS

Our earlier studies showed that efficient heme transport through HutA in *E. coli* requires the *V. cholerae* TonB1 system (68, 124). In contrast, the *V. cholerae* TonB2 system failed to promote hemin uptake via HutA in *E. coli* (data not shown). These data suggest that HutA may have a strong preference for the TonB1 system. Similar results were obtained when *hutR* was expressed in *E. coli* (data not shown). To establish whether the observed dependence of HutA and HutR on the TonB1 system in *E. coli* reflects a true specificity for the TonB1 system, a series of receptor mutants was created in DHH11, a TonB-deficient derivative of *V. cholerae* CA40130. DHH11 was unable to use any of the TonB-dependent substrates tested unless supplied with a set of *tonB* system genes. We had shown earlier that either *V. cholerae* TonB system facilitates vibriobactin transport; however, only the TonB2 system promotes enterobactin transport (149). These two iron sources were therefore included to demonstrate proper function of the TonB system expressed in each strain tested. The TonB specificities of HutA and HutR were investigated by constructing a *hutR or hutA* mutation in the *tonB*

mutant background. Because the contribution of HasR to hemin utilization was relatively minor compared to HutA and HutR (Table 3, page 79, and Table 6), it was not necessary to eliminate *hasR* expression from these strains. A *tonB, hutR* mutant ARM213 used hemin efficiently when provided with either the *tonB1* or the *tonB2* system genes on a plasmid (Table 6), suggesting that HutA does not have an absolute specificity for TonB1. Hemin utilization was similarly restored to the *tonB, hutA* mutant ARM215 when either set of *tonB* genes was supplied on a plasmid, suggesting that HutR also functions with both TonB systems (Table 6). In both cases, strains carrying the TonB1 system genes had larger zones of growth around hemin than strains carrying the TonB2 system genes. Although overexpression of the heme transporters HutBCD encoded by the *tonB1* plasmid may have contributed to the growth observed, this does not fully account for the growth advantage of strains expressing the *tonB1* genes. A plasmid carrying only *tonB1exbB1exbD1* (pAMT12) conferred hemin utilization in the parental strain DHH11 as efficiently as a *tonB1exbB1exbD1* plasmid carrying additionally *hutBCD* (pAMT112) (Table 6). Taken together, these data indicate that TonB1 may facilitate heme transport through either HutA or HutR more efficiently than TonB2.

To study the TonB-dependence of heme transport through HasR, a *hutA, hutR* mutant was created in the *tonB*-deficient strain DHH11. This mutant, ARM219, exhibited a small amount of growth around hemin in a bioassay when supplied with the *tonB2* genes, but not when supplied with the *tonB1* genes (Table 6). Heme uptake through HasR must therefore specifically require TonB2.

Table 6. TonB-dependence of HutA, HutR, and HasR.

Strain[b]	Heme receptor(s) present	TonB system present	Zone of growth (mm) after 48 hours[a]			
			$FeSO_4$	Hemin	Vibriobactin[c]	Enterobactin[d]
DHH11/pWKS30	HutA, HutR, HasR	None	25	NG	NG	NG
DHH11/pAMT112	HutA, HutR, HasR	TonB1	24	15	28	NG
DHH11/pAMT12	HutA, HutR, HasR	TonB1	25	15	27	NG
DHH11/pOUT11	HutA, HutR, HasR	TonB2	24	11	28	30
ARM213/pAMT112	HutA, HasR	TonB1	24	15	29	NG
ARM213/pOUT11	HutA, HasR	TonB2	25	11	28	30
ARM215/pAMT112	HutR, HasR	TonB1	23	11	27	NG
ARM215/pOUT11	HutR, HasR	TonB2	23	8	28	30
ARM219/pAMT112	HasR	TonB1	23	NG	27	NG
ARM219/pOUT11/pACYC184	HasR	TonB2	24	6 (faint)	26	28
ARM219/pOUT11/pAMH20	HutA, HasR	TonB2	23	12	26	27
ARM219/pOUT11/pAMR14	HutR, HasR	TonB2	23	12	27	29

a. The indicated strains were seeded at 10^6 cells per ml into molten L agar containing 100 μg EDDA per ml. Iron sources were spotted onto the solidified agar as follows: 20 μl 10 mM $FeSO_4$; 5 μl CA401 overnight culture (vibriobactin); 5 μl DH5α overnight culture (enterobactin); 5 μl 50 μM hemin. NG=No growth. Plates were incubated for 24 hrs at 37°C, then for 24 hrs at room temperature, and the diameter of each zone of growth was measured. The assays were repeated at least 3 times. Although the relative differences between strains were consistent between experiments, the absolute values for each strain varied due to subtle changes in the media and assay conditions, and thus only one representative experiment is shown.

b. All strains are derived from CA40130, and thus are Vib^-.

c. Vibriobactin utilization shows proper TonB function.

d. Enterobactin utilization specifically demonstrates TonB2 function.

TonB2 facilitated hemin utilization more efficiently when either HutA or HutR was present than when only HasR was present (Table 6). These data support the above conclusion that TonB2 is able to promote heme transport through both HutA and HutR. However, to establish conclusively that TonB2 interacts productively with HutA and HutR, the *tonB, hutA, hutR* mutant ARM219 was supplied with the *tonB2* system genes and tested for hemin utilization in the presence or absence of a *hutA* or *hutR* clone. Plasmid-encoded HutA or HutR significantly increased the level of TonB2-dependent hemin utilization in these strains (Table 6). These data confirm that both receptors can use TonB2; nevertheless, HutA and HutR may both require TonB1 for optimal heme transport.

9. ROLE OF HEME UPTAKE FOR GROWTH OF *V. CHOLERAE IN VIVO*

The infant mouse model of *V. cholerae* infection (166) was used to investigate the role of heme uptake for growth of *V. cholerae* in the mammalian host. Five-day old BALB/c mice were inoculated intragastrically with equal numbers of the triple heme receptor mutant ARM932 and its Vib^- parental strain CA40130N. The ability of the heme receptor mutant to compete with the parental strain *in vivo* was determined by counting the numbers of viable ARM932 and CA40130N cells recovered from the intestines after 24 hours. The mean competitive index of ARM932 to CA40130N did not deviate significantly from 1.0 (mean = 0.94; range = 0.8-1.2; data collected from seven mice), indicating that the heme uptake mutant did not have an *in vivo* growth defect compared to its

Vib$^-$ parental strain. These data suggest that iron sources in addition to heme are available to *V. cholerae* colonizing the infant mouse.

10. SOURCES OF HEME IN THE MARINE ENVIRONMENT

The presence of three functional heme receptors in *V. cholerae* suggests that heme is an important nutrient for this organism. *V. cholerae* has been isolated from a variety of marine organisms, including crustaceans and molluskan shellfish (137), and it is possible that hemolymph from these animals can provide *V. cholerae* with heme-iron. To determine if heme sources from potential marine hosts can be used efficiently by *V. cholerae*, iron-starved *V. cholerae* was tested for ability to use hemoglobin preparations from two marine invertebrates, *Barbatia reeveana* (117) and *Urechis caupo* (56), as sources of iron. The parental strain CA40130N used both sources of heme, whereas the triple heme receptor mutant ARM932 was unable to grow using either type of hemoglobin as an iron source (Table 7). These data indicate that *V. cholerae* can readily assimilate heme derived from these invertebrates, and thus may be able to take advantage of the abundant sources of heme present in the marine environment.

Table 7. Utilization of marine invertebrate hemoglobin by *V. cholerae.*

Strain	Relevant Phenotype[b]	Growth after 48 hours[a]		
		$FeSO_4$	*Barbatia reeveana* hemoglobin	*Urechis caupo* hemoglobin
CA40130N	parent	+	+	+
ARM932	$HutA^-$, $HutR^-$, $HasR^-$	+	–	–

a. The indicated strains were seeded at 10^6 cells per ml into molten L agar containing 100 μg EDDA per ml. Iron sources were spotted onto the solidified agar as follows: 20 μl 10 mM $FeSO_4$; 5 μl marine invertebrate hemoglobin preparation (50 μM heme content). Plates were incubated for 24 hrs at 37°C, then for 24 hrs at room temperature. + indicates a zone of growth > 15 mm; – indicates no growth around the iron source.

b. All strains are derived from CA40130N and thus are Vib^-.

B. Characterization of domains involved in the interaction between TonB and TonB-dependent receptors

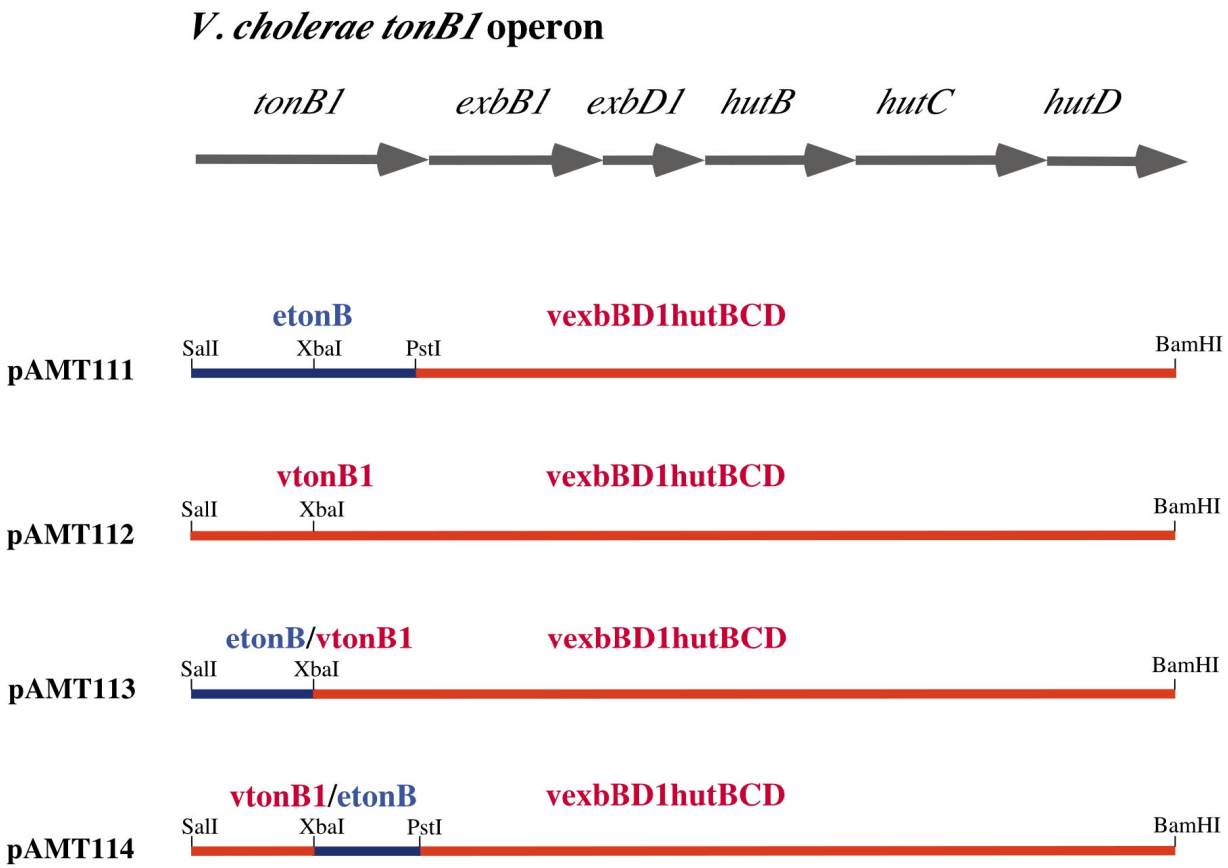
Data presented in this dissertation and in Seliger *et al.*, 2001, indicate that *V. cholerae* TonB1 may be specific for a subset of *V. cholerae* outer membrane receptors. The specificity of TonB1 for particular receptors has been demonstrated also by studies in *E. coli*. The *V. cholerae tonB1, exbB1, exbD1* genes do not complement an *E. coli tonB* mutation, suggesting that *V. cholerae* tonB1 does not recognize *E. coli* TonB-dependent receptors. Further, the ability of the *V. cholerae* heme receptor HutA to mediate efficient heme utilization in *E. coli* requires the *V. cholerae tonB1* system genes (68, 124). Thus, the *E. coli* TonB and *V. cholerae* TonB1 systems are not functionally interchangeable, and the hypothesis can be made that the *V. cholerae* TonB1 protein does not recognize or functionally interact with *E. coli* outer membrane receptors, and vice versa. The specificity exhibited by *E. coli* and *V. cholerae* TonB proteins for outer membrane receptors of their own species is consistent with a direct interaction between TonB and the receptors. To investigate the basis for the receptor specificity exhibited by TonB1, and to define the region(s) of the TonB proteins involved in the TonB-receptor interaction, a series of TonB chimeras was constructed between *E. coli* TonB and *V. cholerae* TonB1.

1. CONSTRUCTION OF HYBRID TONB GENES

The hybrid *tonB* genes were created by exchanging equivalent *E. coli tonB* and *V. cholerae tonB1* fragments using unique restriction sites engineered into the genes by PCR (Fig. 5, page 57). These restriction sites did not alter the efficacy or the specificity of the TonB proteins when compared with the corresponding wild type TonB in functional assays (data not shown). The *V. cholerae* TonB1 N-terminus does not recognize the native *E. coli* ExbB and ExbD proteins (124), and thus it was necessary to include the *V. cholerae exbBD1* genes in all constructs encoding the N-terminal domain of *V. cholerae* TonB1. In addition, heme uptake via the HutA/TonB1 pair in *E. coli* was more efficient when the heme transport genes *hutBCD* were expressed as well (data not shown). Therefore, to maintain consistency, all native and chimeric *tonB* genes were inserted in place of *V. cholerae tonB1* in the context of the entire *V. cholerae tonB1, exbBD1, hutBCD* operon (Fig. 11). To ensure translation of *V. cholerae* genes cloned behind *E. coli tonB*, a Shine-Dalgarno consensus sequence was introduced by PCR immediately upstream of *exbB1*. Expression of the *V. cholerae* genes cloned in this manner was tested by complementation of *V. cholerae exbB* or *exbD* mutations (data not shown). The level of expression of the hybrid TonBs was not determined. It is possible that the growth phenotype associated with a hybrid TonB could be due to overexpression or poor expression of the hybrid TonB protein rather than to receptor specificity. For example, the absence of growth could be due to poor expression of a TonB hybrid, whereas enhanced growth could be attributable to overexpression of a poorly functioning hybrid. This issue remains to be resolved.

Figure 11. *E. coli* and *V. cholerae tonB* gene fusions.

The restriction sites shown were engineered in by PCR, and the fusions were created by cutting and ligating the relevant fragments. The *hutBCD* genes encode *V. cholerae* heme-specific transport proteins that were found to increase the efficiency of heme utilization in the *E. coli* strain carrying these constructs. Blue indicates sequences derived from *E. coli*, and red indicates sequences derived from *V. cholerae.*


V. cholerae tonB1 operon
tonB1
exbB1
exbD1
hutB
hutC
hutD
pAMT111
etonB
vexbBD1hutBCD
SalI
XbaI
PstI
BamHI
pAMT112
vtonB1
vexbBD1hutBCD
SalI
XbaI
BamHI
pAMT113
etonB/vtonB1
vexbBD1hutBCD
SalI
XbaI
BamHI
pAMT114
vtonB1/etonB
vexbBD1hutBCD
SalI
XbaI
PstI
BamHI

2. FUNCTIONAL ANALYSIS OF *E. COLI* AND *V. CHOLERAE* TONB CHIMERAS INVOLVING THE C-TERMINAL DOMAIN OF TONB

TonB is composed of three functional domains: the N-terminal domain, involved in the association with the inner membrane; the central proline rich domain, which plays a role in spanning the periplasm; and the C-terminal domain, which mediates interactions with the outer membrane. To study the role of the C-terminal domain in receptor recognition, chimeras were created that switched this domain between *E. coli* TonB and *V. cholerae* TonB1. The chimeras were constructed by introducing an *Xba*I site into each *tonB* gene in region encoding the central proline-rich domain in each TonB. Whereas the *Xba*I mutation in the *E. coli tonB* gene was silent, the mutation in *V. cholerae* TonB1 resulted in the insertion of a Ser residue, thus making *V. cholerae* TonB1 more similar to *E. coli* TonB. The mutations did not alter the efficacy or specificity of the TonB proteins, as determined by comparisons with the corresponding wild type TonB in functional assays (data not shown). To determine the specificity of the native and hybrid TonBs in *E. coli*, *tonB* constructs were expressed in the *E. coli tonB, entF* mutant ARM100. This enterobactin synthesis mutant was used in order to reduce background growth in halo assays due to enterobactin-mediated utilization of iron sources in the media. This was particularly important for distinguishing between growth due to utilization of heme as an iron source, and growth due to enterobactin-mediated scavenging of free iron in the hemin preparations. The *tonB* constructs were tested for ability to confer heme utilization through *V. cholerae* HutA, and utilization of enterobactin or ferrichrome via the *E. coli*

receptors FepA or FhuA, respectively (Table 8 and Table 9). Heme utilization was only observed in strains expressing *V. cholerae hutA* (Table 8 and Table 9), and expression of *hutA* from a plasmid did not interfere with enterobactin or ferrichrome utilization (Table 9). Native *E. coli* TonB (construct pAMT111) permitted uptake of enterobactin and ferrichrome through the *E. coli* receptors FepA and FhuA, respectively, but did not mediate efficient heme uptake through the *V. cholerae* receptor HutA, suggesting that *E. coli* TonB interacts poorly with HutA (Table 9 and Fig. 12). In contrast, *V. cholerae* TonB1 (construct pAMT112) was unable to facilitate transport through any of the *E. coli* receptors tested, but was able to promote heme uptake via HutA, demonstrating that *V. cholerae* TonB1 has an absolute specificity for *V. cholerae* receptors (Table 9 and Fig. 13). Thus, both *E. coli* TonB and *V. cholerae* TonB1 exhibit a degree of specificity for receptors of their own species. Replacing the carboxy-terminal 121 amino acids of *E. coli* TonB with the equivalent region from *V. cholerae* TonB1 produced a functional TonB (construct pAMT113) that allowed transport through *V. cholerae* HutA, but not through the *E. coli* receptors (Table 9 and Fig. 14). This *E. coli* TonB/*V. cholerae* TonB1 (e/v1) hybrid is therefore functionally equivalent to *V. cholerae* TonB1. Conversely, replacing the carboxy-terminal 125 residues of *V. cholerae* TonB1 with the equivalent region from *E. coli* TonB produced a functional TonB (construct pAMT114) that permitted transport through *E. coli* FepA and FhuA, but did not mediate efficient heme uptake through *V. cholerae* HutA. Thus, this *V. cholerae* TonB1/*E. coli* TonB (v1/e) hybrid is functionally equivalent to wild type *E. coli* TonB (Table 9 and Fig 15).

Table 8. Role of the Carboxy-terminal domain of TonB in TonB-receptor interaction specificity.

