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Novel Colicin F_Y of *Yersinia frederiksenii* Inhibits Pathogenic *Yersinia* Strains via YiuR-Mediated Reception, TonB Import, and Cell Membrane Pore Formation

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A novel colicin type, designated colicin F_Y, was found to be encoded and produced by the strain *Yersinia frederiksenii* Y27601. Colicin F_Y was active against both pathogenic and nonpathogenic strains of the genus *Yersinia*. Plasmid YF27601 (5,574 bp) of *Y. frederiksenii* Y27601 was completely sequenced. The colicin F_Y activity gene (*cfyA*) and the colicin F_Y immunity gene (*cfyI*) were identified. The deduced amino acid sequence of colicin F_Y was very similar in its C-terminal pore-forming domain to colicin Ib (69% identity in the last 178 amino acid residues), indicating pore forming as its lethal mode of action. Transposon mutagenesis of the colicin F_Y-susceptible strain *Yersinia kristensenii* Y276 revealed the *yiuR* gene (ykris001_4440), which encodes the YiuR outer membrane protein with unknown function, as the colicin F_Y receptor molecule. Introduction of the *yiuR* gene into the colicin F_Y-resistant strain *Y. kristensenii* Y104 restored its susceptibility to colicin F_Y. In contrast, the colicin F_Y-resistant strain *Escherichia coli* TOP10F′ acquired susceptibility to colicin F_Y only when both the *yiuR* and *tonB* genes from *Y. kristensenii* Y276 were introduced. Similarities between colicins F_Y and Ib, similarities between the Cir and YiuR receptors, and the detected partial cross-immunity of colicin F_Y and colicin Ib producers suggest a common evolutionary origin of the colicin F_Y-YiuR and colicin Ib-Cir systems.

Colicins are proteinaceous antimicrobial agents produced by *Escherichia coli* strains and other related species and genera of the family *Enterobacteriaceae*. All of them share a similar molecular structure comprising three domains for colicin translocation, receptor binding, and lethal effect (11). To date, more than 20 various colicin types have been described on the molecular level (11, 52, 57, 64, 68). Although most of the colicin types were identified among *E. coli* strains, bacteriocins with a molecular structure similar to that of colicins have been found among strains of the genera *Citrobacter*, *Escherichia*, *Klebsiella*, *Pseudomonas*, *Salmonella*, *Shigella*, *Yersinia*, and others (11, 32, 43, 61, 68).

Colicins are directed against closely related strains of the producer's species, and therefore, colicinogenic strains are believed to have a selective advantage compared to related noncolicinogenic strains. In general, colicins are directed against both commensal and pathogenic *E. coli* strains, except for colicin Js, which is active against enteroinvasive *E. coli*, just as *Shigella* strains (28, 65). Although the precise general role of colicins in the human gut is unknown, there is increasing evidence for bacteriocin-enhanced *E. coli* colonization of the gastrointestinal tract (21), for the role of colicins in bacterial virulence (e.g., colicin E1 [62]), and for colicin's role in the probiotic phenotype of *E. coli* strains (14, 63).

Three of 17 species of the genus *Yersinia* (*Enterobacteriaceae*) are known as important human pathogens (*Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*), while the other species comprise nonpathogenic strains or opportunistic pathogens (30, 42, 46, 47, 70, 71, 73). Production of bacteriocins has already been described in two pathogenic (*Y. pestis* and *Y. pseudotuberculosis*) and in two nonpathogenic (*Y. intermedia* and *Y. kristensenii*) *Yersinia* species (4, 5, 8, 60, 77). However, only pesticin I has been characterized on the molecular level as being active against strains of *Y. pestis*, *Y. pseudotuberculosis*, and *E. coli* C6 (Φ) (4, 8, 29, 54, 55, 80).

In this communication, we describe a novel colicin type (F_Y) isolated from a strain of *Yersinia frederiksenii*, its complete plasmid sequence (pYF27601), the corresponding receptor, translocation routes to susceptible strains, and activity spectra against strains of related species.

MATERIALS AND METHODS

Bacterial strains. Colicin F_Y producer *Y. frederiksenii* strain Y27601 was obtained from The National Reference Laboratory for *Salmonellae*, National Institute of Public Health, Prague, Czech Republic. The following *Yersinia* strains tested as possible indicators for this colicin were obtained from the same institution: *Y. frederiksenii* (strains Y62, Y71, Y81, Y172, Y284, Y296, Y26851, Y27334, Y27411, Y27477, Y27601, Y27627, and Y27829), *Y. intermedia* (strains Y67, Y223, Y308, Y418, Y498, Y546, Y22377, Y25448, and Y27471), *Y. kristensenii* (strains Y104, Y276, Y281, Y330, Y476, Y541, Y599, Y610, Y611, Y612, Y613, Y614, Y615, Y27637, and Y29196), *Y. aldovae* (strains Y551, Y552, Y20198, Y21698, Y22412, and Y25525), *Y. rohdei* (strains Y80, Y88, Y137, and Y559), *Y. ruckerii* (strains Y136, Y22505, Y28544, Y28545, Y28590, and Y28631), *Y. pseudotuberculosis* (strains 3Ye06, 4Ye06, 1Ye09, 3Ye09, Y140, Y241, Y384, Y16953, Y19236, Y20462, Y20723, Y22721, Y26579, Y28790, and Y207240), and *Y. enterocolitica* (strains 1Ye03, 5Ye03, 15Ye03, 1Ye06, 5Ye06, 7Ye06, 3Ye07, 6Ye07, 7Ye07, 1Ye08, 2Ye08, 3Ye08, 7Ye08, 8Ye08, 4Ye09, 7Ye09, and 1Ye10). Fourteen other tested strains of the species *Y. enterocolitica* (strains Y7587, Y7782, Y7886, Y8008, Y8472, Y8703, Y8773, Y8886, Y9081, Y9102, Y9464, Y9949, Y9953, and Y10141) were isolated from patients at the Faculty Hospital Brno-Bohunice in Brno, Czech Republic. The standard colicin indicator strains used were *E. coli* K-12 Row,

