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# Analysis of the *Erwinia chrysanthemi* Ferrichrysobactin Receptor Gene: Resemblance to the *Escherichia coli fepA-fes* Bidirectional Promoter Region and Homology with Hydroxamate Receptors

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**The *fct cbsCEBA* operon from the *Erwinia chrysanthemi* 3937 chrysobactin-dependent iron assimilation system codes for transport and biosynthetic functions. The sequence of the *fct* outer membrane receptor gene was determined. The *fct* promoter region displays a strong resemblance to the *Escherichia coli* bidirectional intercistronic region controlling the expression of the *fepA-entD* and *fes-entF* operons. An apparent Fur-binding site was shown to confer iron regulation on an *fct*::*lac* fusion expressed on a low-copy-number plasmid in a Fur-proficient *E. coli* strain. The *fct* gene consists of an open reading frame encoding a 735-amino-acid polypeptide with a signal sequence of 38 residues. The Fct protein has 36% sequence homology with the *E. coli* ferrichrome receptor FhuA and the *Yersinia enterocolitica* ferrioxamine receptor FoxA. On the basis of secondary-structure predictions and these homologies, we propose a two-dimensional folding model for Fct.**

Under iron-limiting conditions, most bacteria produce and excrete low-molecular-mass iron-binding compounds called siderophores and internalize their ferric complexes through specific cognate transport proteins (30). All of the functions involved in high-affinity-iron uptake are coordinately regulated by iron availability (3). This control is achieved in many bacteria by the Fur protein, which, in the presence of ferrous iron as cofactor, acts as a transcriptional repressor by binding to operator-specific sequences (Fur or Iron boxes) (10).

In response to iron starvation, *Erwinia chrysanthemi* 3937, a plant-pathogenic enterobacterium, synthesizes two siderophores, chrysobactin {*N*-[$N^2$-(2,3-dihydroxybenzoyl)-D-lysyl]-L-serine [32]} and achromobactin (24) of still unknown structure. Iron acquisition mediated by chrysobactin is essential for this pathogen to disseminate throughout its host plant. The ferrichrysobactin outer membrane receptor gene *fct* and the *cbs* genes (which stand for ferrichrysobactin transport and chrysobactin biosynthesis, respectively) involved in the four primary steps of chrysobactin biosynthesis are clustered in an operon (13, 14). The chrysobactin *fct cbsCEBA* operon is transcribed into a polycistronic mRNA of about 8 kb only in iron-depleted cells (12). Masclaux and Expert (25) showed recently that this polycistronic transcription unit was turned on in planta.

We report here the sequence and analysis of the *fct* gene and the characterization of the *fct cbsCEBA* operon promoter and operator region.

**Nucleotide sequence of *fct*.** To locate the promoter of the *fct cbsCEBA* operon present on the 6.5-kb genomic segment of plasmid pTF6 (14), we generated a series of *fct*::*lacZ* fusions by mutagenesis with the Tn*5*-B20 transposon (39). One of the constructions (*fct34*::*lacZ*) (25) was subcloned in pUC18, and serial shortenings (200 to 250 bp) of the *fct* upstream region were carried out with the Pharmacia double-stranded nested deletion kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). LacZ activity of the subclones bearing deletions was checked on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) agar medium. The smallest subclone containing an active promoter included a 1.4-kb *E. chrysanthemi* genomic fragment. This fragment was sequenced by the dideoxynucleotide chain termination method with the Sequenase version 2.0 kit (US Biochemical Co.) and [α-$^{35}$S]dATP (Amersham Co.). To sequence the *fct* downstream region, a second Tn*5*-B20 insertion was subcloned. A sequence of 2,800 bp was determined on both strands with synthetic oligonucleotide primers (data not shown). Data were analyzed with the software package UWGCG provided by BIZANCE (11).