ARM100 ($TonB^-$, $EntF^-$) transformed with:	Relevant TonB characteristics[b]	Zone of growth (mm) after 24 hours[a]			
		$FeSO_4$	Ferrichrome	Enterobactin	Hemin
pWKS30ΔX	$TonB^-$	20	NG	NG	NG
pAMT111	e TonB*X*	22	16	25	NG
pAMT112	v1 TonB*X*	20	NG	NG	NG
pAMT113	$e_{1\text{-}119}/v1_{122\text{-}245}$TonB*X*	20	NG	NG	NG
pAMT114	$v1_{1\text{-}121}/e_{120\text{-}239}$TonB*X*	22	16	26	NG

a. The indicated strains were seeded at 10^6 cells per ml into molten L agar containing 100 μg EDDA per ml. Iron sources were spotted onto the solidified agar as follows: 20 μl 10 mM $FeSO_4$; 5 μl 40 μM Ferrichrome; 5 μl DH5α overnight culture (enterobactin); 5 μl 50 μM hemin. NG = No growth. Plates were incubated for 24 hrs at 37°C, and the diameter of each zone of growth was measured. The assays were repeated at least 3 times. Although the relative differences between strains were consistent between experiments, the absolute values for each strain varied due to subtle changes in the media and assay conditions, and thus only one representative experiment is shown.

b. *E. coli* TonB*X* is denoted by e TonB*X*; *V. cholerae* TonB1*X* is denoted by v1 TonB*X*.

Table 9. Role of the C-terminal domain of TonB in specificity for *E. coli* and *V. cholerae* receptors.

		Zone of growth (mm) after 24 hours[a]			
ARM100 ($TonB^-$, $EntF^-$)/ pAMH20[c] transformed with:	Relevant TonB characteristics[b]	$FeSO_4$	Ferrichrome	Enterobactin	Hemin
pWKS30ΔX	$TonB^-$	20	NG	NG	NG
pAMT111	e TonB*X*	20	13	22	10 (faint)
pAMT112	v1 TonB*X*	19	NG	NG	12
pAMT113	$e_{1\text{-}119}/v1_{122\text{-}245}$TonB*X*	18	NG	NG	12
pAMT114	$v1_{1\text{-}121}/e_{120\text{-}239}$TonB*X*	22	14	23	10 (faint)

a. The indicated strains were seeded at 10^6 cells per ml into molten L agar containing 100 μg EDDA per ml. Iron sources were spotted onto the solidified agar as follows: 20 μl 10 mM $FeSO_4$; 5 μl 40 μM Ferrichrome; 5 μl DH5α overnight culture (enterobactin); 5 μl 50 μM hemin. NG = No growth. Plates were incubated for 24 hrs at 37°C, and the diameter of each zone of growth was measured. The assays were repeated at least 3 times. Although the relative differences between strains were consistent between experiments, the absolute values for each strain varied due to subtle changes in the media and assay conditions, and thus only one representative experiment is shown.

b. *E. coli* TonB*X* is denoted by e TonB*X*; *V. cholerae* TonB1*X* is denoted by v1 TonB*X*.

c. Encodes the *V. cholerae* outer membrane receptor for heme, HutA.

Figure 12. *E. coli* TonB-dependent transport of iron substrates in *E. coli* ARM100.

The *E. coli* TonB, ExbB, ExbD complex facilitates transport of ferrichrome through the *E. coli* receptor FhuA, but does not promote efficient heme uptake via the *V. cholerae* receptor HutA in this *E. coli* strain.

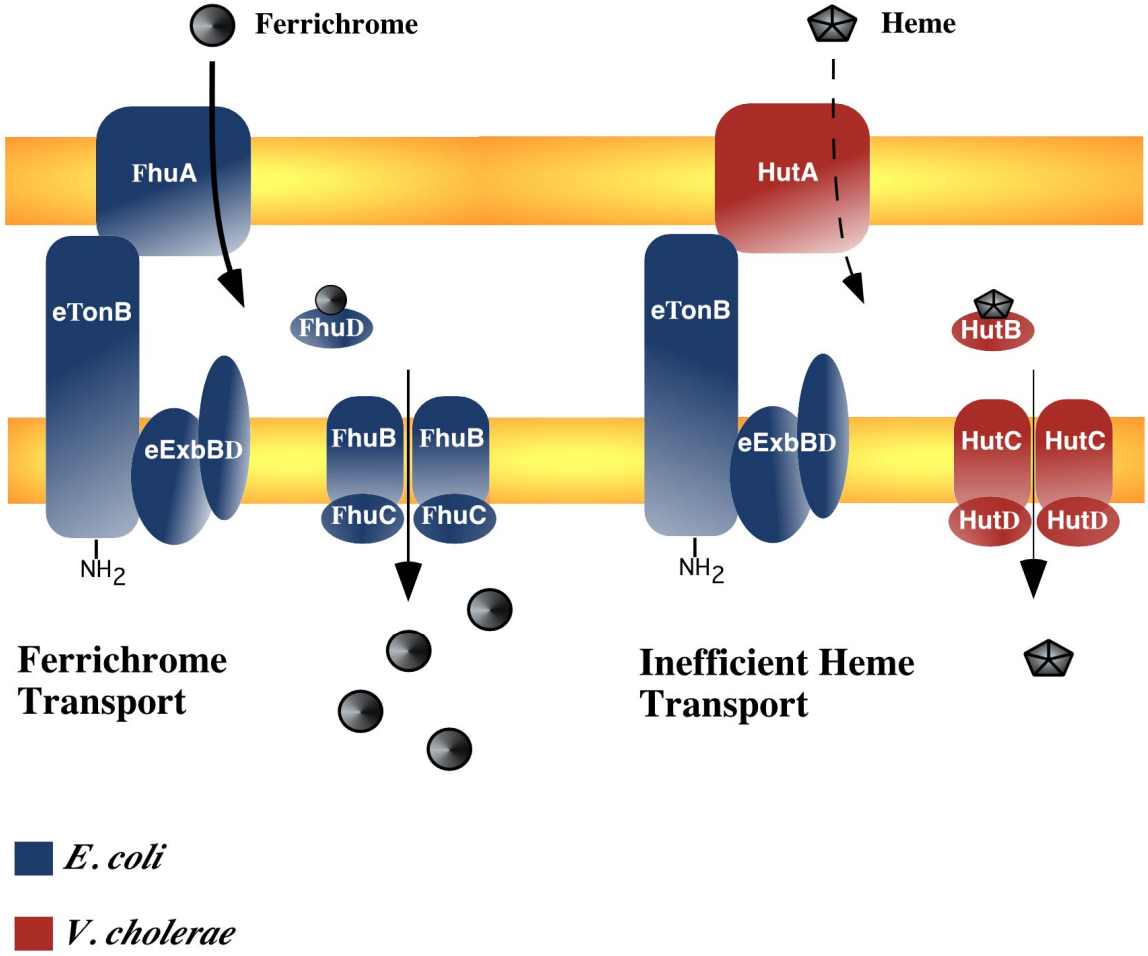

Ferrichrome
Heme
FhuA
HutA
eTonB
FhuD
eTonB
HutB
eExbBD
FhuB
FhuB
eExbBD
HutC
HutC
FhuC
FhuC
HutD
HutD
NH2
NH2
Ferrichrome Transport
Inefficient Heme Transport
E. coli
V. cholerae

Figure 13. *V. cholerae* TonB1-dependent transport of iron complexes in *E. coli*.

The *V. cholerae* TonB1, ExbB1, ExbD1 complex permits heme uptake via the *V. cholerae* receptor HutA, but does not facilitate ferrichrome transport through the *E. coli* receptor FhuA in this *E. coli* strain.

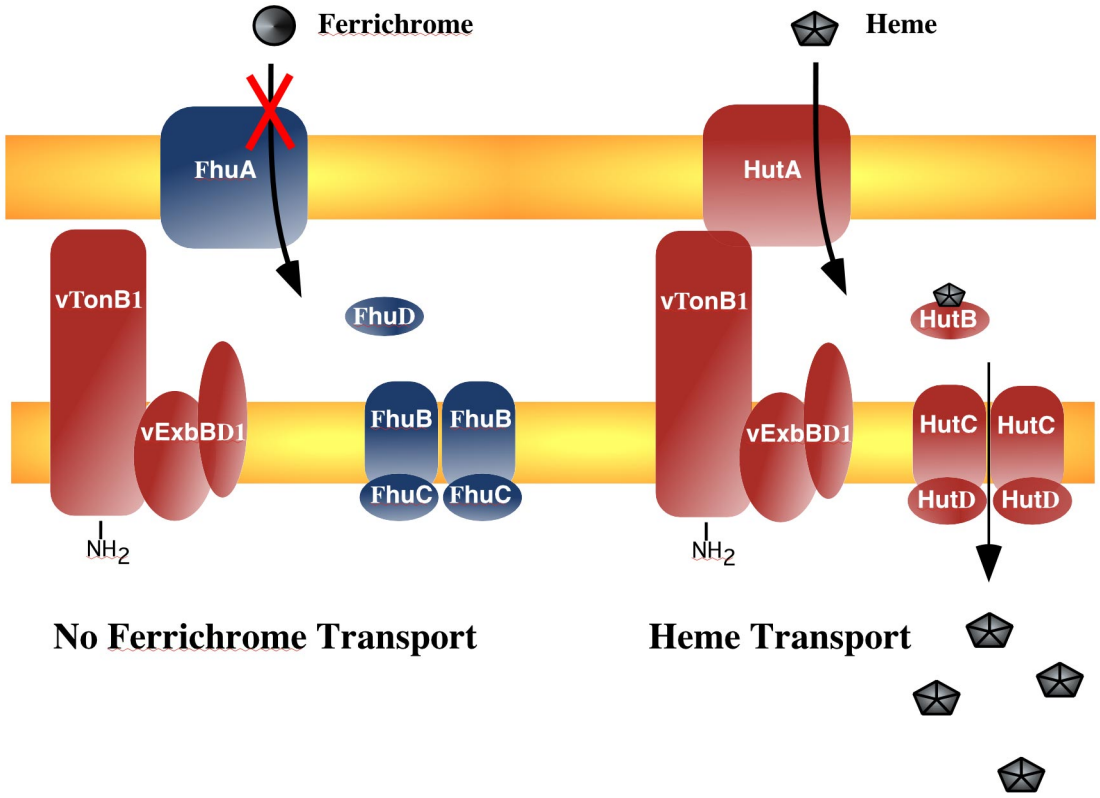

Ferrichrome
Heme
FhuA
HutA
vTonB1
FhuD
vTonB1
HutB
vExbBD1
FhuB
FhuB
vExbBD1
HutC
HutC
FhuC
FhuC
HutD
HutD
NH2
NH2
No Ferrichrome Transport
Heme Transport

Figure 14. Transport of iron complexes via a chimeric e/v1 TonB protein in *E. coli.*

A chimeric TonB protein, composed of the N-terminal domain of *E. coli* TonB fused to the C-terminal domain of *V. cholerae* TonB1, permits heme transport through *V. cholerae* HutA, but not ferrichrome transport through *E. coli* FhuA. Thus, this chimera is functionally equivalent to *V. cholerae* TonB1.

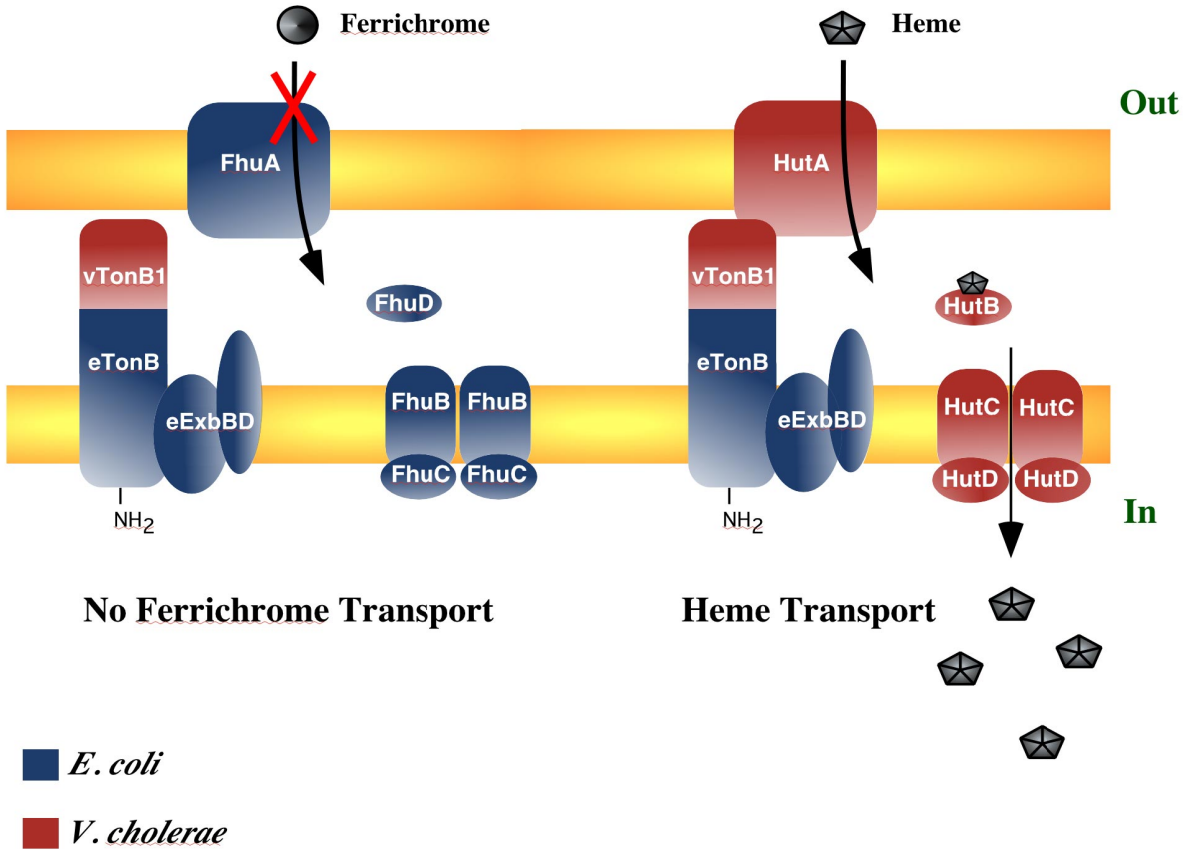
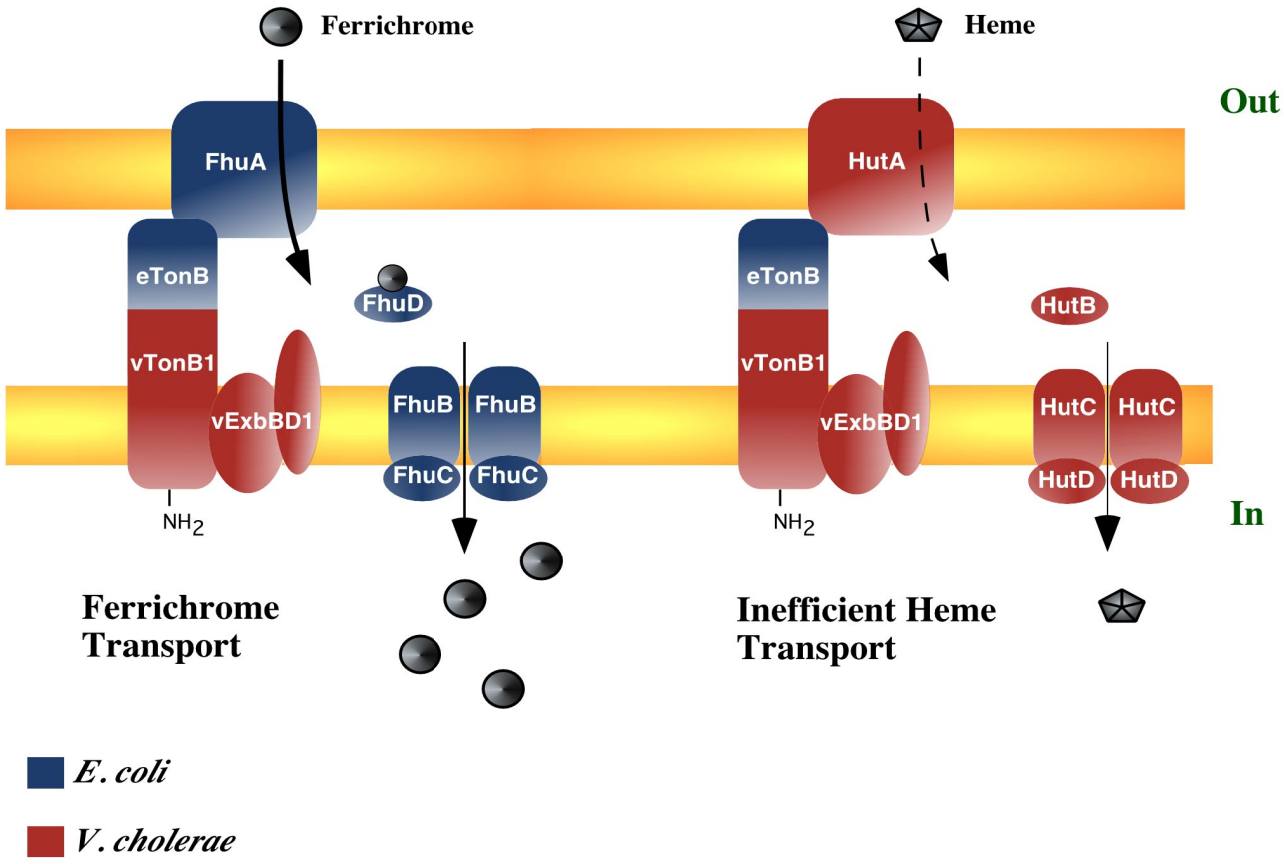

Ferrichrome
Heme
Out
FhuA
HutA
vTonB1
eTonB
eExbBD
NH2
FhuD
FhuB
FhuC
HutB
HutC
HutD
In
No Ferrichrome Transport
Heme Transport
E. coli
V. cholerae

Figure 15. Transport of iron complexes via a chimeric v1/e TonB protein in *E. coli.*

A chimeric TonB protein, composed of the N-terminal domain of *V. cholerae* TonB1 fused to the C-terminal domain of *E. coli* TonB, permits ferrichrome transport through *E. coli* FhuA, but only weak heme uptake through *V. cholerae* HutA. Thus, this chimera is functionally equivalent to *E. coli* TonB.


Ferrichrome
Heme
Out
FhuA
HutA
eTonB
vTonB1
vExbBD1
FhuD
FhuB
FhuB
FhuC
FhuC
NH2
eTonB
vTonB1
vExbBD1
HutB
HutC
HutC
HutD
HutD
NH2
In
Ferrichrome Transport
Inefficient Heme Transport
E. coli
V. cholerae

These data suggest that a region within the carboxy-terminal half of TonB is involved in determining the specificity of the interaction with outer membrane receptors.