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TABLE 1 Strains, plasmids, and primers used in this study

Plasmid, strain, or primer	Description or relevant characteristic(s)	Source or reference
Plasmids		
pYF27601	Colicinogenic plasmid from *Y. frederiksenii* Y27601	This study
pNKBOR	Suicide vector	58
pCR 2.1-TOPO	Commercial cloning vector	Invitrogen
pBAD-A	Commercial expression vector	Invitrogen
pDS1006	pCR 2.1-TOPO carrying *cfyA* to *cfyI* from *Y. frederiksenii* Y27601	This study
pDS1068	pBAD-A carrying *cfyA* to *cfyI* from *Y. frederiksenii* Y27601	This study
pDS1088	pCR 2.1-TOPO carrying *yiuBCR* from *Y. kristensenii* Y276	This study
pDS1082	pCR 2.1-TOPO carrying *yiuR* from *Y. kristensenii* Y276	This study
pDS1091	pCR 2.1-TOPO carrying *yiuR* and *tonB* from *Y. kristensenii* Y276	This study
Strains		
E. coli DH5α *pir*	DH5α−derived strain expressing the R6K π protein	58
E. coli DH10B	Commercial cloning strain	25
E. coli TOP10F′	Commercial expression strain	Invitrogen
PK11.1 to PK11.12	12 strains of *E. coli* DH10B containing Tn*7* insertion in pYF27601	This study
Primers		
NKBORout3	5′-AACAAGCCAGGGATGTAACG-3′	This study
NKBORout4	5′-GCAGGGCTTTATTGATTCCA-3′	This study
colYF-XhoI-F	5′-AGGACTCGAGATGACAGATTATAAAGATGTTGATCCG-3′	This study
immYF	5′-AGGACTCGAGATGGATATTAGATACTATATAAAAAATATA-3′	This study
YE1459SD-F	5′-ACCGAAATAAATGAGCCTATCCACTGAAT-3′	This study
YE1461SD-F	5′-ACCGAAATAAATGGCTAAGGCCTTTAGG-3′	This study
YE1461-R	5′-TTAGAAATCGTAGCTGGCGCCCAC-3′	This study
infusionYE2222F	5′-CTGGCGGCCGCTCGAGATGCAGCTAAATAAATTTTTCTTGGGTCGACGGC-3′	This study
infusionYE2222R	5′-AATTGGGCCCTCTAGATTAGTCCATTTCCGTCGTGCCGCCAATT-3′	This study

C6 (Φ), B1, and P400 and *Shigella sonnei* strain 17 (62, 67). A list of the other strains, plasmids, and primers used in this work is shown in Table 1.

Culture media. TY medium consisting of 8 g/liter tryptone (Hi-Media, Mumbai, India), 5 g/liter yeast extract (Hi-Media), and 5 g/liter sodium chloride in water was used throughout the study. TY agar consisted of a base layer (1.5%, wt/vol) and a top layer (0.75%, wt/vol). For protease sensitivity testing, 0.005% (wt/vol) trypsin (Sigma, St. Louis, MO) was added to 1.5% agar. Chloramphenicol (0.025 g/liter; Sigma), kanamycin (0.050 g/liter; Sigma), or ampicillin (0.100 g/liter; Sigma) was added for selection or maintenance of plasmids. For induction of colicin synthesis, mitomycin C (0.0005 g/liter; Sigma) or L-(+)-arabinose (0.2 g/liter; Sigma) was added to the culture medium 4 h prior to bacterial harvesting.

Detection of colicin production. Detection of colicin production was performed as described previously (69). Briefly, the agar plates were inoculated by a stab of the producer being tested and incubated at 37°C for 48 h. The macrocolonies were killed using chloroform vapors (30-min exposure), and each plate was then overlaid with a thin layer of top agar containing 10^8 cells of an indicator strain. The plates were then incubated at 37°C overnight, and zones of growth inhibition were read.

Preparation of crude colicin extracts and colicin activity assays. Strains *Y. frederiksenii* Y27601 and PK11.3 (Table 1) were used for colicin F_Y production. A 20-fold-diluted overnight TY culture of a colicinogenic strain, induced by mitomycin C, was incubated for an additional 4 h and centrifuged for 15 min at 4,000 × *g*; the sediment was resuspended in 5 ml of distilled water, washed twice in distilled water, and sonicated. The resulting bacterial lysate was centrifuged for 15 min at 4,000 × *g*, and the supernatant was used as a crude colicin preparation. Antibacterial colicin activity was tested by spotting of 10-fold serial dilutions of crude colicins on agar plates with inoculated indicator strains. The indicator bacteria (10^8 cells) were added to the 3-ml top layer of TY agar and poured on a TY plate. The reciprocal of the highest dilution of the colicin-containing cell lysate or purified colicin solution causing growth inhibition of susceptible bacteria was considered the colicin titer (in arbitrary units). The data represent the average results of three independent experiments.

