JOURNAL OF BACTERIOLOGY, Nov. 1997, p. 7103–7110 Vol. 179, No. 22
0021-9193/97/$04.00+0

Unusual Structure of the *tonB-exb* DNA Region of *Xanthomonas campestris* pv. campestris: *tonB*, *exbB*, and *exbD1* Are Essential for Ferric Iron Uptake, but *exbD2* Is Not

HEINRICH-G. WIGGERICH, BÄRBEL KLAUKE, REINHARD KÖPLIN,† URSULA B. PRIEFER,‡ AND ALFRED PÜHLER*

Lehrstuhl für Genetik, Fakultät für Biologie, Universität Bielefeld, D-33501 Bielefeld, Germany

Received 29 May 1997/Accepted 25 August 1997

The nucleotide sequence of a 3.6-kb *Hin*dIII-*Sma*I DNA fragment of *Xanthomonas campestris* pv. campestris revealed four open reading frames which, based on sequence homologies, were designated *tonB*, *exbB*, *exbD1*, and *exbD2*. Analysis of translational fusions to alkaline phosphatase and β-galactosidase confirmed that the TonB, ExbB, ExbD1, and ExbD2 proteins are anchored in the cytoplasmic membrane. The TonB protein of *X. campestris* pv. campestris lacks the conserved (Glu-Pro)$_n$ and (Lys-Pro)$_m$ repeats but harbors a 13-fold repeat of proline residues. By mutational analysis, the *tonB*, *exbB*, and *exbD1* genes were shown to be essential for ferric iron import in *X. campestris* pv. campestris. In contrast, the *exbD2* gene is not involved in the uptake of ferric iron.

Iron is the fourth most abundant element of the earth's crust. The irreplaceable role of iron in almost all oxidation and reduction processes makes it of particular importance to all organisms. Under aerobic conditions iron is rapidly oxidized to the ferric ion. Due to the extremely low solubility of Fe(III) (10^{-18} M) at physiological pHs, iron is one of the factors that most limits bacterial growth. The common strategy of bacteria to overcome this problem is the utilization of siderophores with high affinity for ferric iron (45). Fe(III) is complexed by these chelators, resulting in water-soluble complexes which are transported into the cell (41). The outer membrane of gram-negative bacteria is impermeable to hydrophilic molecules that are too large to pass through the water-filled porin channels. Therefore, specific energy-coupled import systems have been established by bacteria. The acquisition of Fe(III) siderophores and vitamin B_{12} in various gram-negative bacteria is mediated via an energy-coupled process, in which outer membrane receptors and the cytoplasmic membrane proteins TonB and ExbBD are involved. In current models the TonB protein induces a conformational change of the receptor proteins which causes the release of bound ligands into the periplasmic space. Since the translocation into the periplasm is energy dependent, TonB is considered to transduce the cytoplasmic membrane proton force to the receptor proteins (10). The receptor proteins and also group B colicins possess a conserved sequence motif which has been termed the TonB box. The interaction of the TonB box with a binding site in the TonB protein was confirmed by mutational analysis and cross-linking experiments (22, 31, 39, 50). Two auxiliary proteins, ExbB and ExbD, which form a complex with TonB, are necessary for TonB-dependent uptake. Both proteins are required for TonB-dependent energy transduction (2). Like TonB, ExbD is anchored via a single N-terminal hydrophobic segment in the cytoplasmic membrane and the remainder extends into the periplasmic space (28). The ExbB protein is predicted to span the cytoplasmic membrane three times. Only the N terminus and a short loop are located in the periplasm, while most of the protein is in the cytoplasm (29). Moreover, ExbB was shown to be directly involved in energy transduction (51). In addition, infections by bacteriophages ϕ80 and T1 are mediated by the TonB system (20).

Apart from being involved in import processes across the outer membrane, the TonB system is involved in signal transduction from the cell surface to the cytoplasm. This was shown for the induction of the transcription of the ferric citrate and pseudobactin transport genes of *Escherichia coli* and *Pseudomonas putida*, respectively (24, 32).

A second import pathway for macromolecules identified in *E. coli* is the Tol import system, on which the import of group A colicins and the infection by filamentous phages depends (59). The TolABQR proteins build the central structure of the Tol import system. ExbB and ExbD proteins are highly homologous and partially interchangeable with the TolQ and TolR proteins, respectively (11, 14, 16).

In addition to that of *E. coli*, the *tonB* genes of the gram-negative bacteria *Salmonella typhimurium*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Yersinia enterocolitica*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Neisseria meningitidis*, being pathogenic or opportunistic to animals and humans, have been cloned and sequenced (summarized in reference 54). The *exbB* and *exbD* genes were cloned and sequenced from *E. coli*, *P. putida* WCS358, *H. influenzae*, and *N. meningitidis* (9, 16, 25, 54).

Xanthomonas campestris pv. campestris is the causal agent of the black rot disease of crucifers (60). After infection of a plant with *X. campestris* pv. campestris, two different types of reactions can be observed, depending on the susceptibility of the particular plant. If the plant is susceptible, the infection gives rise to black rot disease symptoms (compatible interaction). In contrast, a hypersensitive reaction is induced in a resistant plant or a nonhost plant (incompatible interaction). The hypersensitive reaction is a local defense response characterized by rapid tissue necrosis in the inoculated area. Previously, we

* Corresponding author. Mailing address: Lehrstuhl für Genetik, Fakultät für Biologie, Universität Bielefeld, Postfach 100131, D-33501 Bielefeld, Germany.

† Present address: Department of Microbiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada.

‡ Present address: RWTH Aachen, Botanisches Institut, Ökologie des Bodens, D-52074 Aachen, Germany.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. coli		
294	*thi pro hsdR* Pro	5
BL21(DE3)(pLysS)	*E. coli* B F^- *dcm ompT hsdS*(r_{B^-} m_{B^-}) *gal* λ (DE3) [pLysS Cm^r]	Stratagene
NM522 mutS	*thi supE* Δ(*hsd*MS-*mcrB*)5 Δ(*lac-pro*AB) [*mutS*::Tn*10*] F′ [*pro*AB^+ *lacI*^{q} *lacZ*ΔM15]	Pharmacia
S17-1	*E. coli* 294 RP4-2-Tc::Mu-Km::Tn*7*	49
X. campestris		
B100	DSM 1526 Sm^r	24
B100-3.3	Tn*5*-*lacZ*-induced mutant	This work
B100-5.05	*tonB*; ΩKm(*cat*)-induced mutant	This work
B100-6.01	ΩKm(*cat*)-induced mutant	This work
B100-7.03	*exbB*; ΩKm(*cat*)-induced mutant	This work
B100-9.01	*exbD1*; ΩKm(*cat*)-induced mutant	This work
B100-9.21	*exbD1*; *aacC1*-induced mutant	This work
B100-11.03	*exbD2*; ΩKm(*cat*)-induced mutant	This work
Plasmids		
pAB4000	pUK21; *phoA-aacC1* translational fusion cassette	6
pBK2	pSUP102-Km Gm^r; 10-kb *Sma*I wild-type fragment	53
pBK11	pJFF350 Km^r carrying a promoterless *cat* gene	
pCRSkript Amp SK+	Ap^r T7 promoter	Stratagene
pHGW2	pBK11 Km^r ΔoriT IS*1** ΩKm(*cat*) *Bam*HI cassette	This work
pHGW12-1	pSVB30 Ap^r; *phoA-aacC1* translational fusion cassette	This work
pHGW12-2	pSVB30 Ap^r; *phoA-aacC1* translational fusion cassette	This work
pHGW12-3	pSVB30 Ap^r; *phoA-aacC1* translational fusion cassette	This work
pHGW31	pHIP1 *aacC1* Δ*Bgl*II carrying the multiple cloning site of pSVB30	This work
pHGW100	pSVB30 Ap^r; 3.6-kb *Sma*I-*Hin*dIII wild-type fragment	This work
pHGW100-BGL1 to -11	pHGW100 carrying *Bgl*II sites BGL1 to BGL11	This work
pHGW200	pHGW31; 3.6-kb *Sma*I-*Hin*dIII wild-type fragment	This work
pHGW241	pHGW31 Gm^r $tonB_{Xcc}$	This work
pHGW242	pHGW31 Gm^r $exbB_{Xcc}$	This work
pHGW243	pHGW31 Gm^r $exbD1_{Xcc}$	This work
pHGW258	pBCSK+ Cm^r Gm^r; 3.1-kb *Xba*I-*Bst*EII fragment of plasmid pHGW100-BGL2b carrying *aacC1* gene in BGL2b site	This work
pHGW300	pR102B Ap^r Gm^r; 3.6-kb *Sma*I-*Hin*dIII wild-type fragment	This work
pHIP1	pK18 Km^s Gm^r	7
pJFF350	Km^r ΩKm	18
pKS9I	pBR325 Cm^r Ap^r; *lacZYA-aacC1* translational fusion cassette	K. Schüddekopf
pKS10I	pBR325 Cm^r Ap^r; *lacZYA-aacC1* translational fusion cassette	K. Schüddekopf
pKS11I	pBR325 Cm^r Ap^r; *lacZYA-aacC1* translational fusion cassette	K. Schüddekopf
pMS246	pSVB30 Ap^r Gm^r; *aacC1* cassette	8
pRU884	pME305 Km^r Ap^r Tc^r *trfAts tra*$^+$ Tn*1736* Km	57
pSVB30	pUC8 Ap^r Gm^s	3
pUC21	*lacZ*α Km^r	58