To characterize further the TonB-receptor interaction, TonB hybrids involving smaller portions of the C-termini were tested to define a minimal region required to switch the TonB receptor-specificity from one species to another. A set of TonB chimeras was created in which the C-terminal 66 amino acids from each TonB protein were switched. This domain swap did not include the conserved Gln_{160} residue previously implicated in the direct interaction between *E. coli* TonB and the TonB box of the BtuB receptor in *E. coli* (7, 25). The hybrid genes were constructed by introducing a *Xho*I site into each *tonB* gene and exchanging equivalent fragments. The *Xho*I mutation resulted in an Ile to Leu change in both TonB proteins; however, this change did not adversely affect the function of either TonB in functional assays (Table 10). The native and hybrid *tonB* constructs were tested as described above. pAMT124, encoding the v1/e TonB chimera, conferred the ability to use enterobactin and ferrichrome, but did not allow efficient heme utilization. Thus, this chimera exhibited the specificities associated with *E. coli* TonB, indicating that the C-terminal 66 amino acids of *E. coli* TonB are sufficient for recognizing *E. coli* receptors. The reciprocal e/v1 chimera, encoded by pAMT123, did not have any TonB activity, suggesting that an important functional domain was disrupted in this hybrid.

A set of chimeras was created in which the C-terminal 31 amino acids were switched between *E. coli* TonB and *V. cholerae* TonB1. These hybrids were

Table 10. Analysis of chimeras involving the C-terminal 66 residues of *E. coli* TonB and *V. cholerae* TonB1.

ARM100 (TonB$^-$, EntF$^-$)/ pAMH20[c] transformed with:	Relevant TonB characteristics[b]	Zone of growth (mm) after 24 hours[a]		
		$FeSO_4$	Enterobactin	Hemin
pWKS30ΔX	TonB$^-$	15	NG	NG
pAMT121	e TonB*XXh*	19	20	10 (faint)
pAMT122	v1 TonB*XXh*	16	NG	13
pAMT123	$e_{1\text{-}172}/v1_{179\text{-}245}$TonB*XXh*	17	NG	NG
pAMT124	$v1_{1\text{-}178}/e_{173\text{-}239}$TonB*XXh*	17	21	11 (faint)

a. The indicated strains were seeded at 10^6 cells per ml into molten L agar containing 100 μg EDDA per ml. Iron sources were spotted onto the solidified agar as follows: 20 μl 10 mM $FeSO_4$; 5 μl DH5α overnight culture (enterobactin); 5 μl 50 μM hemin. NG = No growth. Plates were incubated for 24 hrs at 37°C, and the diameter of each zone of growth was measured. The assays were repeated at least 3 times. Although the relative differences between strains were consistent between experiments, the absolute values for each strain varied due to subtle changes in the media and assay conditions, and thus only one representative experiment is shown.

b. *E. coli* TonB*XXh* is denoted by e TonB*XXh*; *V. cholerae* TonB1*XXh* is denoted by v1 TonB*XXh*.

c. Encodes the *V. cholerae* outer membrane receptor for heme, HutA.

constructed by engineering unique *Mlu*I sites into the *tonB* genes and switching the relevant fragments. The *Mlu*I mutation resulted in Glu to Asp and Ile to Leu changes in *V. cholerae* TonB1, and a Met to Leu change in *E. coli* TonB. These amino acid changes did not influence the activities of the TonB proteins in functional assays; however, neither of the chimeric TonBs exhibited any TonB activity, indicating that this switch disrupts important structural or functional motifs (Table 11). All other attempts at switching fewer than 66 residues resulted in non-functional chimeras, suggesting that this C-terminal region constitutes an important functional domain of TonB that relies on many specific intramolecular interactions to form properly.

3. IDENTIFICATION OF A MINIMAL REGION OF *V. CHOLERAE* TONB1 REQUIRED TO CONFER SPECIFICITY FOR *V. CHOLERAE* RECEPTORS

To identify the minimal region of *V. cholerae* TonB1 necessary to confer the specificities associated with the TonB1 protein, hybrids were created between *E. coli* TonB and *V. cholerae* TonB1 that included progressively fewer than 120 TonB1 C-terminal residues. The *tonB* genes encoding these hybrids were engineered without the insertion of restriction sites, and thus no changes in the amino acid sequences of the TonB proteins were incurred. The chimera encoded by pAMT173, containing the N-terminal 158 residues of *E. coli* TonB joined to the C-terminal 88 residues of *V. cholerae* TonB1, allowed heme utilization via *V. cholerae* HutA, but did not permit enterobactin utilization via *E. coli* FepA, or ferrichrome utilization via *E. coli* FhuA, indicating that this chimera is specific for

Table 11. Analysis of chimeras involving the C-terminal 31 residues of *E. coli* TonB and *V. cholerae* TonB1.

ARM100 (TonB$^-$, EntF$^-$)/ pAMH20[c] transformed with:	Relevant TonB characteristics[b]	Zone of growth (mm) after 24 hours[a]		
		$FeSO_4$	Enterobactin	Hemin
pWKS30ΔX	TonB$^-$	15	NG	NG
pAMT131	e TonB*XM*	17	20	11 (faint)
pAMT132	v1 TonB*XM*	16	NG	NG
pAMT133	$e_{1\text{-}208}/v1_{215\text{-}245}$TonB*XM*	16	NG	NG
pAMT134	$v1_{1\text{-}214}/e_{209\text{-}239}$TonB*XM*	17	NG	NG

a. The indicated strains were seeded at 10^6 cells per ml into molten L agar containing 100 μg EDDA per ml. Iron sources were spotted onto the solidified agar as follows: 20 μl 10 mM $FeSO_4$; 5 μl DH5α overnight culture (enterobactin); 5 μl 50 μM hemin. NG = No growth. Plates were incubated for 24 hrs at 37°C, and the diameter of each zone of growth was measured. The assays were repeated at least 3 times. Although the relative differences between strains were consistent between experiments, the absolute values for each strain varied due to subtle changes in the media and assay conditions, and thus only one representative experiment is shown.

b. *E. coli* TonB*XM* is denoted by e TonB*XM*; *V. cholerae* TonB1*XM* is denoted by v1 TonB*XM*.

c. Encodes the *V. cholerae* outer membrane receptor for heme, HutA.

V. cholerae receptors (Table 12). This chimera therefore contains the region of *V. cholerae* TonB1 involved in determining specificity.

The portion of TonB1 encoded by pAMT173 includes a short stretch of amino acids that does not align well with *E. coli* TonB, or with *V. cholerae* TonB2 (Fig. 16). To determine if specificity determinants are contained within this region of TonB1, an e/v1 chimera lacking these residues was constructed by joining the C-terminal 81 amino acids of TonB1 to the N-terminal 158 amino acids of *E. coli* TonB. This chimera, encoded by pAMT183, exhibited all the expected TonB1 specificities, and had wild type levels of TonB1 activity (Table 12), indicating that the missing residues were not required for TonB1 activity or specificity. These data demonstrate that the C-terminal 81 amino acids of TonB1 are sufficient to confer the specificities exhibited by *V. cholerae* TonB1. Because the chimera encoded by pAMT183 is shorter than the chimera encoded by pAMT173, the data also imply that, in *E. coli*, the activities of the TonB chimeras are not sensitive to small variations in length.

4. ISOLATION OF A *V. CHOLERAE* TONB1 POINT MUTANT CAPABLE OF INTERACTING WITH *E. COLI* RECEPTORS

To pinpoint specific residues important for determining the narrow receptor specificity of *V. cholerae* TonB1, *E. coli* ARM100 ($EntF^-$; $TonB^-$) carrying a plasmid encoding the *V. cholerae* TonB1 complex was grown in iron-limiting media containing only enterobactin or ferrichrome as iron sources. As expected, no growth was visible around either iron source after 24 hours, since

Table 12. Identification of a minimal *V. cholerae* TonB1 C-terminal domain required for specificity.

ARM100 (TonB$^-$, EntF$^-$)/ pAMH20[c] transformed with:	Relevant TonB characteristics[b]	Zone of growth (mm) after 48 hours[a]		
		$FeSO_4$	Enterobactin	Hemin
pWKS30ΔX	TonB$^-$	15	NG	NG
pAMT111	e TonB*X*	20	28	10 (faint)
pAMT112	v1 TonB*X*	16	NG	13
pAMT173	$e_{1\text{-}158}/v1_{158\text{-}245}$TonB*X*	19	NG	12
pAMT183	$e_{1\text{-}158}/v1_{165\text{-}245}$TonB*X*	17	NG	12

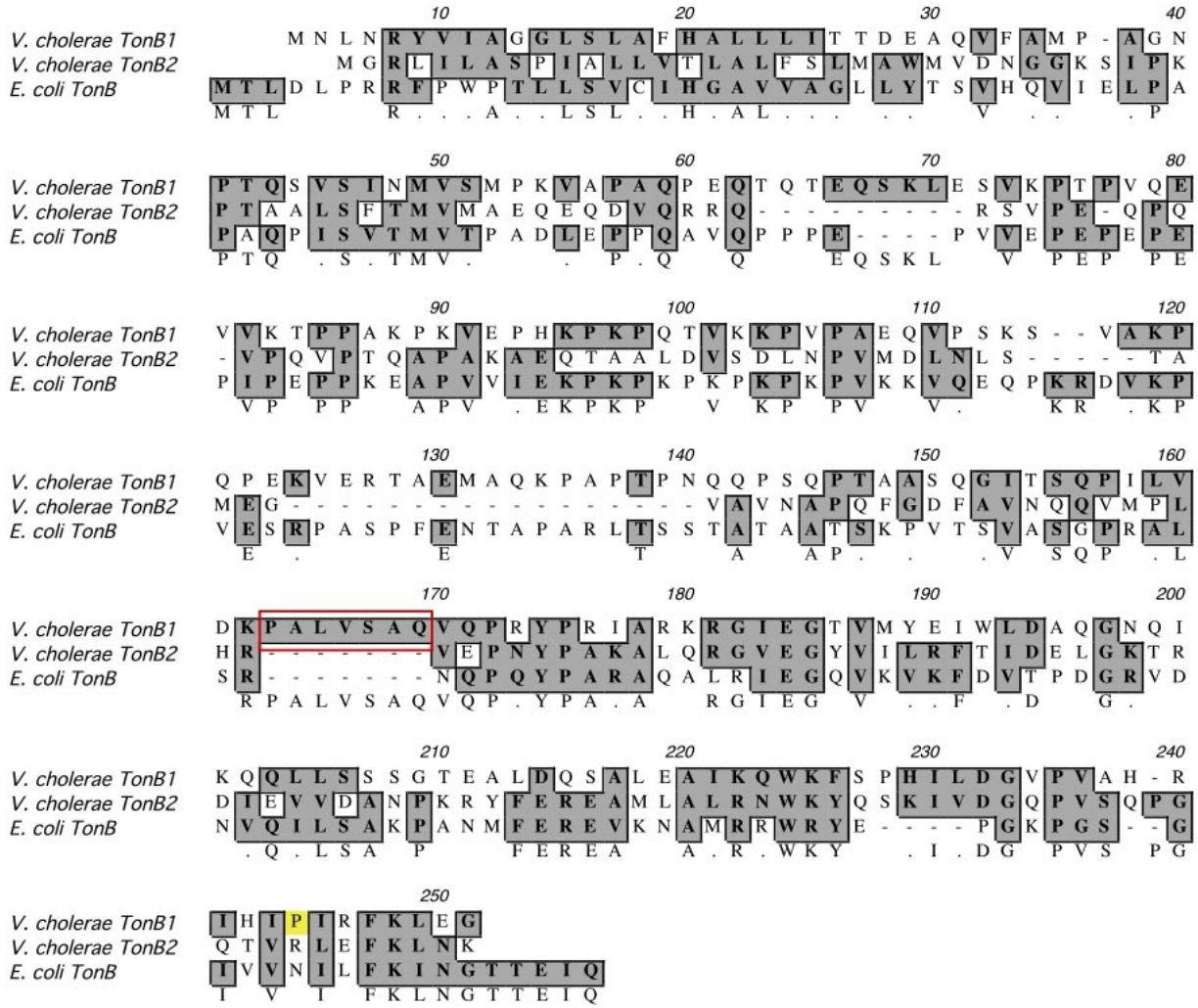
a. The indicated strains were seeded at 10^6 cells per ml into molten L agar containing 100 μg EDDA per ml. Iron sources were spotted onto the solidified agar as follows: 20 μl 10 mM $FeSO_4$; 5 μl DH5α overnight culture (enterobactin); 5 μl 50 μM hemin. NG = No growth. Plates were incubated for 24 hrs at 37°C, then for 24 hrs at room temperature, and the diameter of each zone of growth was measured. The assays were repeated at least 3 times. Although the relative differences between strains were consistent between experiments, the absolute values for each strain varied due to subtle changes in the assay conditions, and thus only one representative experiment is shown.

b. *E. coli* TonB*X* is denoted by e TonB*X*; *V. cholerae* TonB1*X* is denoted by v1 TonB*X*.

c. Encodes the *V. cholerae* outer membrane receptor for heme, HutA.

Figure 16. Alignment of *V. cholerae* TonB1, *V. cholerae* TonB2 and *E. coli* TonB.

The amino acid sequences of *V. cholerae* TonB1, *V. cholerae* TonB2 and *E. coli* TonB were aligned using CLUSTAL-W. The region of TonB1 included in the chimera encoded by pAMT173, but not in the chimera encoded by pAMT183, is boxed in red. The site of the Pro237Thr gain-of-function mutation in *V. cholerae* TonB1 is highlighted in yellow.


V. cholerae TonB1
V. cholerae TonB2
E. coli TonB

V. cholerae TonB1 does not recognize either the enterobactin receptor FepA or the ferrichrome receptor FhuA in *E. coli*. However, after further incubation at room temperature for up to one week, a small number of colonies became visible in the agar surrounding the iron sources, possibly as a result of mutations facilitating the productive interaction between *V. cholerae* TonB1 and *E. coli* FepA or FhuA. To analyze potential mutants, single colonies were tested again in halo assays to verify the transport phenotype. A mutant was obtained which exhibited growth around both enterobactin and ferrichrome, suggesting that this strain expressed a *V. cholerae* TonB1 variant capable of interacting with multiple *E. coli* receptors. To verify that a mutation(s) had occurred in *V. cholerae tonB1*, the *tonB1* plasmid was retransformed into a fresh background. The newly transformed strain was able to use both enterobactin and ferrichrome (Table 13). Sequencing of the *tonB1* gene on the plasmid now designated pAMT112.m1 showed that it carried a mutation resulting in a Pro to Thr substitution at residue 238 TonB1*X*. This residue corresponds to position 237 in the wild type TonB1 protein, and therefore the designation 237* will be used to describe this position in TonB1*X*. Pro_{237} is very close to the C-terminus of TonB1 (Fig. 16). This suggests that either there is a receptor interaction site at the very C-terminus of TonB1, or mutations in this region influence an interaction domain(s) elsewhere in TonB1.

Table 13. Specificity of a *V. cholerae* TonB1 Pro237Thr mutant.

ARM100 (TonB$^-$, EntF$^-$) transformed with:	Relevant TonB characteristics	Zone of growth (mm) after 24 hours[a]		
		$FeSO_4$	Ferrichrome	Enterobactin
pWKS30ΔX	TonB$^-$	20	NG	NG
pAMT111	eTonB*X*	22	16	26
pAMT112	vTonB1*X*	20	NG	NG
pAMT112.m1	vTonB1*X* $_{P237*T}$	20	13	22

a. The indicated strains were seeded at 10^6 cells per ml into molten L agar containing 100 μg EDDA per ml. Iron sources were spotted onto the solidified agar as follows: 20 μl 10 mM $FeSO_4$; 5 μl 40 μM Ferrichrome; 5 μl DH5α overnight culture (enterobactin. NG = No growth. Plates were incubated for 24 hrs at 37°C, and the diameter of each zone of growth was measured. The assays were repeated at least 3 times. Although the relative differences between strains were consistent between experiments, the absolute values for each strain varied due to subtle changes in the media and assay conditions, and thus only one representative experiment is shown.

b. e denotes *E. coli*; v denotes *V. cholerae.*

5. FUNCTIONAL ANALYSIS OF *V. CHOLERAE* AND *E. COLI* HEME RECEPTOR CHIMERAS INVOLVING THE N-TERMINAL TONB BOX DOMAIN

The TonB box region of TonB-dependent receptors has been proposed to be important for the productive interaction with TonB. A comparison of the known *V. cholerae* and *E. coli* TonB boxes reveals that the *V. cholerae* HutA TonB box differs significantly from the *E. coli* TonB boxes, particularly at positions that are highly conserved among the *E. coli* TonB boxes (Fig. 17). Thus, the specificity exhibited by *V. cholerae* TonB1 and *E. coli* TonB for certain receptors may in part be due to a requirement for a particular TonB box sequence. To investigate this hypothesis, chimeras were constructed between *E. coli* and *V. cholerae* receptors in which the TonB box heptapeptides were switched. The chimeras were then tested for ability to function with either *V. cholerae* TonB1 or *E. coli* TonB in iron utilization assays in *E. coli* ARM100.

To test whether the presence of the HutA TonB box is sufficient to allow TonB1 to recognize an *E. coli* receptor, the TonB box of the *E. coli* heme receptor ChuA was replaced with the HutA TonB box. The resulting ChuA (HutA TonB box) chimera was tested for dependence on either *V. cholerae* TonB1 or *E. coli* TonB. Table 14 shows that ChuA (HutA TonB box) facilitated heme uptake using *E. coli* TonB, but not *V. cholerae* TonB1, indicating that the TonB-dependence of this chimera is identical to that of the wild type ChuA protein. The level of heme utilization via the chimeric receptor was similar to that observed for wild type ChuA; thus, binding and transport of heme was not compromised by the TonB box substitution (Table 14). Taken together, these data suggest that

Figure 17. Predicted *E. coli* and *V. cholerae* receptor TonB boxes

The proposed TonB box (underlined) and the residue immediately preceding the TonB box heptapeptide are shown for several *E. coli* and *V. cholerae* receptors. *E. coli* sequences are shown in blue and *V. cholerae* sequences are shown in red. The *V. cholerae* vibriobactin receptor ViuA does not contain an N-terminal sequence with homology to any of the predicted *E. coli* or *V. cholerae* TonB boxes, as is indicated by a question mark. The TonB-dependencies of each receptor in its native environment are shown.