Isolation of plasmid YF27601, *in vitro* transposition, and DNA sequencing and annotation. Plasmid DNA was isolated using a QIAprep spin miniprep kit and Qiagen plasmid midikit (Qiagen, Hilden, Germany). The manufacturer's recommendations were followed. Plasmid mutagenesis was performed using an *in vitro* Tn*7* transposition system (GPS-1 genome priming system, New England BioLabs, Beverly, MA) according to the manufacturer's recommendations. The DNA in the vicinity of the inserted Tn*7* transposon was sequenced using primers Tn7RN and Tn7LS. Plasmid DNA of recombinant strains PK11.1 and PK11.2 was used to construct small insert libraries using the pUC18 vector. Ninety-six colonies were dideoxy terminator sequenced using pUC18 primers for each pPK11.1 and pPK11.2 template. DNA sequencing was performed using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Gene predictions and annotations were performed using Glimmer software (version 3.02; http://www.cbcb.umd.edu/software/glimmer/) (16) and GeneMark software (http://exon.biology.gatech.edu/) (41). The Lasergene program package (DNAStar, Madison, WI) was used for manipulation and assembly of the sequence data.

Chromosome mutagenesis and identification of receptor mutants. The chromosomal *in vivo* mutagenesis protocol (58) was used for transposon inactivation of the colicin F_Y receptor gene of susceptible *Y. kristensenii* Y276. Plasmid NKBOR was isolated from the *E. coli* DH5α *pir* strain and subsequently electroporated to *Y. kristensenii* Y276. Recombinant colonies were placed on 1.5% TY agar containing kanamycin (0.050 g/liter) and colicin F_Y (200 μl of crude sterile lysate, 100 arbitrary units). The resulting colonies were picked and cultivated in liquid TY medium overnight, and then their susceptibility to colicin F_Y was verified using the colicin activity assays described above. The standard cetyltrimethylammonium bromide method was used to isolate the chromosomal DNA from colicin F_Y-resistant mutants. Phenol-chloroform extraction was used to purify the chromosomal DNA (59). Chromosomal DNA (1 to 5 mg) was digested using 5 U of EcoRI (New England BioLabs) (or alternatively the SpeI or EciI enzyme) at 37°C for 3 h, and the restriction digestion

was stopped by heat inactivation. T4 ligase (New England BioLabs) was added to the digested DNA, and the mixture was incubated at 16°C for 16 h. A ligation mixture was used as a template for PCR amplification using a GeneAmp XL PCR kit (Roche Molecular Systems, Branchburg, NJ) with primers NKBORout3 and NKBORout4. The resulting PCR products were sequenced with these primers by the Sanger method.

Colicin F_Y purification and immunoblot analysis. The colicin F_Y gene (*cfyA*, colicin F_Y activity) was amplified together with the immunity gene (*cfyI*, colicin F_Y immunity) using the colYF-XhoI-F and immYF primers (Table 1). The PCR product (1.7 kb) was cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA), resulting in pDS1006. The *cfyA* and *cfyI* genes were then recloned into pBAD-A vector (Invitrogen), where *cfyA* was fused at its 5′ end to DNA encoding the His tag. The resulting pDS1068 was transformed into the expression strain *E. coli* TOP10F′. Colicin F_Y was prepurified from overnight culture (5 liters) of *E. coli* TOP10F′(pDS1068) using Ni-nitrilotriacetic acid agarose (Qiagen). Prepurified colicin F_Y was further separated from other proteins using the AKTA fast protein liquid chromatography system (GE Healthcare, Fairfield, CT) and MonoQ 5/50 GL columns (GE Healthcare). Purified colicin F_Y samples were mixed with 2× Laemmli sample buffer and boiled for 10 min. Samples were separated on SDS-PAGE gels (12%) and electrotransferred (100 V/1 h) onto Immobilon-P transfer membrane (Millipore, Billerica). The His-tagged colicin F_Y was detected using a Penta-His horseradish peroxidase conjugate kit (Qiagen) and a chemiluminescent reagent, Lumi-Light Western blotting substrate (Roche, Branford, CT). The His tag was removed from the colicin F_Y (500 ng) by treatment with 5 U of enterokinase (New England BioLabs) at 25°C for 90 min.

Cloning of colicin F_Y receptor and complementation of resistant bacteria. The *yiuR* gene encoding the receptor of colicin F_Y was amplified from the chromosomal DNA of susceptible strain *Y. kristensenii* Y276 with YE1461SD-F and YE1461-R primers and *Pfu* polymerase (Fermentas, Glen Burnie, MD). The resulting PCR product (1,974 bp) was cloned into the pCR 2.1-TOPO TA cloning vector, and the resulting plasmid (pDS1082) was verified by sequencing and transformed into resistant *Y. kristensenii* Y104, *E. coli* TOP10F′, and *Y. pseudotuberculosis* Y207240. The whole *yiu* locus (*yiuBCR* genes) was cloned similarly using primers YE1459SD-F and YE1461-R, resulting in pDS1088. The *tonB* gene from *Y. kristensenii* Y276 was cloned using an In-Fusion advantage PCR cloning kit (Clontech, Mountain View, CA) using primers infusionYE2222F and infusionYE2222R. The resulting plasmid (pDS1091) was verified by sequencing and transformed into resistant *E. coli* TOP10F′. The colicin F_Y activity assays were used to determine the susceptibility of the transformed bacteria to colicin F_Y.

Construction of phylogenetic trees. The software PAUP* 4b10 (82) and its free graphical user interface PaupUp (version 1.0.3.1. beta; http://www.agro-montpellier.fr/sppe/Recherche/JFM/PaupUp/) were used for construction of phylogenetic trees using the nucleotide sequences of the 3′ ends (~500 bp) of colicin activity genes. The DNA sequences used for tree constructions were aligned using ClustalX software, which is available on-line (version 2.0; http://www.clustal.org/) (40), and ModelTest (version 3.7; http://darwin.uvigo.es/software/modeltest.html) (53) was used to identify the best model of nucleotide substitutions. Phylogenetic trees were constructed by the maximum-likelihood method from the best model identified by ModelTest. TreeView software (version 1.6.6; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) (48) was used for graphical presentations of the corresponding trees.