have isolated a 10-kb *Sma*I DNA region of *X. campestris* pv. campestris involved in the hypersensitive response on the nonhost plant pepper but not required for the pathogenic interaction with host plants. A central region of this fragment was found to contain a plant-inducible transcription unit (53). In this paper, a more detailed characterization of the adjacent 3.6-kb *Hin*dIII-*Sma*I subfragment, which results in the detailed analysis of the *tonB*, *exbB*, *exbD1*, and *exbD2* genes of *X. campestris* pv. campestris, is shown.

MATERIALS AND METHODS

Bacteria and plasmids. The strains and plasmids used in this work are listed in Table 1.

Media and growth conditions. *E. coli* strains were grown in Luria Broth (LB) medium (36) or M9 minimal medium (40) at 37°C. *X. campestris* pv. campestris strains were grown in tryptone-yeast (TY) medium (1) or M9 minimal medium supplemented with 4% glucose and 0.05% Casamino Acids at 30°C. $FeSO_4$ was added to a final concentration of 100 μM if needed. Solid media contained agar at 10 g/liter. For growth under iron-restricted conditions, 2,2′-dipyridyl was added to M9 minimal medium to a final concentration of 200 μM (*E. coli*) or 300 μM (*X. campestris* pv. campestris). Chromazurol S (CAS) agar plates (47) were used to demonstrate siderophore biosynthesis and secretion, which was manifested by orange-halo formation around bacterial colonies. The final concentrations of antibiotics were 200-μg/ml ampicillin (*E. coli*), 20-μg/ml (*E. coli*) or 50-μg/ml (*X. campestris* pv. campestris) kanamycin, 10-μg/ml (*E. coli*) or 30-μg/ml (*X. campestris* pv. campestris) gentamicin, and 800-μg/ml streptomycin (*X. campestris* pv. campestris). 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and 5-bromo-4-chloro-3-indolyl-β-D-phosphate (X-P) were added to LB plates to a concentration of 40 mg/liter.

Electroporation of *X. campestris* pv. campestris and bacterial matings. Electroporations were performed as described by Kamoun and Kado (27). Bacteria to be electroporated were grown in TY medium and regenerated in SOB medium (40), both supplemented with $FeSO_4$ if needed. Bacterial matings were performed on membrane filters (pore size, 0.2 μm) according to the method of Simon et al. (49).

DNA methods, hybridization, and transformation. Plasmid DNA was isolated by using a rapid boiling lysis technique (3). Total DNA was isolated by lysozyme-sarcosyl-proteinase K lysis, followed by phenol-chloroform extraction and isopropanol precipitation (38). Restriction enzymes, T4 ligase, and other commercially available enzymes were obtained from Pharmacia (Freiburg, Germany) and used in accordance with the manufacturer's recommendations. Restriction endonuclease-generated fragments of total DNA were separated by gel electrophoresis and subsequently vacuum blotted on nylon filters as described by Simon

et al. (48). Labeling of the probe (vector and DNA region) and hybridization were done as described elsewhere (48). Preparation of competent *E. coli* cells and transformation with plasmid DNA were carried out as described by Maniatis et al. (36).

Construction of plasmids. Plasmid pHGW31 containing the multiple-cloning site of pSVB30 (3) is a derivative of pHIP1 (7). The *Bgl*II site located within the *aacC1* gene of pHIP1 was removed by oligonucleotide-directed mutagenesis. Plasmid pBK11 carries ΩKm(*cat*), a derivative of the ΩKm (18) equipped with a promoterless chloramphenicol acetyltransferase (*cat*) reporter gene. It was constructed by inserting a 780-bp *Bam*HI fragment of plasmid pRU884 (57) into the partially digested plasmid pJFF350 (18). Plasmid pBK2 was described previously (53). Plasmids pHGW100 and pHGW200, which contain the 3.6-kb *Hin*dIII-*Sma*I *X. campestris* pv. campestris fragment of pBK2, were obtained by cloning the fragments into the multiple-cloning sites of pSVB30 and pHGW31, respectively. The mobilizable suicide vector pHGW300, which harbors the 3.6-kb *Hin*dIII-*Sma*I *X. campestris* pv. campestris fragment, was constructed by digestion of pBK2 with *Hin*dIII and religation.

Plasmid pHGW241 contained the *Hin*dIII-*Eco*RI fragment (*tonB* gene) of pHGW100. Plasmids pHGW242 and pHGW243, which carry the *exbB* and *exbD1* genes from *X. campestris* pv. campestris, respectively, were constructed by cloning the *Bam*HI-*Bgl*II fragment of pHGW100-EXB2b and the *Bgl*II-*Sst*I fragment of pHGW100-EXB4 into pHGW31 (see Fig. 1C).

In order to get a derivative of the ΩKm(*cat*) transposon that can be cloned as a *Bam*HI fragment, plasmid pBK11 was partially digested with *Bam*HI. The resulting overhanging single-stranded DNA was filled in with deoxynucleoside triphosphates by using the Klenow fragment (Pharmacia) and religated. Plasmids which had lost the *Bam*HI site in the ΩKm(*cat*) portion of pBK11 were identified by restriction analysis. The oriT and IS*1** fragments were removed by an additional round of digestion with *Bam*HI and religation. The resulting plasmid was designated pHGW2. After digestion with *Bam*HI, the ΩKm(*cat*) cassette was cloned into the artificially introduced *Bgl*II sites of plasmid pHGW200-BGL. Mutagenized plasmids could be recognized by selection for Km^r and Gm^r. The orientations of the ΩKm(*cat*) insertions within the *X. campestris* pv. campestris DNA fragment were determined by restriction analysis.

For construction of pHGW258, first the 3.1-kb *Bst*EII-*Xba*I fragment of plasmid pHGW100-BGL2b was cloned into plasmid pBCSK+ (Stratagene, Heidelberg, Germany). In a second step, plasmid pMS246 (8) was digested with *Bam*HI and the *aacC1* gene, harboring a P_{out} promoter, was cloned into the artificial *Bgl*II site of the *exbD1* gene.