An open reading frame (ORF) of 2,208 bp extending from bp 269 to 2476 was identified. The ATG start codon is preceded by a good putative Shine-Dalgarno sequence AGGAGG. At the 3′ untranslated region of the *fct* gene, an inverted repeat (5′ stem 2481 to 2491; loop 2492 to 2496; 3′ stem 2497 to 2507) may form an 11-bp hairpin (14 bp if G-T pairs are taken into account) that could act as a transcription terminator. A second ORF starts just downstream from this hairpin (position 2559). The first 230 nucleotides of this ORF show a high degree of homology with the 5′ end of the *E. coli entC* gene (31). This gene is predicted to be *cbsC*, which was identified as the *E. coli entC* counterpart in chrysobactin biosynthesis (13).

The *fct* ORF predicted a 735-amino-acid protein of 81,006 Da. The first 38 amino acids have characteristics typical of a signal sequence. Cleavage of this signal sequence after residues AQA, according to the von Heijne matrix (44), would produce a mature polypeptide of 697 amino acids with a molecular mass of 76,756 Da, which agrees with the size of Fct determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (80 kDa) (14).

**Identification of the *fct cbsCEBA* operon transcription initiation site.** Computer analysis predicted two potential promoters, P1 and P2, located upstream of the *fct* gene (Fig. 1). Two putative Fur-binding sites matching the 5′-GATAATGATA ATCATTATC-3′ consensus sequence at 13 and 12 of 19 positions (10) overlap this promoter region.

To identify the transcriptional start point, we performed a primer extension analysis, using the method described by Uzan

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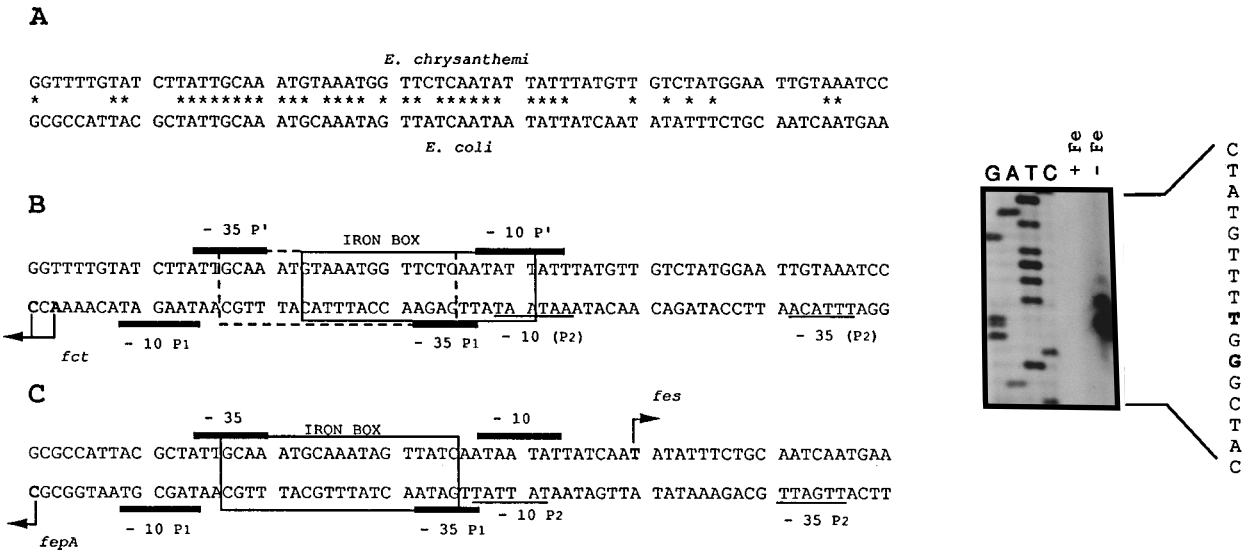