		Ability to function with:	
E. coli		eTonB	vTonB1
FepA	D D T I V V T A	**Yes**	**No**
BtuB	P D T L V V T A	**Yes**	**ND**
FhuA	E D T I T V T A	**Yes**	**No**
ChuA	T E T M T V T A	**Yes**	**No**
$FepA_{D11Y}$	Y D T I V V T A	**Yes**	**Yes**
$ChuA_{T29F}$	F E T M T V T A	**Yes**	**Yes**
V. cholerae		vTonB1	vTonB2
HutA	F D E V V V S T	**Yes**	**Yes**
HutR	Y E E V V V T A	**Yes**	**Yes**
HasR	D E T V T V H G	**No**	**Yes**
ViuA	?	**Yes**	**Yes**
FhuA	L E T L V V T A	**Yes**	**Yes**
BtuB	Q E T V V V T A	**ND**	**ND**
IrgA	D E T M V V T A	**No**	**Yes**
VctA	E Q E N V V I W	**No**	**Yes**

Table 14. TonB-dependence of ChuA and $ChuA_{HutA\ TonB\ box}$ in *E. coli.*

ARM100 ($TonB^-$, $EntF^-$) transformed with:	Relevant Phenotype[b]	Zone of growth (mm) after 48 hours[a]		
		$FeSO_4$	Enterobactin	Hemin
pWKS30ΔX/pChuA	$TonB^-$, $ChuA^+$	21	NG	NG
pAMT111/pChuA	e$TonBX^+$, $ChuA^+$	20	28	16
pAMT112/pChuA	v$TonB1X^+$, $ChuA^+$	21	NG	NG
pAMT111/pAMC22	e$TonBX^+$, $ChuA_{HutA\ TonB\ box}^+$	20	25	15
pAMT112/pAMC22	v$TonB1X^+$, $ChuA_{HutA\ TonB\ box}^+$	21	NG	NG

a. The indicated strains were seeded at 10^6 cells per ml into molten L agar containing 100 μg EDDA per ml. Iron sources were spotted onto the solidified agar as follows: 20 μl 10 mM $FeSO_4$; 5 μl DH5α overnight culture (enterobactin); 5 μl 50 μM hemin. NG = No growth. Plates were incubated for 24 hrs at 37°C, then for 24 hrs at room temperature, and the diameter of each zone of growth was measured. The assays were repeated at least 3 times. Although the relative differences between strains were consistent between experiments, the absolute values for each strain varied due to subtle changes in the media and assay conditions, and thus only one representative experiment is shown.

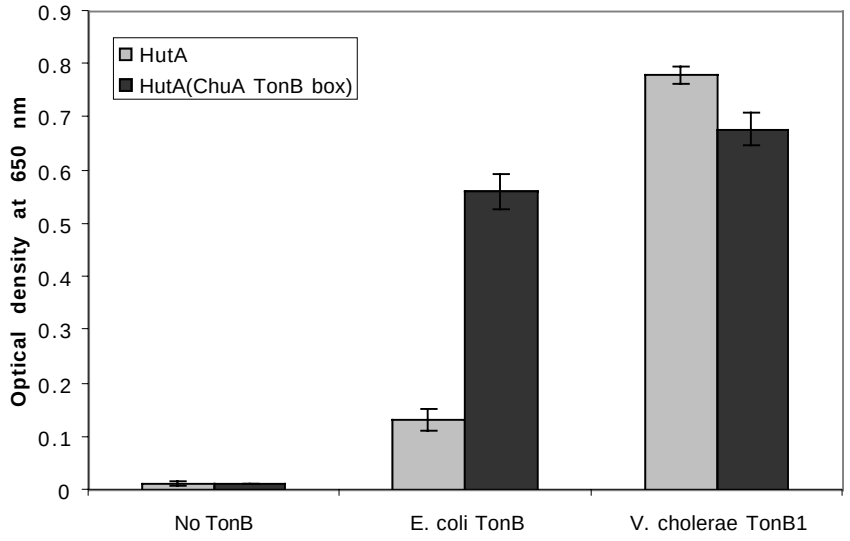
b. e denotes *E. coli*; v denotes *V. cholerae.*

residues apart from, or in addition to, the TonB box heptapeptide are required for the productive interaction with *V. cholerae* TonB1. Because the TonB box substitution did not influence the ability of *E. coli* TonB to recognize ChuA, the data also imply that the ChuA TonB box is not specifically required for the interaction with *E. coli* TonB.

A reciprocal chimera was constructed in which the TonB box of *V. cholerae* HutA was replaced with the TonB box from *E. coli* ChuA. The HutA (ChuA TonB box) chimera was tested for ability to mediate heme utilization using either *V. cholerae* TonB1 or *E. coli* TonB in a broth culture growth assay. This assay method was chosen in order to quantify more accurately the observed growth differences attributable to this particular TonB box substitution. The data show that *E. coli* TonB mediated heme utilization more efficiently through the HutA (ChuA TonB box) chimera than through the wild type HutA receptor, suggesting that the presence of the ChuA TonB box promoted a stronger interaction with *E. coli* TonB (Fig. 18). It is not clear from these data whether *E. coli* TonB specifically recognizes the ChuA TonB box in the context of HutA, or whether the TonB box substitution affects a TonB interaction site elsewhere in the HutA receptor. The level of *V. cholerae* TonB1-mediated heme utilization via the HutA (ChuA TonB box) chimera was essentially unchanged compared with the level achieved via wild type HutA, indicating that the TonB box substitution did not adversely affect either the heme transport functions of HutA or the ability of HutA to interact with *V. cholerae* TonB1 (Fig. 18). These data support the

Figure 18. Heme uptake via HutA carrying the ChuA TonB box.

Strains were inoculated into L broth containing 75 μg EDDA per ml, and 5 μM hemin. The optical density was measured after 24 hrs. Growth in iron-replete media was comparable for all the strains tested, and none of the strains grew in iron-depleted media (data not shown). Each data point represents the mean optical density from three independently grown cultures. Standard deviations are indicated by the error bars.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Optical density at 650 nm
HutA
HutA(ChuA TonB box)
No TonB
E. coli TonB
V. cholerae TonB1

above conclusion that residues elsewhere in the HutA receptor contribute to the specificity of the interaction with *V. cholerae* TonB1.

To test whether a TonB box from an *E. coli* receptor with a different substrate than heme would have a similar effect on HutA as the ChuA TonB box, the *E. coli* enterobactin receptor FepA TonB box was introduced into HutA. This chimera did not function with either *E. coli* TonB or *V. cholerae* TonB1 in an iron utilization assay in *E.coli*, implying that the FepA TonB box is incompatible with HutA (Table 15). This result was somewhat surprising, given that the HutA TonB box is more similar to the FepA TonB box than to the ChuA TonB box (Fig. 17, page 134).

In conclusion, the specificity of the TonB-receptor interaction does not appear to be dictated by specific residues within the TonB box heptapeptide. The presence of a heterologous TonB box within a receptor did not diminish productive interactions with the native TonB protein, implying that other regions(s) of the receptor contain the specificity determinants. However, a heterologous TonB box may influence the productive interaction with TonB by increasing the efficiency of a weak interaction, such as that between *V. cholerae* HutA and *E. coli* TonB, or by eliminating the receptor transport function altogether, as evidenced by the non-functional HutA (FepA TonB box) chimera.

Table 15. TonB-dependence of HutA and $HutA_{FepA\ TonB\ box}$ in *E. coli.*

ARM100 ($TonB^-$, $EntF^-$) transformed with:	Relevant Phenotype[b]	Zone of growth (mm) after 48 hours[a]		
		$FeSO_4$	Enterobactin	Hemin
pWKS30ΔX/pAMH20	$TonB^-$, $HutA^+$	20	NG	NG
pAMT111/pAMH20	e$TonBX^+$, $HutA^+$	20	26	10 (faint)
pAMT112/pAMH20	v$TonB1X^+$, $HutA^+$	19	NG	12
pAMT111/pAMH21	e$TonBX^+$, $HutA_{FepA\ TonB\ box}^+$	20	27	NG
pAMT112/pAMH21	v$TonB1X^+$, $HutA_{FepA\ TonB\ box}^+$	20	NG	NG

a. The indicated strains were seeded at 10^6 cells per ml into molten L agar containing 100 μg EDDA per ml. Iron sources were spotted onto the solidified agar as follows: 20 μl 10 mM $FeSO_4$; 5 μl DH5α overnight culture (enterobactin); 5 μl 50 μM hemin. NG = No growth. Plates were incubated for 24 hrs at 37°C, then for 24 hrs at room temperature, and the diameter of each zone of growth was measured. The assays were repeated at least 3 times. Although the relative differences between strains were consistent between experiments, the absolute values for each strain varied due to subtle changes in the media and assay conditions, and thus only one representative experiment is shown.

b. e denotes *E. coli*; v denotes *V. cholerae.*

6. ISOLATION OF AN *E. COLI* FEPA MUTANT CAPABLE OF INTERACTING WITH *V. CHOLERAE* TONB1

To identify specific receptor residues involved in recognizing *V. cholerae* TonB1, *E. coli* ARM100 carrying a plasmid encoding the *V. cholerae* TonB1 complex was grown in a halo assay using enterobactin as the sole source of iron. A small number of colonies used enterobactin, possibly as a result of gain-of-function mutations allowing productive interactions between *V. cholerae* TonB1 and the chromosomally encoded *E. coli* FepA. A candidate mutant strain was analyzed by curing the strain of the TonB1 plasmid and retransforming the strain with a plasmid encoding either *V. cholerae* TonB1 or *E. coli* TonB. The freshly transformed mutant strain used enterobactin in the presence of either of the two TonBs, indicating that the mutation did not affect the ability of FepA to recognize *E. coli* TonB (Table 16). Sequencing of the candidate gain-of-function *fepA* allele showed that it contained a mutation resulting in an Asp11Tyr substitution in the mature FepA protein. To verify that the Asp11Tyr mutation was responsible for the productive interaction of FepA with *V. cholerae* TonB1, the mutation was re-constructed within a wild type *fepA* gene on a plasmid. The $fepA_{D11Y}$ plasmid conferred enterobactin utilization in ARM100 carrying the *tonB1* system plasmid, whereas the wild type *fepA* plasmid did not (Table 16). These data confirm the gain-of function phenotype of the $FepA_{D11Y}$ mutation. Interestingly, the substituted residue in $FepA_{D11Y}$ immediately precedes the TonB box in the FepA amino acid sequence, strongly implicating this N-terminal receptor region in the interaction with TonB.

Table 16. TonB-dependence of FepA and $FepA_{D11Y}$ in *E. coli.*

		Zone of growth (mm) after 24 hours[a]		
Strain	Relevant Phenotype[b]	$FeSO_4$	Enterobactin	Ferrichrome
ARM100/pWKS30ΔX	$TonB^-$, $FepA^+$	20	NG	NG
ARM100/pAMT111	e$TonBX^+$, $FepA^+$	22	25	15
ARM100/pAMT112	v$TonB1X^+$, $FepA^+$	20	NG	NG
ARM107/pAMT111	e$TonBX^+$, $FepA_{D11Y}{}^+$	20	25	15
ARM107/pAMT112	v$TonB1X^+$, $FepA_{D11Y}{}^+$	21	22	NG
ARM100/pAMT112/pFepA	v$TonB1X^+$, $FepA^+$	23	NG	NG
ARM100/pAMT112/p$FepA_{D11Y}$	v$TonB1X^+$, $FepA_{D11Y}{}^+$	22	23	NG

a. The indicated strains were seeded at 10^6 cells per ml into molten L agar containing 100 μg EDDA per ml. Iron sources were spotted onto the solidified agar as follows: 20 μl 10 mM $FeSO_4$; 5 μl DH5α overnight culture (enterobactin); 5 μl 50 μM hemin. NG = No growth. Plates were incubated for 24 hrs at 37°C, and the diameter of each zone of growth was measured. The assays were repeated at least 3 times. Although the relative differences between strains were consistent between experiments, the absolute values for each strain varied due to subtle changes in the media and assay conditions, and thus only one representative experiment is shown.

b. e denotes *E. coli*; v denotes *V. cholerae.*

7. ANALYSIS OF AN *E. COLI* CHUA MUTANT CAPABLE OF INTERACTING WITH *V. CHOLERAE* TONB1

To investigate the relevance of the TonB box –1 position for TonB specificity, a comparison of *E. coli* and *V. cholerae* receptor sequences was carried out. The comparison shows that *V. cholerae* HutA and HutR, both of which use *V. cholerae* TonB1, contain an aromatic residue immediately prior to the TonB box (Fig. 17, page 134). In contrast, the *V. cholerae* TonB2-dependent receptors IrgA, VctA, and HasR all carry an acidic residue at this position. Further, none of the *E. coli* receptors contain an aromatic residue at the TonB box –1 position, and these receptors are not recognized by *V. cholerae* TonB1. To test the hypothesis that an aromatic amino acid at the position immediately preceding the TonB box permits interaction with *V. cholerae* TonB1, an aromatic residue was introduced into the *E. coli* heme receptor, ChuA, at this position. ChuA carrying Phe in place of Thr at the TonB box –1 position was tested for dependence on *E. coli* TonB or *V. cholerae* TonB1. Table 17 shows that, whereas *V. cholerae* TonB1 failed to recognize wild type ChuA, TonB1 interacted productively with the $ChuA_{T1F}$ variant, facilitating heme utilization via this mutant receptor. These data suggest that receptor recognition by *V. cholerae* TonB1 is determined by the residue immediately preceding the TonB box, further implicating this region of the receptor in the interaction with TonB. As was observed for the FepA Asp11Tyr mutation, the Thr1Phe mutation in ChuA did not abolish interactions with *E. coli* TonB; heme was used as efficiently via

Table 17. TonB-dependence of ChuA and $ChuA_{T1F}$ in *E. coli.*

ARM100 ($TonB^-$, $EntF^-$) transformed with:	Relevant Phenotype[b]	Zone of growth (mm) after 48 hours[a]		
		$FeSO_4$	Enterobactin	Hemin
pWKS30ΔX/pChuA	$TonB^-$, $ChuA^+$	22	NG	NG
pAMT111/pChuA	e$TonBX^+$, $ChuA^+$	20	28	16
pAMT112/pChuA	v$TonB1X^+$, $ChuA^+$	21	NG	NG
pAMT111/$pChuA_{T1F}$	e$TonBX^+$, $ChuA_{T1F}^+$	22	28	15
pAMT112/$pChuA_{T1F}$	v$TonB1X^+$, $ChuA_{T1F}^+$	21	NG	14

a. The indicated strains were seeded at 10^6 cells per ml into molten L agar containing 100 μg EDDA per ml. Iron sources were spotted onto the solidified agar as follows: 20 μl 10 mM $FeSO_4$; 5 μl 40 μM Ferrichrome; 5 μl DH5α overnight culture (enterobactin); 5 μl 50 μM hemin. NG = No growth. Plates were incubated for 24 hrs at 37°C, then for 24 hrs at room temperature, and the diameter of each zone of growth was measured. The assays were repeated at least 3 times. Although the relative differences between strains were consistent between experiments, the absolute values for each strain varied due to subtle changes in the media and assay conditions, and thus only one representative experiment is shown.

b. e denotes *E. coli*; v denotes *V. cholerae.*

$ChuA_{T1F}$ as via wild type ChuA in the presence of *E. coli* TonB. Thus, *E. coli* TonB appears not to have stringent requirements for the TonB box –1 position.

8. DIFFERENCES IN THE ACTIVITY AND/OR SPECIFICITY OF TONB PROTEINS EXPRESSED IN *E. COLI* VERSUS *V. CHOLERAE*

A functional *V. cholerae* heme transport system can be reconstituted in *E. coli* by supplying both HutA and the *V. cholerae* TonB1 heme transport locus. In contrast, *E. coli* cells expressing HutA and *V. cholerae* TonB2 did not use heme (data not shown), suggesting that HutA may be specific for *V. cholerae* TonB1. However, data presented in this dissertation (Table 6, page 102) clearly demonstrate that *V. cholerae* TonB2 promotes heme uptake via HutA in *V. cholerae*, suggesting that differences in the *E. coli* and *V. cholerae* environments contribute to the observed specificities of *E. coli* and *V. cholerae* TonB proteins.

Poor heme utilization in *E. coli* strains expressing the *V. cholerae* heme receptors HutA or HutR (data not shown) and *E. coli* TonB may signify that these receptors lack several or all of the sites required for functional interactions with *E. coli* TonB. However, it is also possible that the environment of the *E. coli* periplasm influences the ability of *E. coli* TonB to make productive contacts with the *V. cholerae* receptors. To test whether *E. coli* TonB interacts with *V. cholerae* receptors in *V. cholerae*, the *E. coli tonB*, *exbB*, and *exbD* genes were expressed in the *V. cholerae* TonB defective strain DHH11. *E. coli* TonB facilitated near wild type levels of utilization of all the iron sources tested, showing that *E. coli*

TonB recognizes at least one receptor for each of the substrates tested, including one or both of the major *V. cholerae* heme receptors HutA and HutR (Table 18). This suggests that *E. coli* TonB interacts with a similar range of receptors in *V. cholerae* as *V. cholerae* TonB2. *E. coli* TonB also interacted productively with the TonB2-dependent heme receptor HasR. This was demonstrated by expressing a chimeric TonB protein with *E. coli* TonB specificity in the *hutA, hutR* mutant ARM219. The v1/e TonB chimera had the same range of activities in the *V. cholerae* tonB mutant DHH11 as the full-length eTonB protein, and promoted heme uptake via HasR in ARM219 (Table 18). The v1/e chimera was chosen for these experiments in order to circumvent the requirement for the *E. coli exbB, exbD* plasmid, since this chimera carries the *V. cholerae* TonB1 N-terminus and uses *V. cholerae* ExbB1 and ExbD1.

Because of the differences in results obtained in *E. coli* versus *V. cholerae*, it was important to establish whether the specificity exhibited by *V. cholerae* TonB1 in *E. coli* is relevant to the receptor bias displayed by TonB1 in *V. cholerae.* To answer this question, the *V. cholerae* TonB1X_{P237*T} variant, which interacts with *E. coli* receptors, was tested for ability to facilitate enterobactin transport in *V. cholerae,* a process that normally requires the TonB2 complex (149). A halo assay demonstrated that, in addition to carrying out all the normal TonB1 functions, the TonB1X_{P237*T} protein conferred enterobactin utilization in the *tonB* mutant strain DHH11, presumably by interacting with one or both of the *V. cholerae* enterobactin receptors IrgA and VctA (Table 19). These data demonstrate that the C-terminal Pro237Thr substitution in TonB1 effectively

Table 18. Function of *E. coli* TonB in *V. cholerae.*

		Zone of growth (mm) after 24 hours[a]				
Strain/plasmid	TonB present	$FeSO_4$	Ferrichrome	Hemin	Vibriobactin	Enterobactin
DHH11 (Vib^-, $TonB^-$)/						
pWKS30	No TonB	21	NG	NG	NG	NG
pAMT112	vTonB1*X*	20	12	15	20	NG
pOUT11	vTonB2	21	12	11	21	25
peTonB*X*/pKP298[c]	eTonB*X*	20	12	15	18	20
pAMT114	$v1_{1\text{-}121}/e_{120\text{-}239}$TonB*X*	20	12	16	17	19
ARM219 (DHH11 $HutA^-$, $HutR^-$, $HasR^+$)/						
pAMT112	vTonB1*X*	22	12	NG	21	NG
pOUT11	vTonB2	20	11	7 (faint)	21	25
pAMT114	$v1_{1\text{-}121}/e_{120\text{-}239}$TonB*X*	21	12	8 (faint)	19	20

a. The indicated strains were seeded at 10^6 cells per ml into molten L agar containing 100 μg EDDA per ml. Iron sources were spotted onto the solidified agar as follows: 20 μl 10 mM $FeSO_4$; 5 μl CA401 overnight culture (vibriobactin); 5 μl DH5α overnight culture (enterobactin); 5 μl 50 μM hemin. NG=No growth. Plates were incubated for 24 hrs at 37°C, and the diameter of each zone of growth was measured. The assays were repeated at least 3 times. Although the relative differences between strains were consistent between experiments, the absolute values for each strain varied due to subtle changes in the media and assay conditions, and thus only one representative experiment is shown.

b. e denotes *E. coli*; v denotes *V. cholerae.*

c. pKP298 encodes *E. coli* ExbB and ExbD.