Construction of translational fusions to alkaline phosphatase and β-galactosidase. Plasmid pAB4000 (6) carries a *phoA-aacC1* cassette which allows the construction of translational fusions by blunt-end cloning. In a first step of establishing a set of *Bam*HI clonable derivatives of this cassette in all three reading phases, we cloned the *Eco*RV-*Stu*I fragment of the multiple-cloning site of plasmid pUC21 (58) into the *Stu*I site of pAB4000. The *Kpn*I-*Hin*dIII fragment of the resulting plasmid, pHGW10, was then cloned into the multiple-cloning site of the pSVB30 derivative pHGW11. The resulting plasmid, pHGW12-1, harbors the *Bam*HI clonable *phoA-aacC1* cassette in the first reading frame. A second reading frame in plasmid pHGW12-2 was achieved after replacing the *Xba*I-*Kpn*I fragment of pHGW12-1 by an artificial linker made of the two primers 5′-ctagaggatcccccggggtac-3′ and 5′-cccggggatcct-3′. The third reading frame in plasmid pHGW12-3 was obtained after combining the *Sma*I-*Hin*dIII *phoA-aacC1* fragment of pHGW12-3 with the *Sma*I-*Hin*dIII vector portion of pHGW12-1. All reading frames were confirmed by DNA sequencing.

For the construction of translational fusions to the *tonB-exbBD1D2* genes, the 3.6-kb *Hin*dIII-*Sma*I fragment of plasmids pHGW100-BGL1 to pHGW100-BGL11 were cloned into the plasmid pCRSkript SK+ (Stratagene) downstream of the T7 polymerase recognition sequence. After restriction with *Bam*HI, the *phoA-aacC1* cassettes from pHGW12-1, pHGW12-2, and pHGW12-3 and the *lacZYA-aacC1* cassettes from pKS9I, pKS10I, and pKS11I (K. Schüddekopf, Bielefeld, Germany) were cloned into the *Bgl*II sites of the mutated *X. campestris* pv. campestris fragment. The orientations of the insertions within the B100 fragment were determined by restriction analysis.

The plasmids carrying the translational fusions were transferred to the *E. coli* strain BL21(DE3)(pLysS) (Stratagene), which allows the induction of transcription by T7 polymerase after addition of isopropyl-β-D-thiogalactoside (IPTG) to the medium.

β-Galactosidase and alkaline phosphatase were assayed on LB medium supplemented with X-Gal and X-P, respectively. Blue *E. coli* colonies expressing translational fusions were detected after 1 day of growth.

Nucleotide sequence analysis. Nucleotide sequences were determined according to the dideoxy method of Sanger et al. (46) using fluorescent primers and the Automatic Laser Fluorescent DNA sequencer (ALF; Pharmacia). Both strands of the entire 3.6-kb fragment were sequenced by using overlapping sequencing clones. Nucleotide sequence data were evaluated with the sequence analysis programs of Staden (52). The deduced amino acid sequence was compared to the EMBL protein databases by using the FASTA programs (35).

Oligonucleotide-directed mutagenesis. The site-directed mutagenesis of the 3.6-kb *Hin*dIII-*Sma*I *X. campestris* pv. campestris wild-type fragment was performed according to the method of Deng and Nickoloff (15) by in vitro unique-site elimination mutagenesis using suitable pairs of primers (u.s.e. mutagenesis kit; Pharmacia). pHGW100, which carries the 3.6-kb *Hin*dIII-*Sma*I fragment, was used for mutation. The following primers, which are directed to randomly chosen sites, introduce artificial *Bgl*II sites into the *Hin*dIII-*Sma*I fragment: BGL1 (5′-cgctgcggcagatcTacggcgatc-3′), BGL2 (5′-ggcgtgacgtAgatCtcggccatt-3′), BGL3 (5′-gcctctggcagAtcTaccttgacc-3′), BGL4 (5′-cgaacagacAgatcTcggtcatga-3′), BGL5 (5′-gaaccggttgcacAgatcttgatc-3′), BGL6 (5′-gggcatcccagaTcTcggtggtga-3′), BGL7 (5′-atctgcgaaagaTcTgccgacgga-3′), BGL8 (5′-cggcacggtcaagAtcTcggttgc-3′), BGL9 (5′-cgatcaggaAgaTctcaccttgga-3′), BGL10 (5′-ggcgtgacgAgatcTctcggacgc-3′), and BGL11 (5′-gtggataacgagAtCttccgtcatg-3′). The BGL2 primer is able to introduce a *Bgl*II site either at DNA sequence position 1706 or at position 2131. The second primer, 5′-tgcttcaataGGCCtgaaaaagga-3′, converts the *Ssp*I site of the *bla* gene of pHGW100 into a *Stu*I site. The resulting plasmids were designated pHGW100-BGL1 to pHGW100-BGL11. The *Bgl*II site of the *aacC1* gene of pHGW31 was removed by using the oligonucleotide 5′-gcgtagtg agatTtatatctatg-3′. The changed bases are shown in capital letters.

Reverse mutagenesis (homogenotization). Homogenotization was carried out as described by Priefer (44). ΩKm(*cat*)-carrying pHGW200 or pHGW300 and pHGW258 were transferred into the *X. campestris* pv. campestris strain B100. Km^r transelectroporants or, in the case of pHGW300, transconjugants were tested for loss of the vector-encoded gentamicin resistance. Recombination of the ΩKm(*cat*)-carrying region into the genome via double crossover was confirmed by hybridization.

Siderophore utilization. Utilization of different siderophores by *X. campestris* pv. campestris B100 was determined by the reversal of iron-limited growth. Aliquots (0.2 ml) of an overnight culture were seeded on M9 minimal medium containing just enough 2,2′-dipyridyl to inhibit growth completely. Filter paper discs containing the different siderophores were placed on the surfaces of the plates. After 3 days of incubation at 30°C, the plates were scored for bacterial growth. The siderophores coprogen, aerobactin, ferrichrome, ferrioxamine B, ferrioxamine E, and schizokinen were generously provided by K. Hantke, Tübingen, Germany.

Nucleotide sequence accession number. The DNA sequence containing the *X. campestris* pv. campestris *tonB*, *exbB*, *exbD1*, and *exbD2* genes has been assigned GenBank accession no. Z95386.

RESULTS

Sequence analysis of a 3.6-kb *Hin*dIII-*Sma*I DNA fragment of *X. campestris* pv. campestris resulted in the identification of the genes *tonB*, *exbB*, *exbD1*, and *exbD2*. Previously, we have isolated a 10-kb *Sma*I DNA fragment of the *X. campestris* pv. campestris wild-type strain B100, which was shown to contain a plant-inducible transcription unit (53). To further characterize this DNA region, we started to determine the nucleotide sequence of a *Hin*dIII-*Sma*I DNA fragment, representing the right edge of the 10-kb *Sma*I fragment (Fig. 1). The complete nucleotide sequence of this 3,631-bp *Hin*dIII-*Sma*I DNA fragment was determined for both strands. A coding region analysis was carried out with the computer program package of Staden (52). The location of start and termination codons in the different reading phases permitted the identification of four open reading frames (ORFs) that had a high coding probability. The four ORFs, all located on the same DNA strand, were named ORF1 to ORF4. The locations of the ORFs as well as the extents and directions of their coding regions are given in Fig. 1B.

All ORFs start with an ATG start codon. ORF1 is located between nucleotide positions 91 and 762. A putative 5′-AA GAG-3′ ribosome-binding site (55), differing in 1 of 5 nucleotides, was found 11 bp in front of the first ATG codon. The translational product of ORF1 deduced from the DNA sequence is a protein of 223 amino acids (aa) with a molecular weight (MW) of 23,598. This deduced protein possesses a 13-fold repeat of proline from position 66 to position 78. This repeat is the beginning of a segment of 37 aa of the deduced protein with a significantly high proline content of 57.81%. In addition, the overall percentage of proline in the entire translational product (20.2%) is very high.