FIG. 1. Comparison between *fct cbsCEBA* operon promoter region and *E. coli* bidirectional *fepA-fes* control region. (A) Nucleotide alignment of the two promoter regions. (B) Organization of the *fct* promoter region. Promoter P1 and P2 determinants are underlined, and those of promoter P′ are overlined. The Fur-binding sites are boxed. The autoradiogram shows results of the primer extension reactions under iron-replete (+Fe) and -depleted (−Fe) conditions, allowing determination of P1 as the functional promoter. Lanes G, A, T, and C are sequencing ladders; to the right of the autoradiogram is the DNA sequence of this region, with the mRNA migrating positions shown in boldface type. (C) Organization of the *fepA-fes* bidirectional promoters (17). *fepA* promoter determinants are underlined, and those of *fes* are overlined. The functional Fur box is enclosed.

et al. (42). Templates were mRNA transcripts extracted from *E. chrysanthemi* wild-type cells grown in iron-supplemented (+20 μM $FeCl_3$) or -depleted (−Fe) M63 medium (12). A $^{32}$P-labelled 20-mer oligonucleotide complementary to positions 121 to 140 was used for primer extension reactions and sequencing. Extension from this primer yielded two iron-regulated cDNAs comigrating with G and T residues (Fig. 1). Transcription of the *fct cbsCEBA* operon therefore starts at two close C and A nucleotides, suggesting that only the anticipated promoter P1 was functional under the conditions tested.

**Homology between the *fct* promoter and the *E. coli fepA-fes* intercistronic region.** A more thorough inspection of this promoter region predicted the occurrence of a third promoter, P′, in the opposite direction to the P1 and P2 promoters. Moreover, the two iron boxes overlap both the P1 and the P′ potential promoter (Fig. 1B). Such an organization is reminiscent of that found in *E. coli* for the *fepA-entD* and *fes-entF* operons involved in biosynthesis and uptake of the enterobactin siderophore (17, 33). Indeed, two divergent overlapping promoters control the expression of both operons: (i) the *fepA-entD* operon is preceded by two tandemly arranged promoters, P1 and P2, with P1 responsible for most of the *fepA* expression; (ii) the divergent *fes-entF* operon is governed by a single promoter (Fig. 1C); and (iii) a unique experimentally determined Fur-binding site symmetrically overlaps these promoters. In contrast, a weak homology between the *fepA* and *fct* coding sequences was found.

**Fur-dependent regulation of the *fct* gene in *E. coli*.** The presence of a potential Fur-binding site located upstream of the chrysobactin operon led us to determine whether the *E. coli* Fur protein regulates the *fct* gene transcription. For this purpose, the *fct34*::*lacZ* fusion was cloned into the low-copy-number plasmid pACYC184 (34), yielding pCS6.9. Plasmid pCS6.9 was introduced into the *E. coli* strain BN402 and its Fur$^-$ derivative BN4020 (29). In the Fur$^+$ background, iron depletion induced a fivefold increase in β-galactosidase activity (Fig. 2). In the Fur$^-$ strain, the *fct*::*lacZ* fusion was constitutively expressed regardless of the iron concentration (Fig. 2). These results demonstrate a Fur-mediated regulation of the *fct* gene in *E. coli*. Therefore, it is likely that a Fur protein regulates expression of the genes involved in chrysobactin-mediated iron acquisition in *E. chrysanthemi.*

**Homology between Fct and other TonB-dependent receptors.** The amino acid sequence of Fct was compared with all published sequences of TonB-dependent receptors. The *E. coli* ferrichrome receptor FhuA (9) and the *Yersinia enterocolitica* ferrioxamine B receptor FoxA (4) have the highest homology with Fct (36.6 and 36.8%, respectively). These two siderophores are hydroxamate chelators. Only 16.8% homology was found between Fct and the ferrienterobactin receptor