Prediction of protein structures. The positions of external loops in the YiuR protein were predicted by the hidden Markov model method (http://biophysics.biol.uoa.gr/PRED-TMBB/) (2, 3).

Nucleotide sequence accession numbers. The nucleotide sequence of plasmid YF27601 was deposited in the GenBank database under accession number JF937655. The *yiuR* and *tonB* gene sequences of *Y. kristensenii* strain Y276 were deposited under accession numbers JF937653 and JF937654, respectively.

TABLE 2 Inhibitory spectrum of colicin F_Y in the genus *Yersinia*

Yersinia species	No. of colicin F_Y-susceptible strains/no. tested (%)
Y. frederiksenii	3/13 (23.08)
Y. intermedia	2/9 (22.22)
Y. kristensenii	5/15 (33.33)
Y. aldovae	4/6 (66.67)
Y. enterocolitica	30/31 (96.77)
Y. pseudotuberculosis	0/15 (0.00)
Y. rohdei	0/4 (0.00)
Y. ruckeri	0/6 (0.00)

RESULTS

Inhibitory spectrum of colicin F_Y. Colicin F_Y, produced by the strain *Y. frederiksenii* Y27601, inhibited the growth of strains belonging to five of the eight species of the genus *Yersinia* tested, including *Y. frederiksenii* (3 susceptible strains out of 13 tested), *Y. intermedia* (2 out of 9), *Y. kristensenii* (5 out of 15), *Y. aldovae* (4 out of 6), and *Y. enterocolitica* (30 out of 31). Four tested bacterial strains of *Y. rohdei*, six strains of *Y. ruckeri*, and 15 strains of *Y. pseudotuberculosis* were found not to be susceptible to colicin F_Y (Table 2). None of the standard colicin indicator strains K-12 Row, C6 (Φ), B1, P400, and *S. sonnei* 17 (62) were susceptible to this colicin.

Isolation and sequencing of plasmid YF27601. In order to identify the plasmid encoding colicin F_Y, the total plasmid DNA of the strain *Y. frederiksenii* Y27601 was subjected to *in vitro* transposon mutagenesis with Tn*7*. The recombinant DNA was used to transform *E. coli* DH10B. Selection for chloramphenicol resistance resulted in 12 recombinant colonies (named PK11.1 to PK11.12). The PK11.8 strain was excluded because of ambiguous sequencing results. Nine of the remaining 11 colonies were able to inhibit *Y. kristensenii* Y276 indicator bacteria. Plasmid DNA preparations of strains PK11.1 and PK11.2 were used to construct a small insert library. The resulting clones were used for sequencing of the plasmid DNA (96 clones of each plasmid). More than 16× average sequencing coverage was obtained for both pPK11.1 and pPK11.2. In addition, specific oligonucleotides were used to finish the complete plasmid sequence. The complete plasmid DNA (excluding the transposon DNA sequence) comprised 5,574 bp, and the plasmid was named pYF27601 (Fig. 1).

Sequence analysis of pYF27601. The complete sequence of pYF27601 was numbered from the unique BamHI restriction target site starting with its first recognized nucleotide (GGATCC). The pYF27601 sequence comprised 8 predicted open reading frames (ORFs) encoding polypeptides longer than 50 amino acid residues (Table 3). The average G+C content of this plasmid sequence was 50.0%; however, the colicin F_Y-encoding gene and colicin F_Y immunity gene showed lower G+C contents (42.1% and 30.7%, respectively). Three ORF types with predicted functions were found in pYF27601, including ORFs encoding plasmid mobilization (*mobA* and *mobC*) and colicin production (*cfyA* and *cfyI*) and ORFs homologous to ORFs present in insertion sequence elements (*isnA* and *isnB*). A 964-nucleotide (nt) sequence of pYF27601 (nt 5497 to 886) was found to be very similar (77% nucleotide sequence identity) to the origin of replication of plasmid AlvA of *H. alvei* (81). In this 964-nt region of pYF27601, the RNAI and RNAII promoter sequences (−35 and −10 regions)