ORF2, which is separated from ORF1 by an intergenic region of 84 bp, includes 759 bp. Upstream of ORF2 a putative 5′-AGGT-3′ ribosome-binding site was identified, being at an optimal distance of 7 bp from the first base of the ATG start

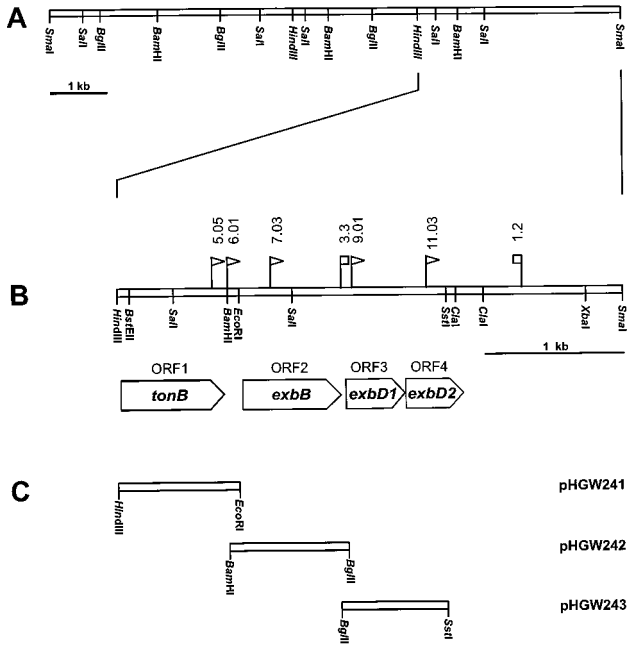

FIG. 1. Genetic and physical maps of the 3.6-kb *Hin*dIII-*Sma*I *X. campestris* pv. campestris DNA fragment involved in the uptake of ferric iron. (A) Partial restriction map of an *X. campestris* pv. campestris DNA region cloned in pBK2 (53). (B) Detailed restriction map of the sequenced *X. campestris* 3.6-kb *Hin*dIII-*Sma*I subfragment. The locations of the genes *tonB*, *exbB*, *exbD1*, and *exbD2*, as determined by sequence analysis, are indicated below the restriction map. (C) Subfragments cloned for complementation of *X. campestris tonB*, *exbB*, and *exbD1* mutants. Symbols: squares, Tn*5-lacZ* insertions, indicating the orientation of the *lacZ* gene; triangles, ΩKm(*cat*) insertions, indicating the orientation of the *cat* gene.

codon. The translational product predicted for this ORF is a 253-aa protein with a MW of 26,752.

A second short intergenic region of 46 bp separates ORF3 from ORF2. ORF3 is located between bp 1665 and 2087. The deduced translational product of ORF3 is a 140-aa protein with a MW of 15,215. At a distance of 8 bp, the ATG start codon is preceded by a putative 5′-AGACGG-3′ ribosome-binding site, which differs in 2 out of 6 nucleotides from the *E. coli* consensus sequence.

The last ORF identified on this 3.6-kb *Hin*dIII-*Sma*I fragment starts 3 bp behind the TAA stop codon of ORF3 and is located between bp 2091 and 2501. The predicted translational product of ORF4 is a 136-aa protein with a MW of 14,997. A putative 5′-AAGGGG-3′ ribosome-binding site, differing in only 1 out of 6 bp from the *E. coli* consensus sequence, is located 10 bp in front of the initiation codon.

No convincing −10 and −35 *E. coli* or *X. campestris* promoter consensus sequences (30, 37) were detected upstream of the ribosome-binding sites. A G+C-rich inverted sequence, which leads to a double-stranded RNA hairpin structure, is located 41 bp downstream of the TAA stop codon of ORF4. This stem-and-loop structure is followed by 6 U nucleotides, typical of *rho*-independent transcription termination (42).

Due to low coding probability and the wide spread of translational termination codons in all three reading phases, no other ORFs were identified downstream of ORF4.

The predicted amino acid sequences of ORF1, ORF2, ORF3, and ORF4 were compared to protein sequences in the EMBL databases. The highest degree of homology of ORF1 was to the deduced sequence of the TonB protein from *S. marcescens* (19), which encodes the central protein of the TonB system, with 67.7% similarity and 28.8% identity. Therefore, we designated ORF1 the *tonB* gene of *X. campestris* pv. campestris.

```
XCCEXBD1  MAFSSGNSGGPMADINVTPLVDVMLVLLIIFIITAPLMSHKVKVELPEANLIQKEDAEKR  60
          ||||·|·· |||||||||||||||||||||·|||·|·· · |·||·  |    ·· ·
XCCEXBD2  MAFSTGGNRGPMADINVTPLVDVMLVLLIIFIVTAPIMTYPIAVDLPQRVLNPPPQTTEP  60

XCCEXBD1  AAPITLAVKEDGSLYWNDEPISKEALESRLSTAAQQTP--QPPLNLRGDRTTKMRTINEI 118
            || | ·  ·····||· |     |····  ··|  |  || |····· ····· ··
XCCEXBD2  PPPIELRIDASNQVFWNNSPTPVAQLQQKMEEVVQADPTNQPELRIDANEDAEYEVMAKV 120

XCCEXBD1  TKIAQGQGMLDVGFVATKEKGQ                                      140
             |··· |  ·||·
XCCEXBD2  LAAAKNSQMKKIGFMQ                                            136
```

FIG. 2. Comparison of the deduced amino acid sequences of the proteins encoded by the *exbD1* and *exbD2* genes of *X. campestris* pv. campestris. Identical residues are marked by vertical lines; similar residues are marked by dots. Amino acids are given in one-letter code.

The amino acid sequence deduced from ORF2 showed 40.6% identity and 71.8% similarity to the translated amino acid sequence of the *exbB* gene of *N. meningitidis* (54). Due to this homology we designated ORF2 the *exbB* gene of *X. campestris* pv. campestris.

Comparison of the deduced amino acid sequences of the proteins encoded by ORF3 and ORF4 with the proteins in the EMBL databases showed the highest degree of homology to the predicted translational product of the *exbD* gene of *N. meningitidis*. Compared to the *N. meningitidis* ExbD protein (54), ORF3 and ORF4 were found to have 39.7 and 36.2% identical residues, respectively. Besides homology to other ExbB and ExbD proteins in the protein databases, we also found lower homology of $ExbB_{Xcc}$ to the *E. coli* TolQ protein and of the deduced amino acid sequences of *exbD1* and *exbD2* to the *E. coli* TolR protein (56).

Since the nucleotide sequence homology between $exbD_{Ec}$ and $tolR_{Ec}$ is 49.7% (16), we also scored ORF3 and ORF4 for nucleotide sequence homology. Using the LFASTA program, we found an identity of 61.6% within the first 360 nucleotides of ORF3 and ORF4, indicating that ORF3 and ORF4 were probably created by gene duplication in *X. campestris* pv. campestris. The deduced amino acid sequences of the proteins encoded by ORF3 and ORF4 showed 38.2% identity and 71.3% similarity to each other (Fig. 2). On the basis of the higher degree of homology of ORF3 and ORF4 to $ExbD_{Nm}$ than to $TolR_{Ec}$ and on the basis of the high level of identity of the nucleotide sequences of ORF3 and ORF4, we designated ORF3 and ORF4 the *exbD1* and *exbD2* genes of *X. campestris* pv. campestris, respectively.