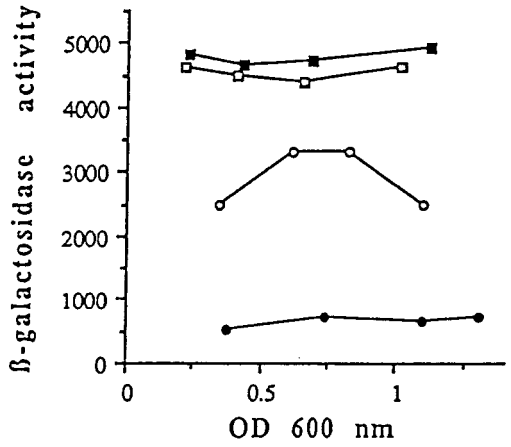


FIG. 2. Expression of the *fct34*::*lacZ* fusion carried by plasmid pCS6.9 in *E. coli*. The wild-type strain BN402 (circles) and its Fur$^-$ derivative BN4020 (squares) were grown in iron-depleted (open symbols) or -replete (closed symbols) M63 medium. β-Galactosidase activity expressed in Miller units was assayed as previously described by Miller (26). OD, optical density.

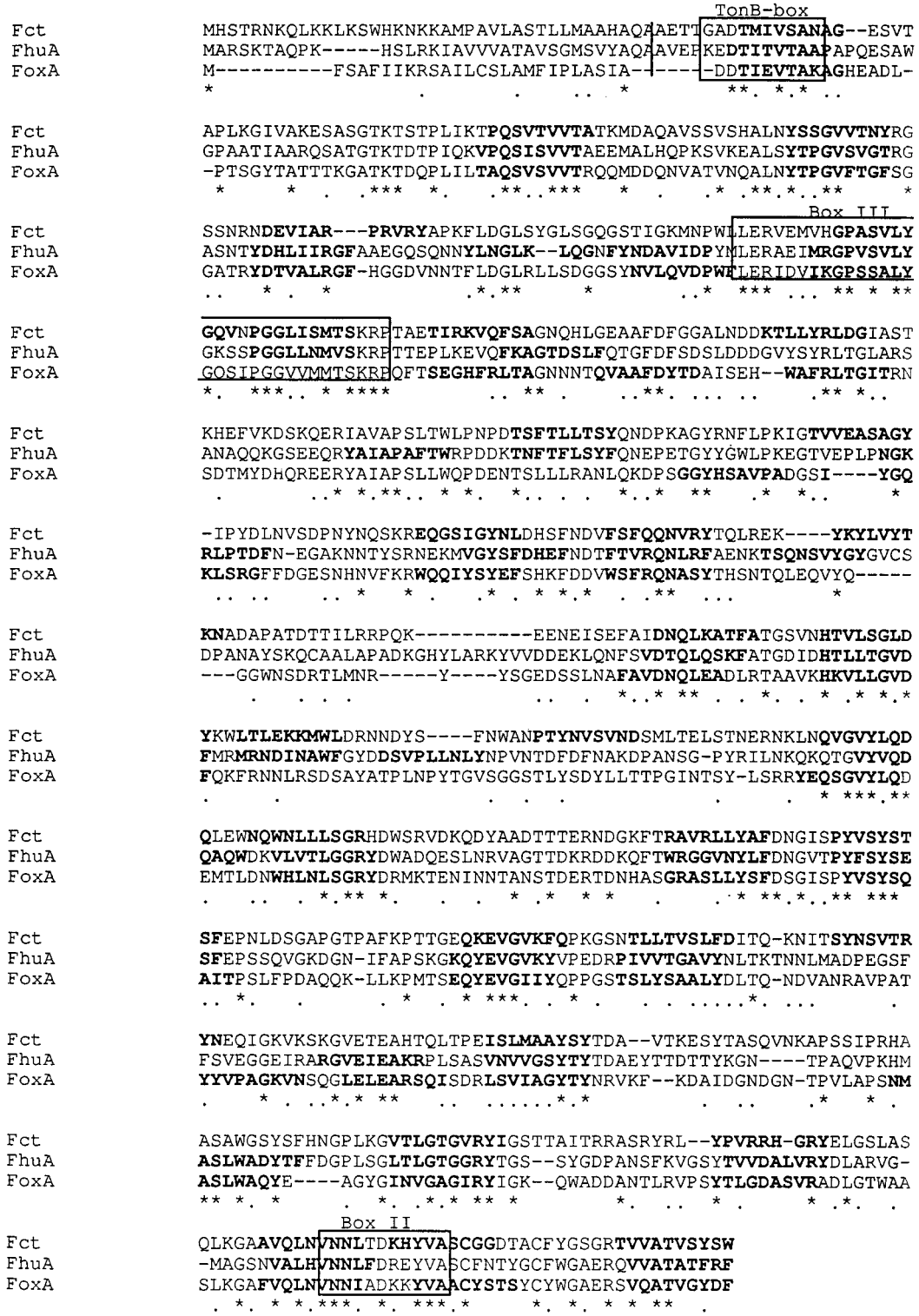


FIG. 3. Multiple alignment of Fct, FhuA, and FoxA proteins. Stars indicate identical amino acids, and dots indicate residues with similar chemical properties. Boldface letters indicate possible membrane-spanning domains. Boxes contain consensus sequences characteristic of TonB-dependent proteins.

FepA, although both siderophores contain the same catechol moiety and have related structures. Other groups have recently reported unexpected similarities between hydroxamate and catechol siderophore receptors (1, 22).

Three sequences characteristic of TonB-dependent proteins (Fig. 3) (4) were found. The presence of a typical TonB box (16) is consistent with the fact that Fct is functional in *E. coli* (14). The last 45 C-terminal amino acids are markedly con-