Table 19. Function of *V. cholerae* TonB1*X*Δ and TonB1X_{P237*T} in *V. cholerae.*

			Zone of growth (mm) after 48 hours[a]			
Strain/plasmid	TonB present	$FeSO_4$	Ferrichrome	Hemin	Vibriobactin	Enterobactin
DHH11 (Vib^-, $TonB^-$)/						
pWKS30	No TonB	21	NG	NG	NG	NG
pAMT112	TonB1*X*	20	12	15	20	NG
pOUT11	TonB2	21	12	11	21	25
pAMT112Δ	TonB1*X*Δ	21	13	15	20	NG
pAMT112.m1	TonB1X_{P237*T}	20	11	15	21	18
ARM219 (DHH11 $HutA^-$, $HutR^-$, $HasR^+$)/						
pAMT112	TonB1*X*	22	12	NG	21	NG
pOUT11	TonB2	20	11	7 (faint)	21	25
pAMT112Δ	TonB1*X*Δ	22	12	NG	22	NG
pAMT112.m1	TonB1X_{P237*T}	22	12	NG	20	18

a. The indicated strains were seeded at 10^6 cells per ml into molten L agar containing 100 μg EDDA per ml. Iron sources were spotted onto the solidified agar as follows: 20 μl 10 mM $FeSO_4$; 5 μl CA401 overnight culture (vibriobactin); 5 μl DH5α overnight culture (enterobactin); 5 μl 50 μM hemin. NG=No growth. Plates were incubated for 24 hrs at 37°C, and the diameter of each zone of growth was measured. The assays were repeated at least 3 times. Although the relative differences between strains were consistent between experiments, the absolute values for each strain varied due to subtle changes in the media and assay conditions, and thus only one representative experiment is shown.

b. All strains are derived from CA40130, and thus are Vib^-.

widens the range of receptors that TonB1 can interact with in *V. cholerae*. The data further imply that the molecular basis for receptor discrimination by TonB1 is similar in *V. cholerae* and in *E. coli*. Interestingly, TonB1*X* carrying the Pro237*Thr mutation did not mediate heme utilization in the *hutA, hutR* mutant ARM219, suggesting that the heme receptor HasR specifically requires features of TonB2 that are not supplied by TonB1 or the $TonB1_{P237T}$ variant (Table 19).

V. cholerae TonB1 is longer than TonB1 by 38 amino acids, raising the possibility that the failure of TonB1 to recognize certain receptors in *V. cholerae* is related to its length, rather than to a lack of specific residues required for the productive interaction with these receptors. To test this hypothesis, a TonB1 mutant containing a 35 amino acid deletion spanning the proline-rich region (149) was tested for ability to mediate transport of various iron sources in *V. cholerae*. Strains expressing TonB1Δ used vibriobactin, ferrichrome, and hemin as sources of iron, indicating that the deletion did not adversely affect TonB1 function under the conditions tested. However, the TonB1Δ mutant, although comparable in length to TonB2, did not facilitate enterobactin utilization, or utilization of hemin via HasR (Table 19). Thus, the receptor specificity exhibited by TonB1 in *V. cholerae* is not likely to be due solely to it being longer than TonB2.

IV. DISCUSSION

V. cholerae encodes three functional heme receptors, HutA, HutR, and HasR. The *V. cholerae* heme receptors share many of the conserved features of TonB-dependent outer membrane transporters, and all three receptors have accordingly been assigned to COG1629 (Clusters of Orthologous Groups) in the National Center for Biotechnology Information phylogenetic classification of proteins encoded in complete genomes (165). COG1629 contains outer membrane receptor proteins, most of which are dedicated to TonB-dependent iron or vitamin B_{12} transport. There are currently seven *V. cholerae* COG1629 members. In addition to the heme receptors, they include ViuA (receptor for vibriobactin), FhuA (receptor for ferrichrome), BtuB (putative receptor for vitamin B_{12}), and IrgA (receptor for enterobactin). At least two other outer membrane proteins involved in iron transport, VctA (receptor for enterobactin; Mey *et al*., manuscript in preparation) and VhrA (putative siderophore receptor, substrate unknown; A. R. Mey, unpublished results) have been found in *V. cholerae* (Table 20). These receptors were not included in the COG database because the *V. cholerae* El Tor 016961 genome database sequence contains inactivating mutations in the genes encoding these receptors. It is striking that three of the nine *V. cholerae* TonB dependent receptors are involved in heme transport, perhaps reflecting the importance of heme acquisition for this pathogen. The presence of multiple receptors for heme substrates is not without precedent

Table 20. *V. cholerae* TonB-dependent receptors

TIGR database ORF name	Receptor name	Substrate
VC0156	BtuB	probably vitamin B_{12}
VC0200	FhuA	ferrichrome
VC0284	VhrA	specific substrate unknown
VC0475	IrgA	enterobactin
VC2211	ViuA	vibriobactin
VCA0064	HutR	heme
VCA0232	VctA	enterobactin
VCA0576	HutA	heme
VCA0625	HasR	heme

among bacterial pathogens. *H. influenzae*, for example, expresses four different TonB-dependent outer membrane proteins involved in heme acquisition (37, 115).

Most of the iron present in the mammalian host is in the form of heme, and thus it is not surprising that the human pathogen *V. cholerae* can use heme as an iron source. The ability to take advantage of host heme reservoirs has been correlated with virulence in several human pathogens, including *N. meningiditis* (159), and the heme auxotrophs *H. influenzae* (78), and *H. ducreyi* (2, 156). However, the relationship between heme acquisition and virulence is more complex in *V. cholerae*, a pathogen that is non-invasive and does not have a requirement for exogenous heme. *V. cholerae* expresses multiple high affinity iron uptake systems, and *V. cholerae* strains defective in all TonB-dependent transport systems were severely impaired in the ability to colonize infant mice, emphasizing the importance of TonB-dependent iron acquisition for the pathogenesis of *V. cholerae* (70, 149). In contrast, a *V. cholerae* strain defective in *hutA* alone was not significantly compromised in growth *in vivo* in a rabbit ileal loop model, or *in vitro* using hemin as the iron source (164). We show here that the ability of a *hutA* mutant to use hemin as an iron source is due to the presence of additional receptors for heme, and not, as previously proposed, to siderophore-mediated iron uptake (164). To completely abolish hemin utilization in *V. cholerae*, it was necessary to create a triple *hutA, hutR, hasR* mutant. Interestingly, this triple heme receptor mutant was not at a competitive disadvantage in the infant mouse model compared to its Vib^- parent, indicating that additional sources of iron are available to *V. cholerae* colonizing the

mammalian host. It is feasible that heme is more important in later stages of the infection when release of heme proteins may occur due to cell sloughing and tissue damage. The ability to take up the released heme and accumulate iron prior to exiting the mammalian host may aid in the survival of the pathogen in the external environment, and thus increase its chances for gaining access to a new host.

Hemoglobins derived from marine organisms may be another source of heme-iron for *V. cholerae*. Studies have shown that a variety of filter-feeding marine organisms accumulate *V. cholerae* to high levels when exposed to seawater containing *V. cholerae* (49, 116). The persistence of *V. cholerae* within these hosts may be determined by the availability of nutrients such as iron. We demonstrate here that *V. cholerae* can use marine invertebrate hemoglobins as a source of iron, and that this iron acquisition requires the presence of a functional heme receptor. Because these hemoglobins readily release their heme under oxidizing conditions, it is difficult to assess in the laboratory whether utilization of these types of hemoglobin by *V. cholerae* requires any specific mechanism for release of the heme moiety. The marine invertebrate hemoglobins are typically much less stable than the vertebrate hemoglobins (A. F. Riggs, personal communication), and thus free heme is potentially available to *V. cholerae* growing in association with its marine hosts. Shellfish infected with *V. cholerae* are often implicated in the transmission of *V. cholerae* to the human host (137), and it is therefore important to gain a better understanding of the survival and growth of this pathogen within marine ecosystems.

The *V. cholerae* heme receptor HutR most resembles HutA and other known *Vibrio* heme receptors. HutR carries the FRAP motif that is well conserved among heme receptors. It should be noted, however, that many non-heme receptors have a functionally similar sequence, such as YKAP in the *E. coli* enterobactin receptor FepA, and this motif may be important for receptor function independent of ligand specificity. HutR has only 50% homology to the NPNL consensus sequence located C-terminal to the FRAP box, and this motif is less well conserved among the *Vibrio* heme receptors. Secondary structure predictions for both HutA and HutR, based on the solved *E. coli* FepA structure, localize the conserved histidine residue found in the FRAP/NPNL region to a putative extracellular loop, consistent with a potential role in heme binding. A similar prediction was made for the location of the corresponding histidine residue in *Y. enterocolitica* HemR, and site-directed mutagenesis confirmed a role for this residue in heme utilization (11).

HasR is most similar to the HasR hemophore receptors from *P. aeruginosa* and *S. marcescens. V. cholerae* HasR has only 50% identity to the FRAP/NPNL consensus motifs, and, unlike *Pseudomonas* and *Serratia* HasR, *V. cholerae* HasR does not contain the conserved histidine residue in the FRAP/NPNL region (data not shown). Thus, HasR may employ a different mechanism for heme binding and/or transport.

HutA and HutR are both efficient heme transporters; however, HutA was required for optimal hemin utilization in our assays. Loss of HutR alone did not affect the ability of *V. cholerae* to grow using hemin as the sole iron source,

whereas loss of HutA resulted in a measurable decrease in hemin utilization. HutA may have a higher affinity for its substrate, or may be a more effective transporter than HutR. It is also possible that HutA is expressed more efficiently in low iron conditions. HutA, but not HutR, was visible in outer membrane preparations from wild type cells grown in low iron media, consistent with higher expression of *hutA* than *hutR* under these conditions. Although similar levels of expression were detected from the *hutA* and the *hutR* promoters in low iron, *hutR* expression may be subject to regulation not detected in our assays.

HutA was required for hemoglobin utilization under all conditions tested. This suggests that HutA may bind hemoglobin directly; however, a previous experiment demonstrated that *V. cholerae* does not bind hemoglobin to its surface efficiently (S. M. Payne, unpublished results). Furthermore, while both TonB systems promoted heme uptake via HutA, only TonB1 mediated hemoglobin utilization. The ability of TonB2 to facilitate transport via HutA of free heme, but not heme derived from hemoglobin, is difficult to explain if HutA binds both heme and hemoglobin. Whether or not hemoglobin associates with HutA, it is likely that the heme portion is released prior to transport into the cell. Growth curve experiments (Fig. 10, page 96) indicated that the amount of heme released may be limiting, and it is conceivable that only the HutA-TonB1 pair transports heme efficiently enough to sustain growth under limiting conditions.

Expression from the *hutR* operon promoter was strongly induced in low iron conditions. Because genes involved in iron acquisition are typically iron-regulated, the regulation of *hutR* expression by iron was expected. However, the

hutR operon also contains three genes of unknown function, as well as the *ptrB* gene, and the significance of this genetic organization is not clear. Many bacterial heme receptors are encoded within multi-gene loci dedicated to heme transport (58), yet we found no evidence that any of the genes linked to *hutR* participate in utilization of heme or hemoglobin. It is possible that there is redundancy, as is often the case in *V. cholerae*, and, thus, expression of other genes may compensate for the loss of these genes. However, the function of these genes may be unrelated to iron acquisition, even though they are iron-regulated. There are many examples of bacterial virulence genes that are upregulated in response to the low iron levels encountered in the mammalian host (129). Interestingly, *V. cholerae* PtrB has significant homology to the *Trypanosoma cruzi* oligopeptidase B, which is an important virulence factor in this human pathogen (27).

The finding that *V. cholerae* encodes a functional heme receptor with homology to the HasR-type hemophore receptors suggested that *V. cholerae* may also produce a HasA-like hemophore. However, no *hasA* homolog was found in the genome of *V. cholerae* El Tor N16961. The *hasA* gene may have been lost from this strain, or the *hasR* gene, which is typically linked to the *hasA* gene, may have been acquired independently of *hasA*. HasA and HasR are both required for efficient hemoglobin utilization in *Pseudomonas* and *Serratia* species. In contrast, *V. cholerae* HasR did not appear to be necessary for hemoglobin utilization. Thus, if *V. cholerae* encodes a hemophore, it may be structurally and functionally different from the known HasA hemophores.

Heme uptake in *V. cholerae* is a TonB-dependent process, and data presented in this study support earlier observations that both TonB systems participate in heme acquisition. The two TonB systems exhibit differences in heme receptor specificities and in the efficiency of heme substrate utilization via these receptors. These differences may allow *V. cholerae* to maximize heme acquisition under a variety of conditions. TonB1 functions with both of the major heme receptors HutA and HutR, but does not recognize the third receptor HasR. Nonetheless, TonB1 is required for optimal heme acquisition by the cell under standard laboratory conditions, emphasizing that HasR plays only a minor role in heme acquisition under the conditions tested. TonB1 is also required for heme uptake under conditions of high osmolarity (149), indicating that TonB1 may be particularly important for heme acquisition in the marine environment. Further, the TonB1 system is required for the ability of *V. cholerae* to use mammalian hemoglobin as an iron source (149), suggesting a role for the TonB1 system in heme acquisition within the human host as well. The unique contribution of the TonB2 system to heme utilization in *V. cholerae* is to facilitate heme uptake through HasR. The relevance of this contribution is not clear, since no particular heme substrates or assay conditions were identified that specifically required TonB2 or HasR.

The TonB box regions of HutA (68) and HutR are similar to each other and to those found in other *Vibrio* heme receptors, including *V. vulnificus* HupA (106), *V. anguillarum* HupA (GenBank accession no. CAC28362), and *V. mimicus* MhuA (GenBank accession no. BAB33171). In contrast, the

V. cholerae HasR sequence does not correspond well to the other *Vibrio* heme receptors in this region (Fig. 17, page 134). Because TonB1 promotes heme transport through HutA and HutR, but not through HasR, it is possible that TonB1 specifically requires a HutA- or HutR-like TonB box. The conservation of this type of TonB box across many of the *Vibrio* heme receptors may reflect their dependence on a TonB1-like molecule for optimal transport function. This appears to be the case in *V. cholerae,* which relies on the TonB1 system for optimal heme transport, as well as for the utilization of hemoglobin as an iron source. An even more striking example is *V. alginolyticus,* which encodes two TonB systems similar to *V. cholerae* TonB1 and TonB2, and is completely dependent upon expression of its *tonB1* system-like genes for heme utilization (123).

The ability to express *V. cholerae* genes in *E. coli* has been very useful for identifying *V. cholerae* genes involved in iron transport. Nevertheless, it is important to recognize that the activities of *V. cholerae* gene products within the *E. coli* environment may not accurately reflect their activities in *V. cholerae*. The failure of TonB2 to satisfy the TonB requirements of either HutA or HutR in *E. coli* was surprising, given that TonB2 is functional in *E. coli* (124) and was able to facilitate transport through both receptors in *V. cholerae.* Seliger *et al*. (149) showed that heme uptake in *V. cholerae* is sensitive to increases in osmolarity and that this sensitivity correlates with the ability of the TonB molecules to span the periplasmic space (149). It is conceivable that subtle differences between the *V. cholerae* and *E. coli* periplasmic environments account

for the failure of the TonB2 complex to mediate heme transport through HutA or HutR in *E. coli*. Some caution should therefore be exercised when interpreting studies of *V. cholerae* iron transport systems expressed in *E. coli*.

TonB1 exhibits a narrower specificity than TonB2. While TonB2 promoted transport through all of the *V. cholerae* and *E. coli* receptors tested in this study, TonB1 recognized none of the *E. coli* receptors and only a subset of *V. cholerae* receptors. The specificity of TonB1 cannot be attributed solely to its longer length compared with TonB2, since a shortened TonB1 had the same specificities as the full-length TonB1. Furthermore, TonB1 exhibited the same specificities in both the *V. cholerae* and *E. coli* environments. Thus, the receptor discrimination displayed by TonB1 likely represents a true requirement for particular interaction sites. Such a specificity is consistent with a more specialized role for TonB1 in the life cycle of *V. cholerae*. While TonB2 is encoded on the large chromosome along with the *V. cholerae* housekeeping genes, the TonB1 system genes are located on the smaller replicon, and were probably acquired by horizontal transfer. The *tonB1* locus may have been maintained during the evolution of this pathogen in order to cope with specific environments such as the marine environment, or the human intestinal tract.

Despite the differences in receptor specificities between *V. cholerae* TonB1 and *E. coli* TonB, their comparable lengths and overall sequence similarities made it feasible to construct functional chimeras between these two TonB proteins. The functional domain organization in the two TonB proteins appears to very similar. The N-terminal domain of *V. cholerae* TonB1 is specific

for *V. cholerae* ExbB1 and ExbD1 proteins, consistent with direct interactions between the TonB1 N-terminus and the ExbB1, ExbD1 complex in the inner membrane. This is the case also for the *E. coli* TonB N-terminal domain, which requires a set of *E. coli* ExbB, ExbD accessory proteins in order to function in *V. cholerae* (data not shown). Like *E. coli* TonB, *V. cholerae* TonB1 contains a central proline-rich region. This region appears to be necessary for the TonB molecule to traverse the increased periplasmic space under conditions of high osmolarity, but is otherwise dispensable for TonB function (91, 149). Finally, secondary structure predictions for the *V. cholerae* TonB1 C-terminus indicate that *V. cholerae* TonB1 may contain several of the secondary structural elements found in *E. coli* TonB, including a putative α-helical domain that aligns with the amphipathic helix within the *E. coli* TonB C-terminal dimerization domain (data not shown). This suggests that *V. cholerae* TonB1 may adopt a domain structure at the C-terminus similar to that of *E. coli* TonB.

The C-terminus of TonB determines the specificity of the interaction between TonB and TonB-dependent receptors. In the case of *E. coli* TonB, the C-terminal 66 residues were sufficient for recognizing *E. coli* receptors. These 66 residues may represent the minimal region required to reconstitute a functional *E. coli* TonB C-terminal dimerization domain. Although at least 75 residues were found to participate in *E. coli* TonB dimerization (31), the first nine of these residues may not be required to form the dimer. Alternatively, the residues contributed by *V. cholerae* TonB1 may be similar enough to the corresponding residues in *E. coli* TonB to permit the formation of a functional dimer. *V.*

cholerae TonB1 requires between 66 and 81 residues to form a functional C-terminal domain. Thus, the putative *V. cholerae* TonB1 C-terminal dimer may involve a greater number of residues, or have more stringent amino acid requirements than the *E. coli* TonB dimer.

None of the chimeras involving fusions within the core of the C-terminal dimerization domain of *E. coli* TonB were functional. This is not surprising, given the tightly intertwined nature of the C-terminal dimer. Each TonB molecule contributes three β-strands to the large antiparallel β-sheet covering most of the dimer surface, and these strands are connected by an extensive network of hydrogen bonds (31). It is therefore unlikely that even a small number of amino acid substitutions would be tolerated within this region.