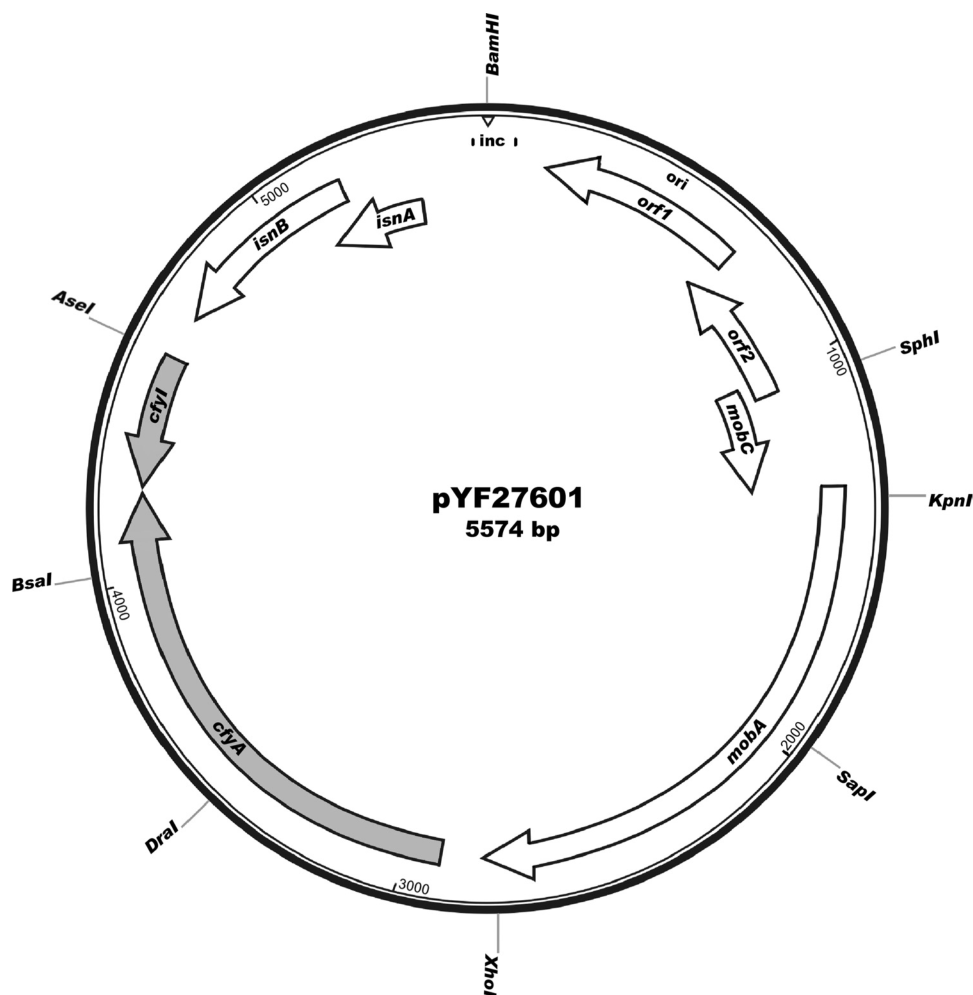

FIG 1 Map of plasmid YF27601 (5,574 bp). The localization and polarity of predicted genes, the positions of several restriction target sites, the RNAI- and RNAII-encoding *inc* (incompatibility) region, and the position of the putative origin of replication (*ori*) are indicated. The colicin F_Y activity and immunity genes are shown as gray arrows.

were identified based on similarity to corresponding sequences of pAlvA and pColE1.

Analysis of the colicin F_Y activity and immunity genes. The colicin F_Y-encoding gene was named *cfyA*, and the corresponding protein CfyA contained 438 amino acid residues with a calculated molecular mass of 49.6 kDa. Colicin F_Y synthesis in PK11.1 and PK11.2 was inactivated by Tn*7* insertion into the *cfyA* gene with insertion target sequences between coordinates 3047 to 3051 and 3613 to 3617, respectively. Upstream from *cfyA*, a ribosome binding site (AGGGA, coordinates on pYF27601, 2897 to 2901), puta-

TABLE 3 Identified ORFs and regulatory regions in pYF27601 and their characteristics

ORF (strand)	Positions	Similar protein/DNA sequence	No. of amino acids	Organism (reference or accession no.)	G+C content (%)	Identity (%)	No. of amino acids/nucleotides aligned
1 (−)	155–682	—[a]	175		58.5		
2 (−)	645–1058	—	137		55.0		
3 (+)	1005–1346	Mobilization protein MobC	113	*H. alvei* (81)	61.1	56	107
4 (+)	1336–2799	Mobilization nuclease MobA	487	*H. alvei* (81)	57.9	52	459
5 (+)	2908–4224	Colicin	438	*A. nasoniae* (83)	42.1	44	436
6 (−)	4238–4576	Immunity protein for colicin Ib	112	*S. sonnei* P9 (NP_052460)	30.7	37	111
7 (−)	4692–5195	IS*1* transposase B	167	*E. coli* K-12 (56)	54.6	100	167
8 (−)	5114–5389	IS*1* transposase A	91	*E. coli* H10407 (13)	53	99	91
(−)	79–108	RNAI promoter (−35 −10 region)		*H. alvei* (81)		93	28
(+)	5504–5532	RNAII promoter (−35 −10 region)		*H. alvei* (81)		62	18

[a] —, no similarity found.