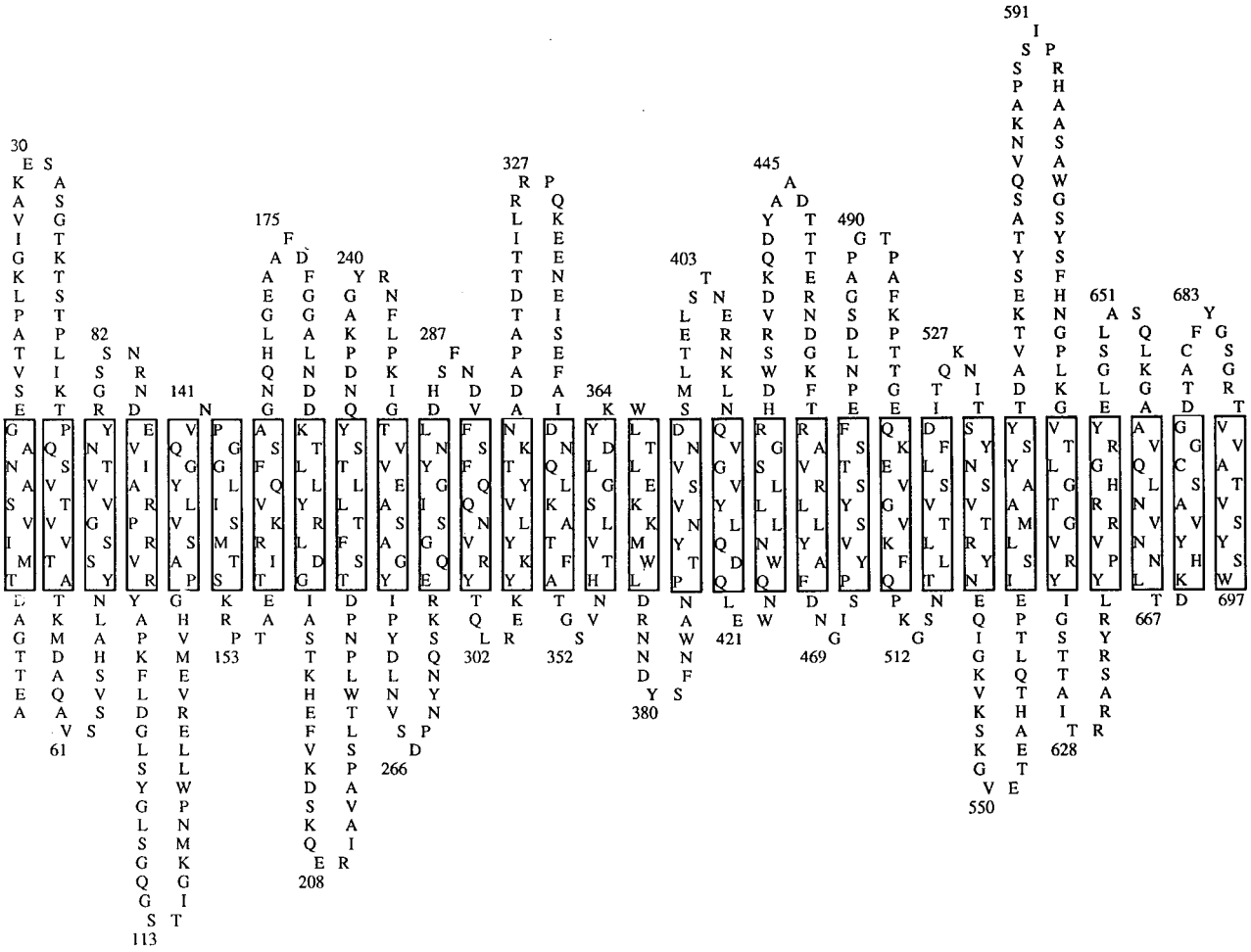

FIG. 4. Hypothetical transmembrane organization of Fct. Boxes contain membrane-spanning β-strands. PP, periplasm; OM, outer membrane.

served between Fct, FhuA, and FoxA. Several studies have shown that the last C-terminal residues confer stability on the protein and are required for an efficient protein export and assembly into the outer membrane (2, 37, 40, 41). However, the Fct C-terminal amino acid is a tryptophan, whereas a phenylalanine is present in most of the outer membrane proteins (40).

**Fct two-dimensional structure.** Structure predictions for ferrisiderophore receptors are based on models constructed for the porin family and the specific channel LamB (7, 18, 43, 45). These models were confirmed by determination of the three-dimensional structure through X-ray diffraction (46). Furthermore, experimental results were incorporated for the FhuA and FepA models (2, 5, 6, 20, 21, 27, 28, 35), while the FoxA model was based on computer prediction, multialignment, and comparison with FepA (4).

Thirty potential transmembrane sequences in Fct were identified according to their ability to form β-sheets by using the Chou-Fassman (8) and GOR (15) methods. Nineteen regions were predicted by both programs. The remaining membrane-spanning segments were identified by one or the other of these algorithms and were based on conserved sequences coming in the multialignment (Fig. 3), since transmembrane domains are usually well conserved (18, 43, 45). Twenty-two membrane-spanning segments were found to be aligned in Fct, FhuA, and FoxA (Fig. 4).