Residues surrounding Glu_{160} in *E. coli* TonB have been implicated in the direct interaction with the TonB box of the BtuB receptor (25). Nevertheless, this stretch of amino acids is not involved in determining specificity for *E. coli* receptors, since a chimera containing the *E. coli* TonB C-terminus, but not the Glu_{160} region, was able to interact productively with all the *E. coli* receptors tested. Further, the Glu_{160} residue, as well as many of the neighboring amino acids, are conserved in *V. cholerae* TonB1, arguing against a role for this region in specificity. However, this does not exclude a role for these residues in the TonB-receptor interaction(s). Although the Glu_{160} region is not required for dimerization, its proximity to the dimerization domain suggests that it could form part of a C-terminal receptor-binding site. Alternatively, the Glu_{160} region may interact with TonB independently of the C-terminal dimerization domain.

In interpreting the results of studies with chimeric TonB proteins, it is important to note that aberrant expression of the hybrid proteins may have contributed to the observed activities and specificities. It is possible that overexpression of a poorly functioning TonB hybrid could permit wild type levels of transport, and could mask any subtle changes in receptor specificity. Conversely, the observed lack of activity of a TonB chimera could be due to poor expression of the hybrid protein rather than to non-productive receptor interactions. Thus, while the results obtained from these hybrid TonB studies are in good agreement with the current understanding that the carboxy terminus is important for receptor recognition, they do not unambiguously subdivide the C-terminal domain further.

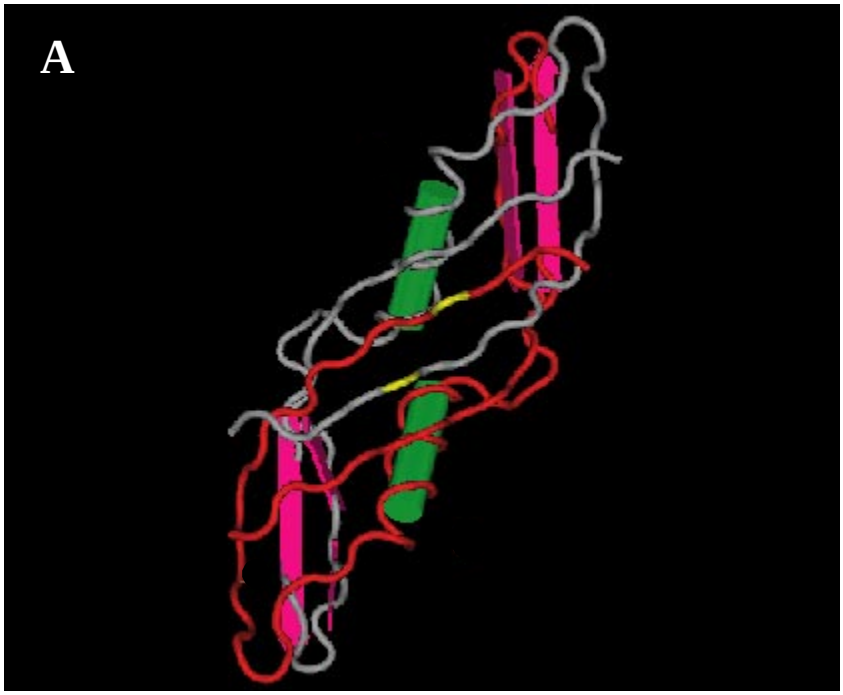
The specificity of *V. cholerae* TonB1 towards receptors in both *V. cholerae* and *E. coli* appears to be determined by the very C-terminal end of the TonB molecule. A Pro237Thr substitution in *V. cholerae* TonB1 resulted in the ability of TonB1 to recognize at least one TonB2-dependent *V. cholerae* receptor, as well as multiple *E. coli* receptors. Based on a CLUSTAL-W alignment, the *V. cholerae* TonB1 Pro_{237} residue corresponds to Asn_{227} in *E. coli* TonB. Interestingly, Asn_{227} is positioned on the β-sheet face of the cylindrical *E. coli* TonB dimer in such a way that the two Asn_{227} residues, one from each TonB molecule, are in close proximity to each other and almost directly opposite the cleft formed by the two α-helices (Fig. 19). The α-helix cleft was proposed as a binding site for outer membrane receptors, since this region exhibits significant flexibility and forms a motif that could feasibly accommodate a receptor

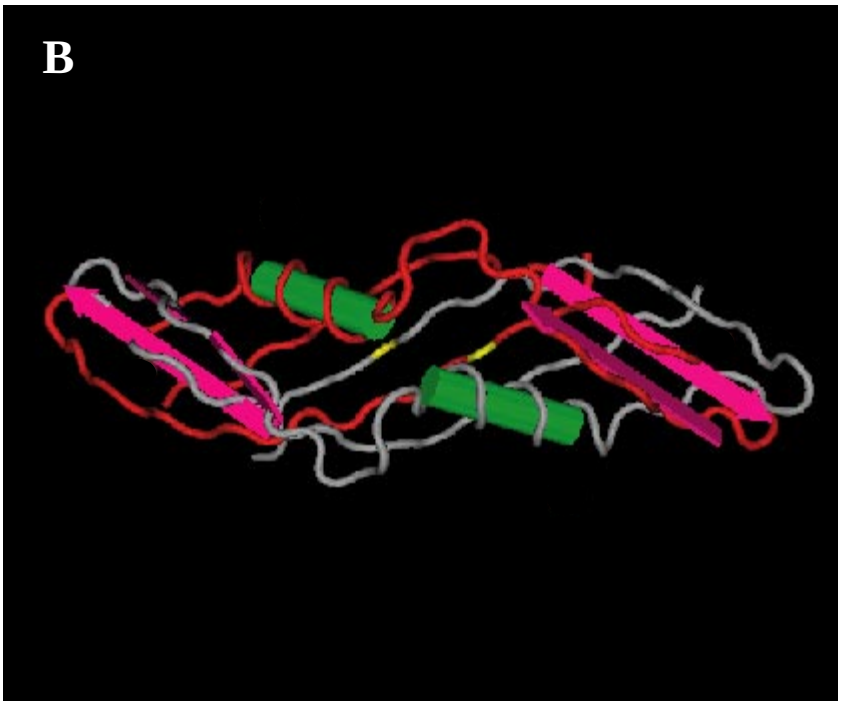
Figure 19. Three-dimensional model of the *E. coli* TonB dimer showing the position of Asn_{227}.

This figure was generated using Cn3D to view the 3-dimensional structure of *E. coli* TonB from the NCBI Entrez Structure database (http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?form=6&db=t&Dopt=s&uid=16883)

A. View from the β-sheet face of the intertwined TonB dimer. The TonB monomers are shown in red and gray, respectively. The two Asn_{227} residues are highlighted in yellow. The figure shows the proximity of the two Asn_{227} residues to each other in the antiparallel β-sheet.

B. View from the α-helix face of the TonB dimer, showing the two highlighted Asn_{227} residues positioned at the bottom of the putative binding cleft formed by the α-helices.


A


B

domain (31). Insertion of a receptor domain into the binding cleft might bring this domain into close contact with the two Asn_{227} residues at the bottom of the putative binding pocket.

While the positioning of the Pro237Thr gain-of-function mutation in *V. cholerae* TonB1 seems logical, the particular relevance of substituting Thr for Pro is less clear. The Thr residue may be able to participate in a hydrogen-bonding pattern similar to that of the equivalent Asn residue in *E. coli* TonB. Alternatively, this substitution may affect other residues involved in receptor binding.

It was surprising that no gain-of-function mutations were isolated in the putative C-terminal α-helix domain in *V. cholerae* TonB1. Not only does the α-helix domain form a putative binding cleft, but these residues are well conserved between *E. coli* TonB and *V. cholerae* TonB2, which have similar specificities, and quite different in *V. cholerae* TonB1. It is possible that this region does contain specificity determinants, but that the β-sheet mutation has a dominant effect on the specificity of the receptor interaction.

The Pro237Thr mutation in *V. cholerae* TonB1 resulted from a single base pair change in *tonB1*. This mutation did not appear to interfere with any of the normal TonB1 functions. Rather, the effect of the mutation was to widen the range of receptors that TonB1 can interact productively with in *V. cholerae*. It seems almost surprising, therefore, that this mutation has not been selected for during the evolution of *V. cholerae* as a pathogen. One possibility is that the mutation permits TonB1 to interact with the enterobactin receptor IrgA, which

normally only recognizes TonB2. Since IrgA is the most abundantly expressed TonB-dependent receptor in *V. cholerae,* interactions with this receptor might sequester the available TonB molecules to such an extent that transport of other substrates, including heme and vibriobactin, is affected. It is also conceivable that the $TonB1_{P237T}$ mutation has an adverse effect on TonB1 function that is not detectable under the assay conditions used.

The TonB box is an important functional domain in TonB-dependent receptors. Several mutations have been identified within this domain of *E. coli* FepA and BtuB that eliminate substrate transport without affecting substrate binding, indicating that this domain may be required for TonB-dependent transport functions (4, 7, 24, 87). A comparison of *E. coli* and *V. cholerae* receptor TonB boxes suggested that this region may contain residues involved in determining the specificity of the TonB-receptor interactions. However, domain-switching experiments showed that the TonB box heptapeptide does not represent a specificity module. *E. coli* ChuA carrying the *V. cholerae* HutA TonB box exhibited the same specificities as wild type ChuA. Substituting the TonB box of HutA with that of ChuA appeared to strengthen the weak interactions with *E. coli* TonB, but interactions with *V. cholerae* TonB1 were unaffected, suggesting that the specificity of the receptor had not changed. Interestingly, HutA did not tolerate insertion of the FepA TonB box, even though there are more conserved residues between the HutA and FepA TonB boxes than between the HutA and ChuA TonB boxes. The mutation responsible for the transport defect in HutA carrying the FepA TonB box may be the Val to Ile substitution at the third

position within the HutA TonB box. All the other FepA residues introduced into HutA were identical to the equivalent ChuA TonB box substitutions, and these did not inhibit transport through HutA. The third residue in the TonB box heptapeptide may be particularly sensitive to mutations, since a variety of substitutions at this position are associated with transport defects also in FepA and BtuB (4, 7, 24, 87).

While the TonB box itself does not determine the specificity of the TonB-receptor interactions, the amino acid residue immediately preceding the TonB box domain had a profound impact on the ability of *E. coli* receptors to recognize *V. cholerae* TonB1. Insertion at this position of a Tyr residue in *E. coli* FepA, or a Phe residue in *E. coli* ChuA, allowed these receptors to interact productively with *V. cholerae* TonB1, suggesting that TonB1 may specifically require an aromatic residue directly prior to the TonB box. This hypothesis is supported by the finding that the *V. cholerae* heme receptors HutA and HutR, both of which recognize TonB1, carry aromatic residues at the TonB box –1 position. Alternatively, the TonB1 specificity may be determined by a restriction against, rather than a requirement for, a certain type of residue in this position. All of the known *V. cholerae* TonB2-dependent receptors, as well as many of the *E. coli* receptors, contain an acidic residue immediately before the TonB box, and perhaps this particular type of amino acid inhibits productive interactions with TonB1. Whatever the case, the data strongly imply that the extreme N-terminus of the receptor is involved in determining the specificity of the TonB interactions. It is likely, therefore, that this receptor region contacts TonB directly. These

findings may necessitate a redefinition of the TonB box functional domain to include the TonB box –1 position.

Although many aspects of TonB-dependent transport across the outer membrane are not yet understood, a model can be proposed that integrates both structural and genetic data relating to this process. Substrate binding at the extracellular face of a TonB-dependent receptor results in a conformational change in the binding loops that is propagated through the receptor to the very N-terminus of the plug domain at the periplasmic face (108). This conformational shift causes the N-terminal TonB box domain to unfold and become available for periplasmic interactions with TonB (109). TonB specifically recognizes the unfolded state of the TonB box, either because of the conformational changes in the TonB box, or because the TonB box is no longer sequestered within the receptor. Specific recognition of the TonB box region by TonB probably involves a binding site in the C-terminal dimerization domain. This binding site may be composed of the C-terminal α-helix binding cleft, as well as specific β-sheet residues at the base of this putative binding pocket. Interactions between the TonB C-terminal dimer and the receptor TonB box domain may cause the plug domain in the receptor to reorganize, and either squeeze against the barrel sides, or exit the barrel pore entirely. This plug domain reorganization is not, however, sufficient for active transport of the substrate. Further interactions with TonB are required, as evidenced by the TonB-dependence of plugless receptor variants (13, 148). By exposing an otherwise secluded TonB binding site in the β-barrel, the rearrangement or removal of the plug domain could be a prerequisite for the

interaction of TonB with the β-barrel domain. It is not currently known which specific region(s) of the TonB C-terminus participate in the β-barrel interactions, although it is conceivable that either the C-terminal dimerization domain, or the Glu_{160} region are involved. Either of these regions might also facilitate repositioning of the TonB box in its sequestered position within the receptor pore (109) after release of the substrate into the periplasm. This, however, is highly speculative. It is not clear at which step in the transport process TonB discharges its energy, but it is likely that energy is required for the reorganization of the plug domain and/or the release of the substrate from its high-affinity binding site within the receptor. Energy may also be required for resetting the receptor after one round of transport. Structural analyses of TonB-receptor complexes trapped at different stages of the transport process may be needed to answer these questions.

In conclusion, data presented in this study demonstrate that *V. cholerae* heme transport involves three separately encoded heme receptors. These receptors share some functional characteristics; however, there are unique features associated with each receptor as well. *V. cholerae* can persist in a variety of environments, both within and outside of the human host, and each receptor may be uniquely adapted for heme acquisition under a particular set of conditions. This study also provides evidence that the specificity exhibited by TonB1 for a subset of *V. cholerae* receptors can be attributed to sequence features near the C-terminus of TonB1 and near the N-terminus of the receptor. This suggests that functional interactions take place between the TonB C-terminal dimerization

domain and a region close to, or overlapping, the TonB box region of the receptor.

References

1. **Ahmer, B. M., M. G. Thomas, R. A. Larsen, and K. Postle.** 1995. Characterization of the exbBD operon of *Escherichia coli* and the role of ExbB and ExbD in TonB function and stability. J. Bacteriol. **177:**4742-7.
2. **Al-Tawfiq, J. A., K. R. Fortney, B. P. Katz, A. F. Hood, C. Elkins, and S. M. Spinola.** 2000. An isogenic hemoglobin receptor-deficient mutant of *Haemophilus ducreyi* is attenuated in the human model of experimental infection. J. Infect. Dis. **181:**1049-54.
3. **Bagg, A., and J. B. Neilands.** 1987. Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol. Rev. **51:**509-518.
4. **Barnard, T. J., M. E. Watson, and M. A. McIntosh.** 2001. Mutations in the *Escherichia coli* receptor FepA reveal residues involved in ligand binding and transport. Mol. Microbiol. **41:**527-36.
5. **Bearden, S. W., J. D. Fetherston, and R. D. Perry.** 1997. Genetic organization of the yersiniabactin biosynthetic region and construction of avirulent mutants in *Yersinia pestis*. Infect. Immun. **65:**1659-68.
6. **Bearden, S. W., and R. D. Perry.** 1999. The Yfe system of *Yersinia pestis* transports iron and manganese and is required for full virulence of plague. Mol. Microbiol. **32:**403-14.

7. **Bell, P. E., C. D. Nau, J. T. Brown, J. Konisky, and R. J. Kadner.** 1990. Genetic suppression demonstrates interaction of TonB protein with outer membrane transport proteins in *Escherichia coli*. J. Bacteriol. **172:**3826-9.

8. **Bjorn, M. J., B. H. Iglewski, S. K. Ives, J. C. Sadoff, and M. L. Vasil.** 1978. Effect of iron on yields of exotoxin A in cultures of *Pseudomonas aeruginosa* PA-103. Infect. Immun. **19:**785-91.

9. **Blanton, K. J., G. D. Biswas, J. Tsai, J. Adams, D. W. Dyer, S. M. Davis, G. G. Koch, P. K. Sen, and P. F. Sparling.** 1990. Genetic evidence that *Neisseria gonorrhoeae* produces specific receptors for transferrin and lactoferrin. J. Bacteriol. **172:**5225-5235.

10. **Boulton, I. C., A. R. Gorringe, J. K. Shergill, C. L. Joannou, and R. W. Evans.** 1999. A dynamic model of the meningococcal transferrin receptor. J. Theor. Biol. **198:**497-505.

11. **Bracken, C. S., M. T. Baer, A. Abdur-Rashid, W. Helms, and I. Stojiljkovic.** 1999. Use of heme-protein complexes by *Yersinia enterocolitica* HemR receptor: histidine residues are essential for receptor function. J. Bacteriol. **181:**6063-6072.

12. **Bradbeer, C.** 1993. The proton motive force drives the outer membrane transport of cobalamin in *Escherichia coli*. J. Bacteriol. **175:**3146-3150.

13. **Braun, M., H. Killmann, and V. Braun.** 1999. The β-barrel domain of FhuΔ5-160 is sufficient for TonB-dependent FhuA activities of *Escherichia coli*. Mol. Microbiol. **33:**1037-1049.

14. **Braun, V.** 1995. Energy-coupled transport and signal transduction through the gram-negative outer membrane via TonB-ExbB-ExbD-dependent receptor proteins. FEMS Microbiol. Rev. **16:**295-307.

15. **Braun, V., K. Hantke, and W. Koster.** 1998. Bacterial iron transport: mechanisms, genetics, and regulation, p. 67-145. *In* A. Sigel and H. Sigel (ed.), Metal Ions in Biological Systems, vol. 35. Marcel Dekker, New York.

16. **Braun, V., and H. Killmann.** 1999. Bacterial solutions to the iron-supply problem. Trends Biochem. Sci. **24:**104-9.

17. **Brickman, T. J., and M. A. McIntosh.** 1992. Overexpression and purification of ferric enterobactin esterase from *Escherichia coli.* Demonstration of enzymatic hydrolysis of enterobactin and its iron complex. J. Biol. Chem. **267:**12350-12355.

18. **Buchanan, S. K., B. S. Smith, L. Venkatramani, D. Xia, L. Esser, M. Palnitkar, R. Chakraborty, D. van der Helm, and J. Deisenhofer.** 1999. Crystal structure of the outer membrane active transporter FepA from *Escherichia coli*. Nat. Struct. Biol. **6:**56-63.

19. **Butterton, J. R., and S. B. Calderwood.** 1994. Identification, cloning, and sequencing of a gene required for ferric vibriobactin utilization by *Vibrio cholerae*. J. Bacteriol. **176:**5631-8.

20. **Butterton, J. R., M. H. Choi, P. A. Carroll, C. W. Wang, and S. B. Calderwood.** 1997. Presented at the 97th General Meeting American Society for Microbiology, Miami, Florida.

21. **Butterton, J. R., M. H. Choi, P. I. Watnick, P. A. Carroll, and S. B. Calderwood.** 2000. *Vibrio cholerae* VibF is required for vibriobactin synthesis and is a member of the family of nonribosomal peptide synthetases. J. Bacteriol. **182:**1731-8.

22. **Butterton, J. R., J. A. Stoebner, S. M. Payne, and S. B. Calderwood.** 1992. Cloning, sequencing, and transcriptional regulation of viuA, the gene encoding the ferric vibriobactin receptor of *Vibrio cholerae*. J. Bacteriol. **174:**3729-38.