By translational fusions to alkaline phosphatase and β-galactosidase, TonB, ExbB, ExbD1, and ExbD2 of *X. campestris* pv. campestris were shown to be anchored in the cytoplasmic membrane. By using the algorithm of Kyte and Doolittle (33), hydropathy profiles of the TonB, ExbB, ExbD1, and ExbD2 proteins of *X. campestris* pv. campestris were carried out. The hydropathy plots revealed one large hydrophobic segment each for TonB, ExbD1, and ExbD2 and three hydrophobic segments for ExbB (Fig. 3A). Therefore, the hydropathic profiles suggested that all four are transmembrane proteins. Despite the difference in amino acid compositions of $TonB_{Xcc}$, $ExbB_{Xcc}$, and $ExbD1D2_{Xcc}$ from those of $TonB_{Sm}$, $ExbB_{Nm}$, and $ExbD_{Nm}$, respectively, the distributions of hydrophobic and hydrophilic protein portions were very similar between the homologs (Fig. 3A). In order to establish the locations of $TonB_{Xcc}$, $ExbB_{Xcc}$, $ExbD1_{Xcc}$, and $ExbD2_{Xcc}$ within the cell, we constructed translational fusions to alkaline phosphatase and β-galactosidase in

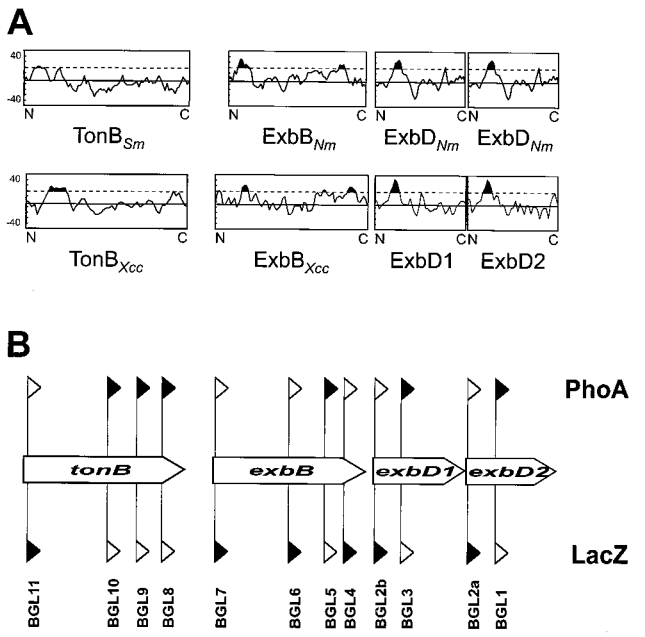

FIG. 3. Expression of translational fusions of the TonB, ExbB, ExbD1, and ExbD2 proteins of *X. campestris* pv. campestris to alkaline phosphatase (PhoA) and β-galactosidase (LacZ) confirm the data achieved from hydropathy profiles. (A) Comparison of the hydropathy profiles of the *X. campestris* pv. campestris TonB, ExbB, ExbD1, and ExbD2 proteins with their homologs from *S. marcescens* and *N. meningitidis* (19, 54). Hydrophobic regions which are considered to be membrane-spanning segments are indicated by filled curves. The mean hydrophobicity along the sequence was calculated as an average of 11 amino acids, according to the method of Kyte and Doolittle (33). (B) Expression of translational fusions of the TonB, ExbB, ExbD1, and ExbD2 proteins of *X. campestris* pv. campestris to alkaline phosphatase (PhoA) and β-galactosidase (LacZ). Promoterless *phoA-aacC1* and *lacZ-aacC1* cassettes were cloned in all three reading frames into artificial *Bgl*II sites of the *Xanthomonas* genes. Enzyme activity exhibited in one of the reading frames is indicated by filled flags, whereas no detectable activity is represented by open flags.

all reading frames by cloning promoterless *lacZYA-aacC1* and *phoA-aacC1* cassettes into *Bgl*II sites introduced into the four genes by oligonucleotide-directed mutagenesis. During the cloning experiments we found that plasmids which carry the *tonB-exbBD1D2* genes downstream of a constitutively expressed promoter and possess an insertion in a *Bgl*II site were highly unstable. In order to overcome this problem, we used the inducible T7 polymerase system for expression studies. Activities of alkaline phosphatase and β-galactosidase were observed only when the fusions were cloned in the reading phases deduced from sequence analysis. The results of the subsequent experiments are summarized in Fig. 3B. Since alkaline phosphatase must be situated outside the cytoplasmic membrane and β-galactosidase must be situated in the cytoplasm to be active (23), we found that TonB$_{Xcc}$ and ExbD1D2$_{Xcc}$ each possess at least one membrane-spanning segment, whereas ExbB$_{Xcc}$ possesses at least two membrane-spanning regions. Because alkaline phosphatase and β-galactosidase activities were both observed in each protein, the gene products of the *tonB-exbBD1D2* genes of *X. campestris* pv. campestris must span the cytoplasmic membrane.

Mutational analysis of the gene cluster *tonB-exbBD1D2* revealed that the genes *tonB*, *exbB*, and *exbD1* are essential for ferric iron uptake but *exbD2* is not. Previously, the ΩKm(*cat*) transposon was found to be a very useful element to introduce stable mutations into genes of *X. campestris* pv. campestris. To further characterize the *tonB-exbBD1D2* gene cluster of *X. campestris* pv. campestris, a fragment-specific mutagenesis of the 3.6-kb *Hin*dIII-*Sma*I wild-type DNA fragment, using a derivative of the ΩKm(*cat*) element located on plasmid pHGW2, which can be cloned as a *Bam*HI fragment, was performed. After digestion with *Bam*HI, plasmid pHGW2 was cloned into the *Bgl*II sites BGL1, BGL2b, BGL6, and BGL8, introduced in the *tonB-exbBD1D2* genes by oligonucleotide-directed mutagenesis, and introduced into the naturally occurring *Bam*HI site located between *tonB* and *exbB*. The orientations of the ΩKm(*cat*) insertions were determined by restriction fragment analysis. For further studies we tried to recombine the ΩKm(*cat*)-carrying regions into the genome of the wild-type strain *X. campestris* pv. campestris B100. All attempts to gain homogenotes failed under normal conditions. Since we mutagenized a gene cluster of *X. campestris* pv. campestris probably involved in the acquisition of ferric iron, we supplemented the selection medium with ferrous iron, which is known to be imported by a TonB-independent pathway in *E. coli* (26). After adding $FeSO_4$ to the medium, we were able to obtain homogenotes. Thereby we could take advantage of directly screening the selection plates during the following days. In addition to the many colonies with wild-type growth rates that formed on the selection plates, slowly growing colonies arose after day 3 of incubation. In hybridization experiments, the fast-growing mutants were found to harbor single-crossover events, whereas the slowly growing mutants were proven to be homogenotes. Out of these homogenotes, we selected the *X. campestris* pv. campestris mutants B100-5.05 (*tonB*), B100-7.03 (*exbB*), B100-9.01 (*exbD1*), and B100-11.03 (*exbD2*) for further characterization. Additionally mutant B100-6.01 with an ΩKm(*cat*) insertion in the intergenic region between *tonB* and *exbB* was chosen (Fig. 1B).

In order to gain more information about the *tonB-exbBD1D2* genes of *X. campestris* pv. campestris, detailed analyses of the ΩKm(*cat*)-induced mutants were carried out. The ability to grow on M9 minimal medium agar plates or in M9 minimal liquid medium with or without ferrous iron was tested. We found that mutants B100-5.05, B100-7.03, and B100-9.01 need ferrous iron to grow (Fig. 4A). Since obviously no uptake of ferric iron occurred in these mutants, the products of the *tonB-exbBD1* genes of *X. campestris* pv. campestris are essential for ferric iron uptake. In contrast, the insertion in the *exbD2* gene had no effect on the utilization of ferric iron (Fig. 4A). The mutant strain B100-11.03 could grow on medium without addition of ferrous iron. Therefore, the *exbD2* gene product is not essential for the uptake of ferric iron. Even the growth on M9 minimal medium without ferrous iron was unaffected in mutant B100-6.01. In addition, the previously characterized mutant B100-3.3, which carries a Tn*5-lacZ* insertion in the translational stop codon of the *exbB* gene maintaining the stop codon (Fig. 1B), was also unaffected in ferric iron uptake (data not shown).