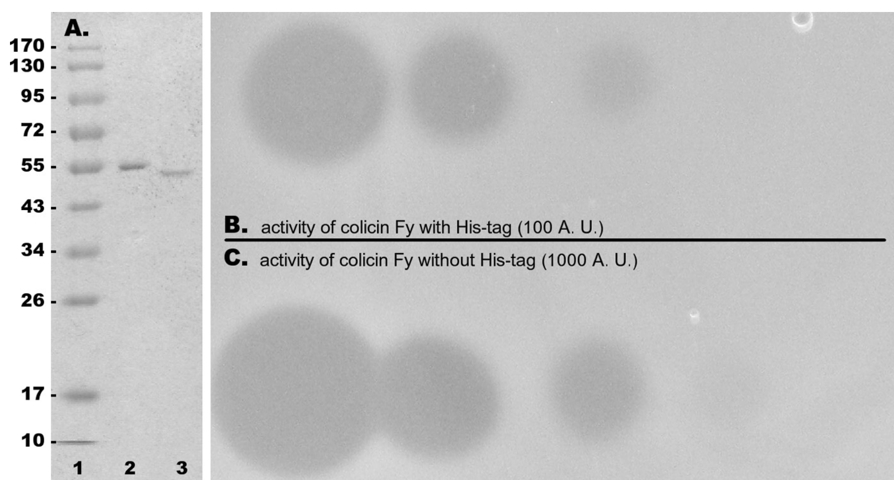

FIG 2 Purification of colicin F_Y and its biological activity. (A) Purification of colicin F_Y containing an N-terminal histidine tag by using Ni and ion-exchange columns. Lane 1, low-molecular-weight protein standard (PageRuler Prestained Protein Ladder, Fermentas); lane 2, purified colicin F_Y with an N-terminal histidine tag; lane 3, purified colicin F_Y with an enterokinase-cleaved N-terminal histidine tag. A 12% polyacrylamide gel was stained with Coomassie brilliant blue. The values on the left are molecular sizes in kilodaltons. (B) Antibacterial activity of purified, His-tagged colicin F_Y on *Y. kristensenii* Y276 indicator strain. (C) Antibacterial activity of enterokinase-treated, purified colicin F_Y. Note that the biological activity of enterokinase-treated, purified colicin F_Y (with the N-terminal His tag removed) was increased approximately by an order of magnitude to 10^3 arbitrary units per μl. Based on the estimated number of colicin F_Y molecules (10^{12} in this purified sample) and the number of lethal colicin units in one arbitrary unit for colicins E1 to E9 (arbitrary unit $= 2 \times 10^8$ lethal units [66]), one lethal unit (i.e., the lowest number of colicin molecules able to kill one susceptible bacterium) of colicin F_Y corresponds to approximately 5 molecules.

tive promoter −10 and −35 regions (TTGACA, 2817 to 2822, and TAGTAT, 2840 to 2845), and a single LexA binding site (CTGTA TGTATATACAG, 2853 to 2868) were found. The *cfyI* gene encoding the colicin F_Y immunity protein was oriented opposite to *cfyA* and a near-consensus promoter (−10 and −35 sequences) was found upstream from the *cfyI* gene (TTGACA, 4660 to 4665, and TAAAAA, 4636 to 4641). In addition, 13-bp inverted repeats were found in the 3′ region of the *cfyI* gene and in the intergenic region of *cfyA*-*cfyI* (4229 to 4241 and 4252 to 4264). These repeats represent potential transcription termination sites.

The deduced amino acid sequence of colicin F_Y revealed relatively low homology in the 260-amino-acid-long N-terminal and central sequence, including 31% amino acid identity with an uncharacterized colicin from *Arsenophonus nasoniae* (CBA74339) and 28% identity with an S-type pyocin domain-containing protein of *Serratia proteamaculans* 568 (YP_001476768), which suggested novel receptor specificity of colicin F_Y. At the N terminus of colicin F_Y, a near-consensus TonB box was found between amino acid residues 42 and 48 (DTMTVTG), indicating a possible interaction between colicin F_Y and the TonB protein. The last 178 amino acid residues, the C-terminal domain of colicin F_Y, showed 69% identity with the C-terminal domain of colicin Ib, encoded in the genome of *Yersinia ruckeri* ATCC 29473 (ZP_04617830), and 57% identity with the C-terminal domain of colicin Ib of *Escherichia fergusonii* EF6 (AF_453413.1) The pore-forming activity of colicin F_Y was verified by lipid bilayer experiments (R. Fišer, unpublished data). The immunity protein of colicin F_Y showed 39% identity in 105 aligned residues to the immunity protein from *A. nasoniae* (CBA74337) and 37% identity in 111 aligned residues to the immunity protein of colicin Ib from *S. sonnei* P9 (NP_052460).

Purification of colicin F_Y. The *E. coli* TOP10F′ strain, containing pDS1068 encoding colicin F_Y with an N-terminal His tag (HT-colicin F_Y), was used to produce larger amounts of HT-colicin F_Y for purification. HT-colicin F_Y was purified (0.1 mg/ml; i.e., approximately 10^{12} molecules per μl) with a corresponding activity of 100 arbitrary units per μl. Removal of the His tag from purified HT-colicin F_Y resulted in 10-fold increased activity of colicin F_Y compared to that of HT-colicin F_Y. This fact indicated the importance of an intact colicin N terminus in its bactericidal activity (Fig. 2).

Identification and characterization of colicin F_Y receptor. Transposon mutagenesis with suicide plasmid NKBOR (58) and selection for resistance to kanamycin and to colicin F_Y resulted in 66 colonies of *Y. kristensenii* Y276. Fifty colonies were further analyzed, and 42 of them showed complete resistance to colicin F_Y (to 100 arbitrary units of colicin F_Y), whereas 8 colonies showed decreases in susceptibility to colicin F_Y of 1 order of magnitude or less (results of transposon mutagenesis are shown in Fig. 3). Twenty-three (out of 50) colonies were sequenced, and 19 of them revealed an insertion in the *yiuR* gene (ykris001_4440; GenBank accession no. ACCA01000005.1). Four clones contained an insertion in one of the two genes upstream from *yiuR* (i.e., the *yiuB* and *yiuC* genes). All four insertions in the *yiuB* or *yiuC* gene showed decreases in susceptibility to colicin F_Y of 1 order of magnitude and no complete resistance, as detected in *yiuR* insertions.

To verify the function of the *yiuR* gene in colicin F_Y susceptibility, a colicin F_Y-resistant strain *Y. kristensenii* Y104 was transformed with pDS1082 harboring the *yiuR* gene. Susceptibility to colicin F_Y was fully restored in this *Y. kristensenii* Y104 *yiuR*$^+$ strain (Table 4). Susceptibility was also restored in *Y. pseudotuberculosis* strain 207240. In contrast, introduction of the *yiuR* gene into the *E. coli* TOP10F′ strain did not result in acquisition of susceptibility to colicin F_Y. However, introduction of the *yiuR* gene together with *tonB* from *Y. kristensenii* (harbored in pDS1091) into *E. coli* resulted in the recombinant strain becoming fully susceptible to colicin F_Y.