23. **Byers, B. R., and J. E. Arceneaux.** 1998. Microbial iron transport: iron acquisition by pathogenic microorganisms. Met Ions Biol Syst **35:**37-66.

24. **Cadieux, N., C. Bradbeer, and R. J. Kadner.** 2000. Sequence changes in the Ton box region of BtuB affect its transport activities and interaction with TonB protein. J. Bacteriol. **182:**5954-5961.

25. **Cadieux N, K. R.** 1999. Site-directed disulfide bonding reveals an interaction site between energy-coupling protein TonB and BtuB, the outer membrane cobalamin transporter. Proc. Natl. Acad. Sci. U.S.A. **96:**10673-8.

26. **Calderwood, S. B., and J. J. Mekalanos.** 1988. Confirmation of the Fur operator site by insertion of a synthetic oligonucleotide into an operon fusion plasmid. J. Bacteriol. **170:**1015-7.

27. **Caler, E. V., S. Vaena de Avalos, P. A. Haynes, N. W. Andrews, and B. A. Burleigh.** 1998. Oligopeptidase B-dependent signaling mediates host cell invasion by *Trypanosoma cruzi*. EMBO J **17:**4975-86.

28. **Cao, Z., Z. Qi, Sprencel, S. M. C. Newton, and P. E. Klebba.** 2000. Aromatic components of two ferric enterobactin binding sites in *Escherichia coli* FepA. Mol. Microbiol. **37:**1306-1317.

29. **Chambers, S. P., S. E. Prior, D. A. Barstow, and N. P. Minton.** 1988. The pMTL nic- cloning vectors. I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. Gene **68:**139-149.

30. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141-1156.

31. **Chang, C., A. Mooser, A. Pluckthun, and A. Wlodawer.** 2001. Crystal structure of the dimeric C-terminal domain of TonB reveals a novel fold. J. Biol. Chem. **276:**27535-40.

32. **Chen, C. J., C. Elkins, and P. F. Sparling.** 1998. Phase variation of hemoglobin utilization in *Neisseria gonorrhoeae*. Infect. Immun. **66:**987-93.

33. **Chu, G. C., K. Katakura, X. Zhang, T. Yoshida, and M. Ikeda-Saito.** 1999. Heme degradation as catalyzed by a recombinant bacterial heme oxygenase (Hmu O) from *Corynebacterium diphtheriae*. J. Biol. Chem. **274:**21319-25.

34. **Coelho, A., J. R. Andrade, A. C. Vicente, and V. J. Dirita.** 2000. Cytotoxic cell vacuolating activity from *Vibrio cholerae* hemolysin. Infect. Immun. **68:**1700-5.

35. **Coelho, A., J. R. Andrade, A. C. Vicente, and C. A. Salles.** 1995. New variant of *Vibrio cholerae* O1 from clinical isolates in Amazonia. J. Clin. Microbiol. **33:**114-8.

36. **Cope, L. D., Z. Hrkal, and E. J. Hansen.** 2000. Detection of phase variation in expression of proteins involved in hemoglobin and hemoglobin-haptoglobin binding by nontypeable *Haemophilus influenzae*. Infect. Immun. **68:**4092-101.

37. **Cope, L. D., R. P. Love, S. E. Guinn, A. Gilep, S. Usanov, R. W. Estabrook, Z. Hrkal, and E. J. Hansen.** 2001. Involvement of HxuC outer membrane protein in utilization of hemoglobin by *Haemophilus influenzae*. Infect. Immun. **69:**2353-63.

38. **Cope, L. D., S. E. Thomas, Z. Hrkal, and E. J. Hansen.** 1998. Binding of heme-hemopexin complexes by soluble HxuA protein allows utilization of this complexed heme by *Haemophilus influenzae*. Infect. Immun. **66:**4511-6.

39. **Cope, L. D., S. E. Thomas, J. L. Latimer, C. A. Slaughter, U. Muller-Eberhard, and E. J. Hansen.** 1994. The 100 kDa haem:haemopexin-binding protein of *Haemophilus influenzae*: structure and localization. Mol. Microbiol. **13:**863-873.

40. **Cornelissen, C. N., M. Kelley, M. M. Hobbs, J. E. Anderson, J. G. Cannon, M. S. Cohen, and P. F. Sparling.** 1998. The transferrin receptor expressed by gonococcal strain FA1090 is required for the experimental infection of human male volunteers. Mol. Microbiol. **27:**611-6.

41. **Cotter, P. A., and V. J. DiRita.** 2000. Bacterial virulence gene regulation: an evolutionary perspective. Annu. Rev. Microbiol. **54:**519-65.

42. **Crosa, J. H.** 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiol. Rev. **53:**517-530.

43. **Daskaleros, P. A., J. A. Stoebner, and S. M. Payne.** 1991. Iron uptake in *Plesiomonas shigelloides*: cloning of the genes for the heme-iron uptake system. Infect. Immun. **59:**2706-2711.

44. **de Lorenzo, V., F. Giovannini, M. Herrero, and J. B. Neilands.** 1988. Metal ion regulation of gene expression. Fur repressor-operator interaction at the promoter region of the aerobactin system of pColV- K30. J. Mol. Biol. **203:**875-84.

45. **De Voss, J. J., K. Rutter, B. G. Schroeder, and C. E. Barry, 3rd.** 1999. Iron acquisition and metabolism by mycobacteria. J. Bacteriol. **181:**4443-51.

46. **Earhart, C. F.** 1996. Uptake and metabolism of iron and molybdenum, p. 1075-1090. *In* F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella.* Cellular and Molecular Biology, 2nd ed, vol. 1. ASM Press, Washington, D. C.

47. **Eick-Helmerich, K., K. Hantke, and V. Braun.** 1987. Cloning and expression of the *exbB* gene of *Escherichia coli* K-12. Mol. Gen. Genet. **206:**246-51.

48. **Escolar, L., J. Perez-Martin, and V. de Lorenzo.** 1998. Binding of the fur (ferric uptake regulator) repressor of *Escherichia coli* to arrays of the GATAAT sequence. J. Mol. Biol. **283:**537-47.

49. **Faghri, M. A., C. L. Pennington, L. S. Cronholm, and R. M. Atlas.** 1984. Bacteria associated with crabs from cold waters with emphasis on the occurrence of potential human pathogens. Appl. Environ. Microbiol. **47:**1054-61.

50. **Farinha, M. A., and A. M. Kropinski.** 1990. Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. J. Bacteriol. **172:**3496-3499.

51. **Faruque, S. M., M. J. Albert, and J. J. Mekalanos.** 1998. Epidemiology, Genetics and Ecology of Toxigenic *Vibrio cholerae*. Microbiol. Mol. Biol. Rev. **62:**1301-1314.

52. **Ferguson, A. D., E. Hofmann, J. W. Coulton, K. Diederichs, and W. Welte.** 1998. Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide. Science **282:**2215-2220.

53. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. **115:**717-722.

54. **Freter, R., P. C. M. O'Brien, and M. S. Macsai.** 1981. Role of chemotaxis in the association of motile bacteria with intestinal mucosa: in vivo studies. Infect. Immun. **34:**234-240.

55. **Gardner, E. W., S. T. Lyles, and C. E. Langkford.** 1964. A comparison of virulence in *Vibrio cholerae* strains for embryonated egg. J. Infect. Dis. **114:**412.

56. **Garey, J. R., and A. F. Riggs.** 1986. The hemoglobin of *Urechis caupo*. The cDNA-derived amino acid sequence. J. Biol. Chem. **261:**16446-50.

57. **Genco, C. A., and P. J. Desai.** 1996. Iron acquisition in the pathogenic Neisseria. Trends Microbiol. **4:**179-84.

58. **Genco, C. A., and D. W. Dixon.** 2001. Emerging strategies in microbial haem capture. Mol. Microbiol. **39:**1-11.

59. **Genco, C. A., B. M. Odusanya, and G. Brown.** 1994. Binding and accumulation of hemin in *Porphyromonas gingivalis* are induced by hemin. Infect. Immun. **62:**2885-92.

60. **Ghigo, J. M., S. Letoffe, and C. Wandersman.** 1997. A new type of hemophore-dependent heme acquisition system of *Serratia marcescens* reconstituted in *Escherichia coli*. J. Bacteriol. **179:**3572-9.

61. **Goldberg, M. B., S. A. Boyko, and S. B. Calderwood.** 1990. Transcriptional regulation by iron of a *Vibrio cholerae* virulence gene and homology of the gene to the *Escherichia coli* fur system. J. Bacteriol. **172:**6863-70.

62. **Goldberg, M. B., V. J. DiRita, and S. B. Calderwood.** 1990. Identification of an iron-regulated virulence determinant in *Vibrio cholerae,* using Tn*phoA* mutagenesis. Infect. Immun. **58:**55-60.

63. **Gray-Owen, S. D., and A. B. Schryvers.** 1996. Bacterial transferrin and lactoferrin receptors. Trends Microbiol. **4:**185-91.

64. **Griffiths, G. L., S. P. Sigel, S. M. Payne, and J. B. Neilands.** 1984. Vibriobactin, a siderophore from *Vibrio cholerae*. J. Biol. Chem. **259:**383-385.

65. **Guerinot, M. L.** 1994. Microbial iron transport. Annu. Rev. Microbiol. **48:**743-72.

66. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557-580.

67. **Heidelberg, J. F., J. A. Eisen, W. C. Nelson, R. A. Clayton, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, L. Umayam, S. R. Gill, K. E. Nelson, T. D. Read, H. Tettelin, D. Richardson, M. Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I. Dragoi, P. Sellers, L. McDonald, T. Utterback, R. D. Fleishmann, W. C. Nierman, O. White, S. L. Salzberg, H. O. Smith, R. R. Colwell, J. J. Mekalanos, J. C. Venter, and C. M. Fraser.** 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature **406:**477-483.

68. **Henderson, D. P., and S. M. Payne.** 1994. Characterization of the *Vibrio cholerae* outer membrane heme transport protein HutA: sequence of the gene, regulation of expression, and homology to the family of TonB-dependent proteins. J. Bacteriol. **176:**3269-3277.

69. **Henderson, D. P., and S. M. Payne.** 1993. Cloning and characterization of the *Vibrio cholerae* genes encoding the utilization of iron from haemin and haemoglobin. Mol. Microbiol. **7:**461-469.

70. **Henderson, D. P., and S. M. Payne.** 1994. *Vibrio cholerae* iron transport systems: role of heme and siderophore iron transport in virulence and identification of a gene associated with multiple iron transport systems. Infect. Immun. **62:**5120-5125.

71. **Henderson, D. P., E. E. Wyckoff, C. E. Rashidi, H. Verlei, and A. L. Oldham.** 2001. Characterization of the *Plesiomonas shigelloides* Genes Encoding the Heme Iron Utilization System. J. Bacteriol. **183:**2715-23.

72. **Herrington, D. A., R. H. Hall, G. Losonsky, J. J. Mekalanos, R. K. Taylor, and M. M. Levine.** 1988. Toxin, toxin-coregulated pili, and the *toxR* regulon are essential for *Vibrio cholerae* pathogenesis in humans. J. Exp. Med. **168:**1487-92.

73. **Higgs, P. I., P. S. Myers, and K. Postle.** 1998. Interactions in the TonB-dependent energy transduction complex: ExbB and ExbD form homomultimers. J. Bacteriol. **180:**6031-8.

74. **Hill, C., and B. Harnish.** 1981. Inversions between ribosomal RNA genes of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **78:**7069-7072.

75. **Howard, S. P., C. Herrmann, C. W. Stratilo, and V. Braun.** 2001. In Vivo Synthesis of the Periplasmic Domain of TonB Inhibits Transport through the FecA and FhuA Iron Siderophore Transporters of *Escherichia coli*. J. Bacteriol. **183:**5885-95.

76. **Idei, A., E. Kawai, H. Akatsuka, and K. Omori.** 1999. Cloning and characterization of the Pseudomonas fluorescens ATP-binding cassette exporter, HasDEF, for the heme acquisition protein HasA. J. Bacteriol. **181:**7545-51.

77. **Inouye, M., and J. P. Guthrie.** 1969. A mutation which changes a membrane protein of *E. coli*. Proc. Natl. Acad. Sci. U.S.A. **64:**957-961.

78. **Jarosik, G. P., J. D. Sanders, L. D. Cope, U. Muller-Eberhard, and E. J. Hansen.** 1994. A functional tonB gene is required for both utilization of heme and virulence expression by *Haemophilus influenzae* type b. Infect. Immun. **62:**2470-7.

79. **Jaskula, J. C., T. E. Letain, S. K. Roof, J. T. Skare, and K. Postle.** 1994. Role of the TonB amino terminus in energy transduction between membranes. J. Bacteriol. **176:**2326-38.

80. **Kadner, R. J.** 1990. Vitamin B12 transport in *Escherichia coli*: energy coupling between membranes. Mol. Microbiol. **4:**2027-33.

81. **Kampfenkel, K., and V. Braun.** 1992. Membrane topology of the *Escherichia coli* ExbD protein. J. Bacteriol. **174:**5485-7.

82. **Kampfenkel, K., and V. Braun.** 1993. Topology of the ExbB protein in the cytoplasmic membrane of *Escherichia coli*. J. Biol. Chem. **268:**6050-7.

83. **Kaper, J. B., J. Morris, J.G., and M. M. Levine.** 1995. Cholera. Clinical Microbiol. Rev. **8:**48-86.

84. **Kingsley, R., W. Rabsch, P. Stephens, M. Roberts, R. Reissbrodt, and P. H. Williams.** 1995. Iron supplying systems of *Salmonella* in diagnostics, epidemiology and infection. FEMS Immunol. Med. Microbiol. **11:**257-64.

85. **Kirn, T. J., M. J. Lafferty, C. M. Sandoe, and R. K. Taylor.** 2000. Delineation of pilin domains required for bacterial association into microcolonies and intestinal colonization by *Vibrio cholerae*. Mol. Microbiol. **35:**896-910.

86. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**59-64.

87. **Larsen, R. A., D. Foster-Hartnett, M. A. McIntosh, and K. Postle.** 1997. Regions of *Escherichia coli* TonB and FepA proteins essential for *in vivo* physical interactions. J. Bacteriol. **179:**3213-21.

88. **Larsen, R. A., and K. Postle.** 2001. Conserved residues Ser(16) and His(20) and their relative positioning are essential for TonB activity, cross-linking of TonB with ExbB, and the ability of TonB to respond to proton motive force. J. Biol. Chem. **276:**8111-7.

89. **Larsen, R. A., M. G. Thomas, and K. Postle.** 1999. Protonmotive force, ExbB and ligand-bound FepA drive conformational changes in TonB. Mol. Microbiol. **31:**1809-24.

90. **Larsen, R. A., M. G. Thomas, G. E. Wood, and K. Postle.** 1994. Partial suppression of an *Escherichia coli* TonB transmembrane domain mutation (delta V17) by a missense mutation in ExbB. Mol. Microbiol. **13:**627-640.

91. **Larsen, R. A., G. E. Wood, and K. Postle.** 1993. The conserved proline-rich motif is not essential for energy transduction by *Escherichia coli* TonB protein. Mol. Microbiol. **10:**943-953.

92. **Le Cam, E., D. Frechon, M. Barray, A. Fourcade, and E. Delain.** 1994. Observation of binding and polymerization of Fur repressor onto operator-containing DNA with electron and atomic force microscopes. Proc. Natl. Acad. Sci. U.S.A. **91:**11816-20.

93. **Lee, S. H., D. L. Hava, M. K. Waldor, and A. Camilli.** 1999. Regulation and temporal expression patterns of *Vibrio cholerae* virulence genes during infection. Cell **99:**625-34.

94. **Letain, T. E., and K. Postle.** 1997. TonB protein appears to transduce energy by shuttling between the cytoplasmic membrane and the outer membrane in *Escherichia coli* [published erratum appears in Mol. Microbiol. 1997 Aug;25(3):617]. Mol. Microbiol. **24:**271-83.

95. **Letoffe, S., C. Deniau, N. Wolff, E. Dassa, P. Delepelaire, A. Lecroisey, and C. Wandersman.** 2001. Haemophore-mediated bacterial haem transport: evidence for a common or overlapping site for haem-free and haem-loaded haemophore on its specific outer membrane receptor. Mol. Microbiol. **41:**439-50.

96. **Letoffe, S., J. M. Ghigo, and C. Wandersman.** 1994. Iron acquisition from heme and hemoglobin by a *Serratia marcescens* extracellular protein. Proc. Natl. Acad. Sci. U.S.A. **91:**9876-80.

97. **Letoffe, S., J. M. Ghigo, and C. Wandersman.** 1994. Secretion of the *Serratia marcescens* HasA protein by an ABC transporter. J. Bacteriol. **176:**5372-7.

98. **Letoffe, S., F. Nato, M. E. Goldberg, and C. Wandersman.** 1999. Interactions of HasA, a bacterial haemophore, with haemoglobin and with its outer membrane receptor HasR. Mol. Microbiol. **33:**546-55.

99. **Letoffe, S., K. Omori, and C. Wandersman.** 2000. Functional characterization of the HasA(PF) hemophore and its truncated and chimeric variants: determination of a region involved in binding to the hemophore receptor. J. Bacteriol. **182:**4401-5.

100. **Letoffe, S., V. Redeker, and C. Wandersman.** 1998. Isolation and characterization of an extracellular haem-binding protein from *Pseudomonas aeruginosa* that shares function and sequence similarities with the *Serratia marcescens* HasA haemophore. Mol. Microbiol. **28:**1223-34.

101. **Lewis, J. P., J. A. Dawson, J. C. Hannis, D. Muddiman, and F. L. Macrina.** 1999. Hemoglobinase activity of the lysine gingipain protease (Kgp) of *Porphyromonas gingivalis* W83. J. Bacteriol. **181:**4905-13.

102. **Lewis, L. A., and D. W. Dyer.** 1995. Identification of an iron-regulated outer membrane protein of *Neisseria meningitidis* involved in the utilization of hemoglobin complexed to haptoglobin. J. Bacteriol. **177:**1299-306.

103. **Lewis, L. A., M. Gipson, K. Hartman, T. Ownbey, J. Vaughn, and D. W. Dyer.** 1999. Phase variation of HpuAB and HmbR, two distinct haemoglobin receptors of *Neisseria meningitidis* DNM2. Mol. Microbiol. **32:**977-989.

104. **Lewis, L. A., E. Gray, Y.-P. Wang, B. A. Roe, and D. W. Dyer.** 1997. Molecular characterization of *hpuAB*, the haemoglobin-haptoglobin-utilization operon of *Neisseria meningitidis*. Mol. Microbiol. **23:**737-749.

105. **Litwin, C. M., S. A. Boyko, and S. B. Calderwood.** 1992. Cloning, sequencing and transcriptional regulation of the *Vibrio cholerae* fur gene. J. Bacteriol. **174:**1897-1903.

106. **Litwin, C. M., and B. L. Byrne.** 1998. Cloning and characterization of an outer membrane protein of *Vibrio vulnificus* required for heme utilization: regulation of expression and determination of the gene sequence. Infect. Immun. **66:**3134-41.

107. **Litwin, C. M., and S. B. Calderwood.** 1994. Analysis of the complexity of gene regulation by fur in *Vibrio cholerae*. J. Bacteriol. **176:**240-248.