On M9 minimal medium supplemented with ferrous iron, the mutant strains B100-5.05, B100-7.03, and B100-9.01 could grow, although their growth was markedly reduced (Fig. 4B).

To obtain clear results, it was necessary to grow all mutants once on medium without ferrous iron, 2 days prior to their being taken for the actual test; this means that all mutants cultured on medium supplemented with ferrous iron could grow on fresh medium for 2 days without any further addition of iron.

To confirm that all effects measured were due to the insertions located in the investigated genes, we complemented mutants B100-5.05, B100-7.03, and B100-9.01 with the corresponding subfragments of the 3.6-kb *Hin*dIII-*Sma*I wild-type

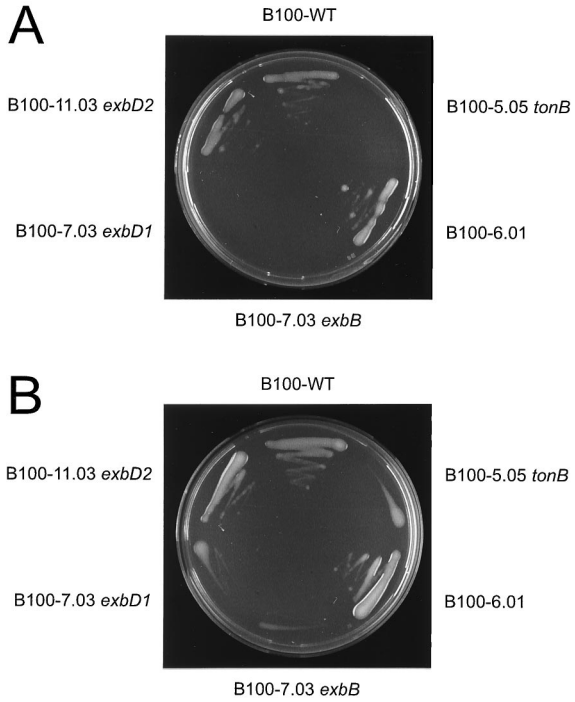

FIG. 4. Dependence on ferrous iron for growth of the *X. campestris* pv. campestris wild-type strain B100 and mutant strains which are affected in the TonB system. (A) Strains were cultured on medium without ferrous iron. The mutant strains B100-5.05, B100-7.03, and B100-9.01, with insertions in the *tonB*, *exbB*, and *exbD1* genes, could not grow on M9 minimal medium, whereas the *exbD2* mutant strain B100-11.03 and the mutant strain B100-6.01 were unaffected. (B) Strains were cultured on medium with supplementation of ferrous iron. Even on this medium the mutant strains B100-5.05, B100-7.03, and B100-9.01 showed markedly reduced growth rates.

DNA fragment (Fig. 1C). Single-crossover events partially restored the ability to take up ferric iron (data not shown).

No promoter sequences with homology to the *E. coli* or *X. campestris* consensus sequences (30, 37) could be detected in front of the *exbD2* gene. To rule out the possibility that the inability of the *exbD1* mutant B100-9.01 to import ferric iron was due to the lack of both ExbD1 and ExbD2 proteins, we introduced a polar mutation in *exbD1* by cloning an *aacC1* gene into the BGL2b *Bgl*II site located in the *exbD1* gene, bringing the *exbD2* gene under the control of the constitutively active P_{out} promoter of the *aacC1* gene. The correct orientation was confirmed by restriction analysis. The *aacC1* gene-carrying fragment was recombined into the genome of the *X. campestris* pv. campestris wild-type strain B100. The resulting homogenote was designated B100-9.21 and could not grow on medium without ferrous iron.

The *X. campestris* pv. campestris *tonB*, *exbB*, and *exbD1* mutants shared an enhanced siderophore production. While several bacteria do not produce their own siderophores, e.g., *Neisseria* and *Haemophilus*, most of the bacteria in which TonB-dependent ferric iron uptake was identified secrete at least one siderophore (61). Due to the need of acquiring enough iron from the environment, the production of siderophores is increased under iron-limiting conditions. To our knowledge a siderophore from an *X. campestris* strain has not been identified so far. In order to examine if *X. campestris* produces siderophores and to determine whether the siderophore production is iron regulated, we used the universal chemical assay for the detection of siderophores as described by Schwyn and Neilands (47). Due to the low-iron conditions of the CAS agar plates the homogenotes with disrupted *tonB*, *exbB*, and *exbD1* genes could only grow on this medium when cultured on M9 medium supplemented with ferrous iron for 2 days before being transferred to the CAS agar plates. After 3 days of incubation, the starvation of iron became visible by the secretion of siderophores. The *X. campestris* pv. campestris wild-type strain B100 produced only small amounts of siderophores. The same was seen with the *exbD2* mutant strain B100-11.03, which was not affected in ferric iron uptake. In contrast, the *tonB* mutant B100-5.05 secreted the highest amounts of siderophores. Strains B100-7.03 and B100-9.01, mutated in *exbB* and *exbD1*, respectively, produced intermediate amounts of siderophores. Even the strain B100-6.01, with an ΩKm(*cat*) insertion in the intergenic region between *tonB* and *exbB*, showed a slightly increased secretion of siderophores (data not shown). These results indicate that the production and secretion of siderophores in *X. campestris* pv. campestris are iron regulated.

In order to obtain some data about the structural nature of the siderophore produced by *X. campestris*, we tried to cross-feed the wild-type strain B100 which was cultured under iron-limiting conditions with several siderophores produced by other microbes. The hydroxamate siderophores tested were aerobactin, coprogen, ferrichrome, ferrioxamine B, ferrioxamine E, and schizokinen, produced, e.g., by *Serratia* sp., *Penicillium* sp., *Ustilago sphaerogena*, *Erwinia herbicola*, *Pseudomonas stutzeri*, and *Bacillus megaterium*, respectively (61). None of these siderophores exhibited a growth-promoting activity on *X. campestris* pv. campestris.

DISCUSSION

In this report, we describe the sequencing and functional analysis of a 3.6-kb *Hin*dIII-*Sma*I DNA fragment of *X. campestris* pv. campestris which was found to be involved in the uptake of ferric iron. DNA sequence analysis of this 3.6-kb *Hin*dIII-*Sma*I fragment revealed the presence of four genes. Based on sequence homologies and similarities in the hydropathic profiles to genes coding for the structural components of the bacterial TonB transport complex, these genes were designated *tonB*, *exbB*, *exbD1*, and *exbD2.*

The inability of the *X. campestris* pv. campestris *tonB*, *exbB*, and *exbD* mutant strains to grow on medium without Fe(II) reflects the general finding that the destruction of *tonB* genes in gram-negative bacteria results in the inhibition of ferric-siderophore uptake. Since the ExbB and ExbD proteins are also required for a functional TonB system, mutations in *exbB* and *exbD* lead to a comparable phenotype (9). For *E. coli* it is known that the homolog proteins TolQ and TolR are able to partially complement the *tonB* phenotype exhibited by *E. coli exbB* and *exbD* mutants (11). Therefore, we conclude that like *P. putida*, *X. campestris* pv. campestris harbors no other proteins able to take over the function of the ExbB and ExbD proteins (9). Even the ExbD2 protein of *X. campestris* pv. campestris failed to substitute for the ExbD1 protein in the TonB-dependent siderophore uptake. It was shown that *X. campestris* pv. campestris is capable of siderophore production and that this siderophore production is iron regulated. An explanation for the inability of *X. campestris* pv. campestris to utilize the foreign ferric siderophores tested might be the lack of specific receptors in the outer membrane or the presence of additional import systems required for the translocation across the cytoplasmic membrane.