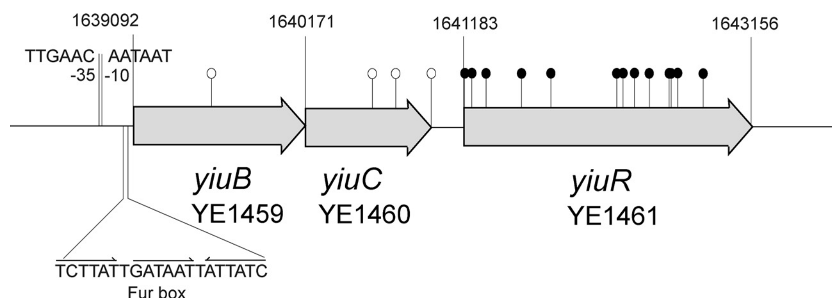

FIG 3 Schematic representation of the *yiuBCR* chromosomal genes of *Y. kristensenii* Y276. Black circles indicate Tn*7* insertions that result in complete resistance to colicin F_Y, while white circles represent a Tn*7* insertions that cause slight decreases in susceptibility (up to 1 order of magnitude) to colicin F_Y. Due to proximity at transposon insertions, six black circles are not shown. A putative Fur box and a putative promoter region (−10 and −35 sequences) upstream from *yiuB* are indicated. Coordinates taken from the genome sequence of *Y. enterocolitica* (75) for each *yiuBCR* gene are indicated. The partial decrease in susceptibility to colicin F_Y caused by insertions in *yiuB* or *yiuC* likely resulted from a decreased rate of *yiuR* transcription from the promoter region upstream of the *yiuB* gene.

DISCUSSION

Although the majority of colicin types have been identified in *E. coli* strains, a number of colicins have been identified for the first time in different enterobacterial strains, including *Citrobacter freundii* CA31 (colicin A; 45), *Serratia marcescens* JF246 (colicin L; 18), *S. sonnei* P9 (colicins E2, Ia, and Ib; 12, 38), *S. sonnei* 7 (colicin Js; 1), and *S. boydii* M592 (colicin U; 27). In addition, several bacteriocins were named differently although the principal features of these proteins are similar to those of colicins, including bacteriocin 28b of *S. marcescens* N28b (79), pesticin I of *Yersinia pestis* A1122 (4, 29), cloacin DF13 of *Enterobacter cloacae* DF13 (72), and S-type pyocins of *Pseudomonas aeruginosa* (reviewed in reference 43). All of the above-mentioned bacteriocins are protease-sensitive proteins with a modular structure containing receptor, translocation, and killing domains similar to those of colicin proteins. Because of the modular proteinaceous structure, the novel, protease-sensitive (data not shown) bacteriocin of *Y. frederiksenii* was named colicin F_Y. The letter F remained unused (the originally identified colicin F was reclassified as colicin E2 [19] in the list of colicin types, and the index $_Y$ stands for <u>*Y*</u>*ersinia*. Strains of *Y. frederiksenii* (both colicin F_Y susceptible and producer strains) were found among non-symptomatic fecal carriers, and these strains are generally considered nonpathogenic or moderately pathogenic (10, 73). Like other colicin producers, strains of *Y. frederiksenii* belong to bacteria living in animal and human guts, and the producer strains may have a selective ecological advantage in microbiocenoses typical for this environment (24, 34).

Colicin F_Y showed an inhibitory effect against strains of several *Yersinia* species (five out of eight tested). Interestingly, the highest number of susceptible strains was found in strains of *Y. enterocolitica*. These strains cause most of the human diarrheal yersinia infections (especially in children), representing 1 to 9% of all cases of diarrhea (44, 84). Colicin F_Y was also active against other non-pathogenic strains of "enterocolitica-like group" (73), including *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, and *Y. aldovae*. This is the first colicin characterized in detail that is active mainly against the pathogenic species *Y. enterocolitica* and related "enterocolitica-like" species. On the other hand, not a single strain of *Y. pseudotuberculosis* was found to be susceptible to colicin F_Y, despite the fact that both *Y. enterocolitica* and *Y. pseudotuberculosis* are primarily gut pathogens; however, the *yiuR* genes of the two species differ significantly, and our results revealed that transfer of *yiuR* from *Y. kristensenii* to strains of *Y. pseudotuberculosis* resulted in acquired susceptibility to colicin F_Y. Therefore, amino acid residues different in both YiuR proteins specify recognition of colicin F_Y by this receptor. Residues interacting with colicin F_Y are likely to be found on externally exposed loops 1 to 11 of the YiuR receptor (Table 5). For comparison, most of the sites of interaction between the receptor domain of colicin Ia and the Cir receptor (homologous to YiuR) were identified in Cir loops L7 and L8 (9). Interestingly, these loops in the YiuR receptor are the longest and most divergent between *Y. kristensenii* and *Y. pseudotuberculosis* (Table 5) and may therefore specify interaction with colicin F_Y.

As shown by multilocus sequencing, *Y. pestis* and *Y. pseudotuberculosis* are very closely related and represent two lineages of the same species (39). *Y. pestis* strains are known to possess a *yiuR* gene (35) that is very closely related to the *yiuR* gene of *Y. pseudotuberculosis*, and therefore, strains of *Y. pestis* are unlikely to be susceptible to colicin F_Y.