108. **Locher, K. P., B. Rees, R. Koebnik, A. Mitschler, L. Moulinier, J. P. Rosenbusch, and D. Moras.** 1998. Transmembrane signaling across the ligand-gated FhuA receptor: crystal structures of free and ferrichrome-bound states reveal allosteric changes. Cell **95:**771-8.

109. **Merianos, H. J., N. Cadieux, C. H. Lin, R. J. Kadner, and D. S. Cafiso.** 2000. Substrate-induced exposure of an energy-coupling motif of a membrane transporter. Nat Struct Biol **7:**205-9.

110. **Miller, J. H.** 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

111. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* required *tox*R. J. Bacteriol. **170:**2575-2583.

112. **Mills, M., and S. M. Payne.** 1997. Analysis of *shuA*, the gene encoding the heme receptor of *Shigella dysenteriae* and analysis of invasion and intracellular multiplication of a *shuA* mutant. Infect. Immun.. **65:**5358-5363.

113. **Mills, M., and S. M. Payne.** 1995. Genetics and regulation of heme iron transport in *Shigella dysenteriae* and detection of an analogous system in *Escherichia coli* O157:H7. J. Bacteriol. **177:**3004-3009.

114. **Moeck, G. S., J. W. Coulton, and K. Postle.** 1997. Cell envelope signaling in *Escherichia coli*. Ligand binding to the ferrichrome-iron receptor fhua promotes interaction with the energy-transducing protein TonB. J. Biol. Chem. **272:**28391-7.

115. **Morton, D. J., P. W. Whitby, H. Jin, Z. Ren, and T. L. Stull.** 1999. Effect of multiple mutations in the hemoglobin- and hemoglobin-haptoglobin-binding proteins, HgpA, HgpB, and HgpC, of *Haemophilus influenzae* type b. Infect. Immun. **67:**2729-39.

116. **Murphree, R. L., and M. L. Tamplin.** 1995. Uptake and retention of *Vibrio cholerae* O1 in the Eastern oyster, *Crassostrea virginica*. Appl. Environ. Microbiol. **61:**3656-60.

117. **Naito, Y., C. K. Riggs, T. L. Vandergon, and A. F. Riggs.** 1991. Origin of a "bridge" intron in the gene for a two-domain globin. Proc. Natl. Acad. Sci. U.S.A. **88:**6672-6.

118. **Neilands, J. B.** 1995. Siderophores: structure and function of microbial iron transport compounds. J. Biol. Chem. **270:**26723-6.

119. **Newton, S. M. C., J. D. Igo, D. C. Scott, and P. E. Klebba.** 1999. Effect of loop deletions on the binding and transport of ferric enterobactin by FepA. Mol. Microbiol. **32:**1153-1165.

120. **Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne.** 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10:**1-6.

121. **Nishina, Y., S.-i. Miyoshi, A. Nagase, and S. Shinoda.** 1992. Significant role of an exocellular protease in utilization of heme by *Vibrio vulnificus*. Infect. Immun. **60:**2128-2132.

122. **O'Brien, I. G., and F. Gibson.** 1970. The structure of enterochelin and related 2,3-dihydroxy-N-benzoylserine conjugates from *Escherichia coli*. Biochim. Biophys. Acta **215:**393-402.

123. **O'Malley, S. M., S. L. Mouton, D. A. Occhino, M. T. Deanda, J. R. Rashidi, K. L. Fuson, C. E. Rashidi, M. Y. Mora, S. M. Payne, and D.**

P. Henderson. 1999. Comparison of the heme iron utilization systems of pathogenic *Vibrios*. J. Bacteriol. **181:**3594-3598.

124. **Occhino, D. A., E. E. Wyckoff, D. P. Henderson, T. J. Wrona, and S. M. Payne.** 1998. *Vibrio cholerae* iron transport: haem transport genes are linked to one of two sets of *tonB, exbB, exbD* genes. Mol. Microbiol. **29:**1493-507.

125. **Ochsner, U. A., Z. Johnson, and M. L. Vasil.** 2000. Genetics and regulation of two distinct haem-uptake systems, *phu* and *has*, in *Pseudomonas aeruginosa*. Microbiology **146:**185-98.

126. **Ogunnariwo, J. A., and A. B. Schryvers.** 1996. Rapid identification and cloning of bacterial transferrin and lactoferrin receptor protein genes. J. Bacteriol. **178:**7326-8.

127. **Olczak, T., D. W. Dixon, and C. A. Genco.** 2001. Binding Specificity of the *Porphyromonas gingivalis* Heme and Hemoglobin Receptor HmuR, Gingipain K, and Gingipain R1 for Heme, Porphyrins, and Metalloporphyrins. J. Bacteriol. **183:**5599-608.

128. **Otto, B. R., S. J. van Dooren, J. H. Nuijens, J. Luirink, and B. Oudega.** 1998. Characterization of a hemoglobin protease secreted by the pathogenic *Escherichia coli* strain EB1. J. Exp. Med. **188:**1091-1103.

129. **Payne, S. M.** 1993. Iron acquisition in microbial pathogenesis. Trends Microbiol. **1:**66-69.

130. **Payne, S. M.** 1988. Iron and virulence in the family Enterobacteriaceae. CRC Crit. Rev. Microbiol. **16:**81-111.

131. **Pollack, J. R., and J. B. Neilands.** 1970. Enterobactin, an iron transport compound from *Salmonella typhimurium*. Biochem. Biophys. Res. Commun. **38:**989-992.

132. **Poole, K., and V. Braun.** 1988. Iron regulation of Serratia marcescens hemolysin gene expression. Infect. Immun. **56:**2967-71.

133. **Postle, K.** 1993. TonB protein and energy transduction between membranes. J. Bioenerg. Biomembr. **25:**591-601.

134. **Postle, K., and J. T. Skare.** 1988. *Escherichia coli* TonB protein is exported from the cytoplasm without proteolytic cleavage of its amino terminus. J. Biol. Chem. **263:**11000-11007.

135. **Pradel, E., N. Guiso, F. D. Menozzi, and C. Locht.** 2000. *Bordetella pertussis* TonB, a Bvg-independent virulence determinant. Infect. Immun. **68:**1919-27.

136. **Pressler, U., H. Staudenmaier, L. Zimmermann, and V. Braun.** 1988. Genetics of the iron dicitrate transport system of *Escherichia coli.* J. Bacteriol. **170:**2716-24.

137. **Rabbani, G. H., and W. B. Greenough, 3rd.** 1999. Food as a vehicle of transmission of cholera. J. Diarrhoeal Dis. Res. **17:**1-9.

138. **Ratledge, C., and L. G. Dover.** 2000. Iron metabolism in pathogenic bacteria. Annu. Rev. Microbiol. **54:**881-941.

139. **Ratliff, M., W. Zhu, R. Deshmukh, A. Wilks, and I. Stojiljkovic.** 2001. Homologues of Neisserial Heme Oxygenase in Gram-Negative Bacteria:

Degradation of Heme by the Product of the *pigA* Gene of *Pseudomonas aeruginosa*. J. Bacteriol. **183:**6394-403.

140. **Reeves, S. A., A. G. Torres, and S. M. Payne.** 2000. TonB is required for intracellular growth and virulence of *Shigella dysenteriae*. Infect. Immun. **68:**6329-6336.

141. **Richardson, A. R., and I. Stojiljokovic.** 1999. HmbR, a hemoglobin-binding outer membrane protein of *Neisseria meningitidis*, undergoes phase variation. J. Bacteriol. **181:**2067-2074.

142. **Rogers, H. J.** 1973. Iron-binding catechols and virulence in *Escherichia coli*. Infect. Immun. **7:**445-456.

143. **Rogers, M. B., J. A. Sexton, G. J. DeCastro, and S. B. Calderwood.** 2000. Identification of an operon required for ferrichrome iron utilization in *Vibrio cholerae*. J. Bacteriol. **182:**2350-3.

144. **Roof, S. K., J. D. Allard, K. P. Bertrand, and K. Postle.** 1991. Analysis of *Escherichia coli* TonB membrane topology by use of PhoA fusions. J. Bacteriol. **173:**5554-5557.

145. **Runyen-Janecky, L. J., M. Hong, and S. M. Payne.** 1999. Virulence plasmid-encoded *impCAB* operon enhances survival and induced mutagenesis in *Shigella flexneri* after exposure to UV radiation. Infect. Immun. **67:**1415-1423.

146. **Salyers, A. A., and D. D. Whitt.** 1994. Bacterial Pathogenesis. American Society of Microbiology, Washington DC.

147. **Schmitt, M. P.** 1997. Utilization of host iron sources by *Corynebacterium diphtheriae*: identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin. J. Bacteriol. **179:**838-45.

148. **Scott, D. C., Z. Cao, Z. Qi, M. Bauler, J. D. Igo, S. M. Newton, and P. E. Klebba.** 2001. Exchangeability of N termini in the ligand-gated porins of *Escherichia coli*. J. Biol. Chem. **276:**13025-33.

149. **Seliger, S., A. Mey, A. Valle, and S. Payne.** 2001. The two TonB systems of *Vibrio cholerae*: redundant and specific functions. Mol. Microbiol. **39:**801-12.

150. **Sigel, S. P., and S. M. Payne.** 1982. Effect of iron limitation on growth, siderophore production and expression of outer membrane proteins of *Vibrio cholerae*. J. Bacteriol. **150:**148-155.

151. **Sigel, S. P., J. A. Stoebner, and S. M. Payne.** 1985. Iron-vibriobactin transport system is not required for virulence of *Vibrio cholerae*. Infect. Immun. **47:**360-362.

152. **Simpson, W., T. Olczak, and C. A. Genco.** 2000. Characterization and expression of HmuR, a TonB-dependent hemoglobin receptor of *Porphyromonas gingivalis*. J. Bacteriol. **182:**5737-5748.

153. **Skare, J. T., B. M. Ahmer, C. L. Seachord, R. P. Darveau, and K. Postle.** 1993. Energy transduction between membranes. TonB, a cytoplasmic membrane protein, can be chemically cross-linked in vivo to the outer membrane receptor FepA. J. Biol. Chem. **268:**16302-8.

154. **Smalley, J. W., A. J. Birss, A. S. McKee, and P. D. Marsh.** 1996. Haemin binding as a factor in the virulence of *Porphyromonas gingivalis.* FEMS Microbiol. Lett. **141:**65-70.

155. **Sroka, A., M. Sztukowska, J. Potempa, J. Travis, and C. A. Genco.** 2001. Degradation of Host Heme Proteins by Lysine- and Arginine-Specific Cysteine Proteinases (Gingipains) of *Porphyromonas gingivalis.* J. Bacteriol. **183:**5609-16.

156. **Stevens, M. K., S. Porcella, J. Klesney-Tait, S. Lumbley, S. E. Thomas, M. V. Norgard, J. D. Radolf, and E. J. Hansen.** 1996. A hemoglobin-binding outer membrane protein is involved in virulence expression by *Haemophilus ducreyi* in an animal model. Infect. Immun. **64:**1724-1735.

157. **Stoebner, J. A., J. R. Butterton, S. B. Calderwood, and S. M. Payne.** 1992. Identification of the vibriobactin receptor of *Vibrio cholerae*. J. Bacteriol. **174:**3270-3274.

158. **Stoebner, J. A., and S. M. Payne.** 1988. Iron-regulated hemolysin production and utilization of heme and hemoglobin by *Vibrio cholerae*. Infect. Immun. **56:**2891-2895.

159. **Stojiljkovic, I., V. Hwa, L. de Saint Martin, P. O'Gaora, X. Nassif, F. Heffron, and M. So.** 1995. The *Neisseria meningitidis* haemoglobin receptor: its role in iron utilization and virulence. Mol. Microbiol. **15:**531-541.

160. **Stojiljkovic, I., J. Larson, V. Hwa, S. Anic, and M. So.** 1996. HmbR outer membrane receptors of pathogenic *Neisseria* spp.: iron- regulated, hemoglobin-binding proteins with a high level of primary structure conservation. J. Bacteriol. **178:**4670-8.

161. **Struyve, M., M. Moons, and J. Tommassen.** 1991. Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. J. Mol. Biol. **218:**141-8.

162. **Tacket, C. O., R. K. Taylor, G. Losonsky, Y. Lim, J. P. Nataro, J. B. Kaper, and M. M. Levine.** 1998. Investigation of the roles of toxin-coregulated pili and mannose- sensitive hemagglutinin pili in the pathogenesis of *Vibrio cholerae* O139 infection. Infect. Immun. **66:**692-5.

163. **Takase, H., H. Nitanai, K. Hoshino, and T. Otani.** 2000. Requirement of the *Pseudomonas aeruginosa tonB* gene for high-affinity iron acquisition and infection. Infect. Immun. **68:**4498-504.

164. **Tashima, K. T., P. A. Carroll, M. B. Rogers, and S. B. Calderwood.** 1996. Relative importance of three iron-regulated outer membrane proteins for in vivo growth of *Vibrio cholerae*. Infect. Immun. **64:**1756-1761.

165. **Tatusov, R. L., M. Y. Galperin, D. A. Natale, and E. V. Koonin.** 2000. The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res. **28:**33-6.

166. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of *phoA* gene fusions to identify a pilus colonization factor

coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. U.S.A. **84:**2833-2837.

167. **Thompson, J. M., H. A. Jones, and R. D. Perry.** 1999. Molecular characterization of the hemin uptake locus (*hmu*) from *Yersinia pestis* and analysis of *hmu* mutants for hemin and hemoprotein utilization. Infect. Immun. **67:**3879-3892.

168. **Torres, A. G., and S. M. Payne.** 1997. Haem iron-transport system in enterohaemorrhagic *Escherichia coli* O157:H7. Mol. Microbiol. **23:**825-833.

169. **Torres, A. G., P. Redford, R. A. Welch, and S. M. Payne.** 2001. TonB-Dependent Systems of Uropathogenic *Escherichia coli*: Aerobactin and Heme Transport and TonB Are Required for Virulence in the Mouse. Infect. Immun. **69:**6179-85.

170. **Trucksis, M., J. Michalski, Y. K. Deng, and J. B. Kaper.** 1998. The *Vibrio cholerae* genome contains two unique circular chromosomes. Proc. Natl. Acad. Sci. U.S.A. **95:**14464-14469.

171. **Tuckman, M., and M. S. Osburne.** 1992. In vivo inhibition of TonB-dependent processes by a TonB box consensus pentapeptide. J. Bacteriol. **174:**320-3.

172. **Usher, K. C., E. Ozkan, K. H. Gardner, and J. Deisenhofer.** 2001. The plug domain of FepA, a TonB-dependent transport protein from *Escherichia coli*, binds its siderophore in the absence of the transmembrane barrel domain. Proc. Natl. Acad. Sci. U.S.A. **98:**10676-81.

173. **van der Helm, D.** 1998. The physical chemistry of bacterial outer-membrane siderophore receptor proteins. Met. Ions Biol. Syst. **35:**355-401.

174. **Velayudhan, J., N. J. Hughes, A. A. McColm, J. Bagshaw, C. L. Clayton, S. C. Andrews, and D. J. Kelly.** 2000. Iron acquisition and virulence in *Helicobacter pylori*: a major role for FeoB, a high-affinity ferrous iron transporter. Mol. Microbiol. **37:**274-86.

175. **Wandersman, C., and I. Stojiljkovic.** 2000. Bacterial heme sources: the role of heme, hemoprotein receptors and hemophores. Curr. Opin. Microbiol. **3:**215-220.

176. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195-199.

177. **Weinberg, E. D.** 2000. Microbial pathogens with impaired ability to acquire host iron. Biometals **13:**85-9.

178. **Williams, S. G., S. R. Attridge, and P. A. Manning.** 1993. The transcriptional activator HlyU of *Vibrio cholerae*: nucleotide sequence and role in virulence gene expression. Mol. Microbiol. **9:**751-60.

179. **Wong, J. C., R. Patel, D. Kendall, P. W. Whitby, A. Smith, J. Holland, and P. Williams.** 1995. Affinity, conservation, and surface exposure of hemopexin-binding proteins in *Haemophilus influenzae*. Infect. Immun. **63:**2327-2333.

180. **Wooldridge, K. G., and P. H. Williams.** 1993. Iron uptake mechanisms of pathogenic bacteria. FEMS Microbiol. Rev. **12:**325-348.

181. **Wright, A. C., L. M. Simpson, and J. D. Oliver.** 1981. Role of iron in the pathogenesis of *Vibrio vulnificus* infections. Infect. Immun. **34:**503-7.

182. **Wyckoff, E. E., S. L. Smith, and S. M. Payne.** 2001. VibD and VibH are required for late steps in vibriobactin biosynthesis in *Vibrio cholerae.* J. Bacteriol. **183:**1830-4.

183. **Wyckoff, E. E., J. A. Stoebner, K. E. Reed, and S. M. Payne.** 1997. Cloning of a *Vibrio cholerae* vibriobactin gene cluster: identification of genes required for early steps in siderophore biosynthesis. J. Bacteriol. **179:**7055-7062.

184. **Wyckoff, E. E., A.-M. Valle, S. L. Smith, and S. M. Payne.** 1999. A multifunctional ABC transporter system from *Vibrio cholerae* transports vibriobactin and enterobactin. J. Bacteriol. **181:**7588-7596.

185. **Zhao, Q., and K. Poole.** 2000. A second *tonB* gene in *Pseudomonas aeruginosa* is linked to *exbB* and *exbD* genes. FEMS Microbiol. Lett. **184:**127-132.

186. **Zhu, W., D. J. Hunt, A. R. Richardson, and I. Stojiljkovic.** 2000. Use of heme compounds as iron sources by pathogenic *Neisseriae* requires the product of the *hemO* gene. J. Bacteriol. **182:**439-47.

187. **Zhu, W., A. Wilks, and I. Stojiljkovic.** 2000. Degradation of heme in gram-negative bacteria: the product of the *hemO* gene of *Neisseriae* is a heme oxygenase. J. Bacteriol. **182:**6783-90.

Vita

Alexandra Rebecca Mey was born in Austin, Texas on August 23, 1969, the daughter of Inger and Jacob L. Mey. She moved to Denmark with her family in 1972, where she lived until she was 18 years old. After graduating from Odense Katedralskole Gymnasium (High School), she spent two years traveling in Asia and Europe before coming to the United States in 1989. She entered the University of Texas at Austin in the fall of 1990. From 1993 to 1994 she studied at the University of London, England. She received a Bachelor of Arts degree from the University of Texas in the fall of 1994. She entered the Graduate Program in Cellular and Molecular Biology at the University of Texas at Austin in 1996. In 1997, she married Ygnacio V. Perez.

Publications:

1. **Mey, A. R., and S. M. Payne.** 2001. Haem utilization in *Vibrio cholerae* involves multiple TonB-dependent haem receptors. Mol. Microbiol. **42:**835-49.
2. **Seliger, S., A. Mey, A. Valle, and S. Payne.** 2001. The two TonB systems of *Vibrio cholerae*: redundant and specific functions. Mol. Microbiol. **39:**801-12.

Permanent address: 1100 West 29th St., Austin, TX 78756

This dissertation was typed by the author.