Many extracellular and periplasmic loops have been experimentally determined for FhuA (5, 19–21, 27). These data were incorporated into the Fct-FhuA alignment to predict loop localization for Fct. Six β-strands were perfectly aligned between the two proteins and shared many conserved residues from positions 415 to 523 for Fct (Fig. 3). We assumed that these six transmembrane β-strands have the same orientation in FhuA and Fct. Schiltz et al. (38) have shown that the C terminus of a *Rhodobacter* porin is on the periplasmic side of the outer membrane. Since the C-terminal region is well conserved among the porin and the siderophore receptor families (43), we considered the last Fct loop to be extracellular. Furthermore, we assumed an identical orientation for the first Fct β-strand corresponding to the conserved TonB box than for all receptor proteins. According to these predictions, we proposed a transmembrane organization for Fct (Fig. 4) in which membrane-spanning region length was arbitrarily set to 9 amino acids. Multialignment revealed two close regions in Fct which did not display any significant homology with the two other receptors (positions 307 to 337 and 363 to 410). The eighth external loop of FhuA, corresponding to the 307 to 337 region, is particularly exposed to the cell surface (21) and controls FhuA permeability (20). Indeed, excision of residues 322 to 355 converted FhuA into an open channel (19). The corresponding loops in Fct and FoxA have to be examined for their role in receptor activity and specificity.

**Nucleotide sequence accession number.** The sequence of the *E. crysanthemi fct* gene has been submitted to the EMBL GenBank (accession number, X87967).

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