All four genes identified, *tonB*, *exbB*, *exbD1*, and *exbD2*, were found to be clustered in *X. campestris* pv. campestris. The *tonB*,

exbB, and *exbD* genes of *N. meningitidis* are arranged in a comparable way (54). The *tonB*, *exbB*, and *exbD* genes of *P. putida* WSC358 and *H. influenzae* are clustered too, but in *P. putida* and *H. influenzae*, the *tonB* gene is located behind the *exbB* and *exbD* genes (9, 25). In *E. coli* the *tonB* gene is separated from the operon formed by *exbB* and *exbD* (4). The TonB, ExbB, and ExbD proteins of gram-negative bacteria are known to form the central structure of the TonB-dependent uptake pathway (43). In contrast to all bacteria investigated for the presence of TonB systems so far, two *exbD* genes were exclusively identified in *X. campestris* pv. campestris.

Upstream of the *X. campestris* pv. campestris *tonB*, *exbB*, *exbD1*, and *exbD2* genes, no promoter regions with convincing homology to the *E. coli* or *X. campestris* pv. campestris consensus sequences were found (30, 37). Additional experiments are necessary to detect the promoter sequences which are used in vivo. In addition, no sequence homology to the *fur* box could be found in front of the *tonB-exbBD1D2* genes of *X. campestris* pv. campestris. The *fur* box is a DNA sequence at which the iron-loaded Fur protein binds (13). Therefore, the regulation of the *tonB*, *exbB*, *exbD1*, and *exbD2* genes may be independent of the presence of a *fur* box. On the other hand, it cannot be ruled out that a *fur* box is located further upstream of the *Hin*dIII site preceding *tonB*.

Polar insertions in the intergenic region between the *tonB* and *exbB* genes and between the *exbB* and the *exbD1* genes had no effect on growth on media not supplemented with iron. These data clearly indicate that the *tonB*, *exbB*, and *exbD1* genes of *X. campestris* pv. campestris can be expressed independently. Since we could not identify a potential promoter region in front of the *exbD2* gene, this gene may be coregulated with *exbD1*.

By analyzing the expression of translational fusions of the TonB, ExbB, ExbD1, and ExbD2 proteins of *X. campestris* pv. campestris to alkaline phosphatase and β-galactosidase coding regions, we were able to demonstrate that these proteins are anchored in the cytoplasmic membrane by their N termini. For $TonB_{Xcc}$, $ExbD1_{Xcc}$, and $ExbD2_{Xcc}$, at least one membrane-spanning segment was identified for each protein. These results agreed with data obtained from hydropathic profiles and were in accordance with those being published for TonB and ExbD proteins (9, 12, 28). For $ExbB_{Xcc}$, at least two membrane-spanning segments were found, whereas three were deduced from the hydropathic profile. Since three membrane-spanning segments were found for $ExbB_{Ec}$ (29), additional reporter gene fusions are required to determine the correct topology of $ExbB_{Xcc}$.

A proline-rich region, consisting of $(Glu\text{-}Pro)_n$ and $(Lys\text{-}Pro)_m$ repeats, was found to be necessary for spanning the periplasm in the *E. coli* TonB (34). These repeats are highly conserved among all TonB proteins characterized so far. Although the amino acid sequence of $TonB_{Xcc}$ contains 20.2% proline residues, the conserved repeats mentioned above are not present. Instead, $TonB_{Xcc}$ harbors a repeat of 13 proline residues. A closer look at the structure mediated by this repeat should give new insights into the function of TonB proteins. The deduced amino acid sequences of the *exbD1* and *exbD2* genes show significant homologies. In respect to this, one can speculate that the ExbD2 protein is able to replace the ExbD1 protein in the TonB complex. Interestingly, we were able to show that *exbD2* mutants are not impaired in ferric iron uptake. Moreover, the ExbD2 protein cannot replace the ExbD1 protein in ferric-siderophore uptake. Since the TonB system serves as the central structure of the import pathways of several macromolecules and of signal transduction (21, 43) it is possible that the ExbD2 protein was established by *X. campestris* pv. campestris to increase the scope of activity of the TonB system. In order to ensure that sufficient TonB complexes for the uptake of ferric siderophores are produced, there might be the need for a coregulation of the *exbD1* and *exbD2* genes. Further investigations on *exbD2* will give new insights into the TonB system of *X. campestris* pv. campestris.

Since iron and iron siderophores are discussed as signals in plant-pathogen interactions (17) it will be interesting to examine whether functions encoded by the adjacent gene cluster have any influence on the TonB system in planta.

ACKNOWLEDGMENTS

We thank K. Hantke for providing siderophores.

This work was financially supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 223.

REFERENCES

1. **Aguilar, O. M., D. Kapp, and A. Pühler.** 1985. Characterization of a *Rhizobium meliloti* fixation gene (*fixF*) located near the common nodulation region. J. Bacteriol. **164:**245–254.
2. **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:**4742–4747.
3. **Arnold, W., and A. Pühler.** 1988. A family of high copy-number plasmid vectors with single end-label sites for rapid nucleotide sequencing. Gene **70:**171–179.
4. **Bachmann, B. J.** 1990. Linkage map of *Escherichia coli* K-12, edition 8. Microbiol. Rev. **54:**130–197.
5. **Bachmann, K., M. Patschne, and W. Gilbert.** 1976. Construction of a plasmid carrying the CI gene of bacteriophage G. Proc. Natl. Acad. Sci. USA **73:**4174–4178.
6. **Becker, A., K. Niehaus, and A. Pühler.** 1995. Low-molecular-weight succinoglycan is predominantly produced by *Rhizobium meliloti* strains carrying a mutated ExoP protein characterized by a periplasmic N-terminal domain and a missing C-terminal domain. Mol. Microbiol. **16:**191–203.
7. **Becker, A., S. Rüberg, H. Küster, A. A. Roxlau, M. Keller, T. Ivashina, H. Cheng, G. C. Walker, and A. Pühler.** 1997. The 32-kilobase *exp* gene cluster of *Rhizobium meliloti* directing the biosynthesis of galactoglucan: genetic organization and properties of the encoded gene products. J. Bacteriol. **179:**1375–1384.
8. **Becker, A., M. Schmidt, W. Jäger, and A. Pühler.** 1995. New gentamicin-resistance and *lacZ* promoter-probe cassettes suitable for insertion mutagenesis and generation of transcriptional fusions. Gene **162:**37–39.
9. **Bitter, W., J. Tommassen, and P. J. Weisbeek.** 1993. Identification and characterization of the *exbB*, *exbD* and *tonB* genes of *Pseudomonas putida* WCS358: their involvement in ferric-pseudobactin transport. Mol. Microbiol. **7:**117–130.
10. **Bradbeer, C.** 1993. The proton motive force drives the outer membrane transport of cobalamin in *Escherichia coli*. J. Bacteriol. **175:**3146–3150.
11. **Braun, V.** 1989. The structurally related *exbB* and *tolQ* genes are interchangeable in conferring *tonB*-dependent colicin, bacteriophage, and albomycin sensitivity. J. Bacteriol. **171:**6387–6390.
12. **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.
13. **Braun, V., and K. Hantke.** 1991. Genetics of bacterial iron transport, p. 107–138. *In* G. Winkelmann (ed.), CRC handbook of microbial iron chelates. CRC Press, Inc., Boca Raton, Fla.
14. **Braun, V., and C. Herrmann.** 1993. Evolutionary relationship of uptake systems for biopolymers in *Escherichia coli*: cross-complementation between the TonB-Exb-ExbD and the TolA-TolQ-TolR proteins. Mol. Microbiol. **8:**261–268.
15. **Deng, W. P., and J. A. Nickoloff.** 1992. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. Anal. Biochem. **200:**81–88.
16. **Eick-Helmerich, K., and V. Braun.** 1989. Import of biopolymers into *Escherichia coli*: nucleotide sequences of the *exbB* and *exbD* genes are homologous to those of the *tolQ* and *tolR* genes, respectively. J. Bacteriol. **171:**5117–5126.
17. **Expert, D., C. Enard, and C. Masclaux.** 1996. The role of iron in plant host-pathogen interactions. Trends Microbiol. **4:**232–237.
18. **Fellay, R., H. M. Krisch, P. Prentki, and J. Frey.** 1989. Omegon-Km: a transposable element designed for *in vivo* insertional mutagenesis and cloning of genes in Gram-negative bacteria. Gene **76:**215–226.
19. **Gaisser, S., and V. Braun.** 1991. The *tonB* gene of *Serratia marcescens*: sequence, activity and partial complementation of *Escherichia coli tonB* mutants. Mol. Microbiol. **5:**2777–2787.
20. **Hancock, R. E. W., and V. Braun.** 1976. Nature of energy requirement for the irreversible adsorption of bacteriophages T1 and ϕ80 to *Escherichia coli*. J. Bacteriol. **125:**409–415.

21. **Härle, C., I. Kim, A. Angerer, and V. Braun.** 1995. Signal transfer through three compartments: transcription initiation of the *Escherichia coli* ferric citrate transport system from the cell surface. EMBO J. **145:**1430–1438.
22. **Heller, K. J., R. J. Kadner, and K. Günter.** 1988. Suppression of the *btuB451* mutation by mutations in the *tonB* gene suggests a direct interaction between TonB and TonB-dependent receptor proteins in the outer membrane of *Escherichia coli*. Gene **64:**147–153.
23. **Hennessey, E. S., and J. K. Broome-Smith.** 1993. Gene-fusion techniques for determining membrane-protein topology. Curr. Opin. Struct. Biol. **3:**524–531.
24. **Hötte, B., I. Rath-Arnold, A. Pühler, and R. Simon.** 1990. Cloning and analysis of a 35.3-kilobase DNA region involved in exopolysaccharide production by *Xanthomonas campestris* pv. *campestris*. J. Bacteriol. **172:**2804–2807.
25. **Jarosik, G. P., and E. J. Hansen.** 1995. Cloning and sequencing of the *Haemophilus influenzae exbB* and *exbD* genes. Gene **152:**89–92.
26. **Kammler, M., C. Schön, and K. Hantke.** 1993. Characterization of the ferrous iron uptake system of *Escherichia coli*. J. Bacteriol. **175:**6212–6219.
27. **Kamoun, S., and C. I. Kado.** 1990. A plant-inducible gene of *Xanthomonas campestris* pv. campestris encodes an exocellular component required for growth in the host and hypersensitivity on nonhosts. J. Bacteriol. **172:**5165–5172.
28. **Kampfenkel, K., and V. Braun.** 1992. Membrane topology of the *Escherichia coli* ExbD protein. J. Bacteriol. **174:**5485–5487.
29. **Kampfenkel, K., and V. Braun.** 1993. Topology of the ExbB protein in the cytoplasmic membrane of *Escherichia coli*. J. Biol. Chem. **268:**6050–6057.
30. **Katzen, F., A. Becker, A. Zorreguieta, A. Pühler, and L. Ielpi.** 1996. Promoter analysis of the *Xanthomonas campestris* pv. campetris *gum* operon directing biosynthesis of the xanthan polysaccharide. J. Bacteriol. **178:**4313–4318.
31. **Koebnik, R., A. J. Bäumler, J. Heesemann, V. Braun, and K. Hantke.** 1993. The TonB protein of *Yersinia enterocolitica* and its interaction with TonB-box proteins. Mol. Gen. Genet. **237:**152–160.
32. **Koster, M., W. van Klompenburg, W. Bitter, J. Leong, and P. Weisbeck.** 1994. Role for the outer membrane ferric siderophore receptor PupB in signal transduction across the bacterial cell envelope. EMBO J. **13:**2805–2813.
33. **Kyte, J., and R. K. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
34. **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.
35. **Lipman, D. J., and W. R. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227:**1435–1441.
36. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **McClure, W. R.** 1985. Mechanism and control of transcription initiation in prokaryotes. Annu. Rev. Biochem. **54:**171–204.
38. **Meade, H., S. Long, G. Ruvkun, S. Brown, and F. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149:**114–122.
39. **Mende, J., and V. Braun.** 1990. Import-defective colicin B derivates mutated in the TonB box. Mol. Microbiol. **4:**1523–1533.
40. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50:**715–731.
42. **Platt, T.** 1986. Transcription termination and the regulation of gene expression. Annu. Rev. Biochem. **55:**339–372.
43. **Postle, K.** 1990. TonB and the gram-negative dilemma. Mol. Microbiol. **4:**2019–2025.
44. **Priefer, U. B.** 1989. Genes involved in lipopolysaccharide production and symbiosis are clustered on the chromosome of *Rhizobium leguminosarum* biovar *viciae* VF39. J. Bacteriol. **171:**6161–6168.
45. **Raymond, K. N., G. I. Müller, and B. F. Matzanke.** 1989. Complexation of iron by siderophores. A review of their solution and structural chemistry and biological function. Top. Curr. Chem. **123:**49–58.
46. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
47. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160:**47–56.
48. **Simon, R., B. Hötte, B. Klauke, and B. Kosier.** 1991. Isolation and characterization of insertion sequence elements from gram-negative bacteria by using new broad-host-range, positive selection vectors. J. Bacteriol. **173:**1502–1508.
49. **Simon, R., U. B. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology **1:**784–790.
50. **Skare, J. T., B. M. 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:**16302–16308.
51. **Skare, J. T., and K. Postle.** 1991. Evidence for a TonB-dependent energy transduction complex in *Escherichia coli*. Mol. Microbiol. **5:**2883–2890.
52. **Staden, R.** 1986. The current status and portability of our sequence handling software. Nucleic Acids Res. **14:**217–232.
53. **Steinmann, D., H. G. Wiggerich, B. Klauke, U. Schramm, A. Pühler, and U. B. Priefer.** 1993. Saturation mutagenesis in *Escherichia coli* of a cloned *Xanthomonas campestris* DNA fragment with the *lux* transposon Tn*4431* using the delivery plasmid pDS1, thermosensitive in replication. Appl. Microbiol. Biotechnol. **40:**356–360.
54. **Stojiljkovic, I., and N. Srinivasan.** 1997. *Neisseria meningitidis tonB*, *exbB*, and *exbD* genes: Ton-dependent utilization of protein-bound iron in neisseriae. J. Bacteriol. **179:**805–812.
55. **Stormo, G. D., T. D. Schneider, and L. M. Gold.** 1982. Characterization of transcriptional initiation sites in *E. coli*. Nucleic Acids Res. **10:**2971–2996.
56. **Sun, T.-P., and R. E. Webster.** 1987. Nucleotide sequence of a gene cluster involved in entry of E colicins and single-stranded DNA of infecting filamentous bacteriophages into *Escherichia coli*. J. Bacteriol. **169:**2667–2674.
57. **Ubben, D., and R. Schnitt.** 1987. A transposable promoter and transposable promoter probes derived from Tn*1721*. Gene **53:**127–134.
58. **Vieria, J., and J. Messing.** 1991. New pUC-derived cloning vectors with different selectable markers and DNA replication origins. Gene **100:**189–194.
59. **Webster, R. E.** 1991. The *tol* gene products and the import of macromolecules into *Escherichia coli*. Mol. Microbiol. **5:**1005–1011.
60. **Williams, P. H.** 1980. Black rot, a continuing threat to world crucifers. Plant Dis. **64:**736–742.
61. **Winkelmann, G. (ed.).** 1991. CRC handbook of microbial iron chelates, p. 65–106. CRC Press, Inc., Boca Raton, Fla.