Colicin F_Y was found to be encoded by a low-molecular-weight

TABLE 4 Complementation of colicin F_Y-resistant strains and their susceptibility to colicin F_Y

Strain tested	Susceptibility to colicin F_Y[a]	Relevant genotype
Y. kristensenii Y104	R	Control
Y. kristensenii Y104(pDS1082)	S (2)	*yiuR* from *Y. kristensenii* Y276
Y. kristensenii Y104(pDS1088)	S (2)	*yiuBCR* from *Y. kristensenii* Y276
Y. pseudotuberculosis Y207240	R	Control
Y. pseudotuberculosis Y207240(pDS1082)	S (2)	*yiuR* from *Y. kristensenii* Y276
E. coli TOP10F′	R	Control
E. coli TOP10F′(pDS1082)	R	*yiuR* from *Y. kristensenii* Y276
E. coli TOP10F′(pDS1091)	S (3)	*yiuR* and *tonB* from *Y. kristensenii* Y276

[a] The values in parentheses indicate the reciprocal highest colicin dilutions active on bacteria (e.g., $2 = 10^2$). R, resistance; S, susceptibility.

TABLE 5 Differences in amino acid sequences of YiuR external loops

Loop (position)[a]	Sequence in:	
	Y. kristensenii Y276[c]	*Y. pseudotuberculosis* YPIII[d]
L1 (178–186)	QEDS**N**SGDI	****K****[b]
L2 (213–229	RSEDKIIDGYN**E**QR**L**RN	***********Q**M**
L3 (253–267)	QD**R**N**T**T**A**GR**SV**ALNG	**K*S*P**TL****
L4 (290–314)	GNSTSY**V**QRDETRNPSR**E**MKSVDNI	******I**********Q*******
L5 (337–359)	EELYD**E**GNQL**A**SA**K**DL**T**KLTR**G**S	*****K****P**S**K****W*
L6 (383–391)	DQD**E**NYGTH	***Q*****
L7 (417–450)	RSPDLRQATD**N**WGQ**IT**GG**K–**G**D**PA**I**I**V**GNS**S**LKPE	**********D***LS**GKG*L**L*L***N****
L8 (476–518)	TDFKDKITEVR**R**C**TD**TT**GKAS**GQC**MI**NG**NS**YKFISDR**T**NVDKA	***********N*DI**-NTT***VF**IN*******I*****
L9 (544–563)	TQSEQKSG**Q**F**S**G**K**PLNQMPK	********A*A*Q*******
L10 (587–614)	RGK**T**SEYLNRTS**I**G**TT**TPSYTFVDLGAN	***A********M*SR************
L11 (640–648)	NDKVLDGRR	*********

[a] YiuR is a predicted outer membrane β-barrel with an N-terminal plug and 11 large extracellular loops. External loops were predicted by the hidden Markov model method (http://biophysics.biol.uoa.gr/PRED-MBB/).
[b] Asterisks denote identical amino acid residues in both proteins.
[c] Colicin F_Y-sensitive strain.
[d] Colicin F_Y-resistant strain.

plasmid named YF27601 (5.6 kb). Colicin plasmids of this type encode both Tol-dependent colicins (e.g., colicins A, E1-E9, K, N, S4, U, and Y) and TonB-dependent colicins (e.g., colicins 5 and 10) and have the *kil* gene as part of the colicin-encoding plasmid region. As in pesticin I-encoding plasmids (51, 54), the F_Y-encoding region does not contain the gene for the lytic protein (*kil*). Except of colicin F_Y activity and immunity genes, genes for plasmid maintenance (plasmid mobilization) and the IS*1* sequence (encoding transposase) were found on pYF27601. Like other colicin plasmids, including ColE1, ColJs, ColK, Col-Let, ColE2, etc., the primary role of this plasmid is probably the synthesis of colicin F_Y itself. Based on sequence homology, pYF27601 replicates using the theta mechanism, which is similar to AlvA- and other ColE1-type plasmids (31, 49, 57, 74, 81). However, due to sequence diversity, only the promoter regions of RNAI and RNAII were predicted (Table 3). Although the precise transcription starts of RNAI and RNAII genes are not known, the complementary regions of RNAI and RNAII molecules specified plasmid incompatibility. The overlapping RNAI and RNAII sequences (incompatibility region) differ substantially (~70% identity) between pYF27601 and pColE1 (26, 76). This prediction was verified experimentally; pYF27601 was stably maintained in TOP10F′ bacteria with pCR 2.1-TOPO, a vector containing pColE1-derived replication (data not shown). Therefore, pYF27601 is likely to be compatible with ColE1-like plasmids.

Sequence analysis of the colicin F_Y-encoding region revealed a single LexA binding site (SOS box), suggesting a lower level of SOS induction of colicin F_Y synthesis. In addition to colicin F_Y, other colicin gene clusters (e.g., those encoding colicins Ia, Ib, cloacin DF13, and some klebicins) have been shown to possess a single LexA binding site. Colicin synthesis under a single LexA repressor is increased under noninducing conditions with a lower response on SOS induction (22). In fact, experimental induction of the SOS response did not lead to a detectable increase in colicin F_Y synthesis (data not shown). The *cfyI* gene encoding the colicin F_Y immunity protein was found to be oriented opposite to the *cfyA* gene, a situation common in all colicin types that does not require proportional synthesis of colicins and the corresponding immunity proteins, including pore-forming colicins, peptidoglycan-degrading pesticin I and colicin M (inhibitor of murein synthesis). In contrast to other colicin immunity genes of pore-forming colicins, the nearly consensus promoter (−10 and −35 sequences) upstream from *cfyI* suggests relatively strong transcription of the *cfyI* gene.

Colicin F_Y and its immunity protein showed the highest similarity to colicin and immunity protein from *A. nasoniae* and to colicin Ib and colicin Ib immunity protein Ib, respectively. *A. nasoniae* is an entomopathogenic bacterium (*Enterobacteriaceae*) related to bacteria of the genera *Photorhabdus*, *Proteus*, *Serratia*, and *Yersinia* (15). However, the colicin-encoding region in the genome of *A. nasoniae* was predicted without further characterization (83). The most closely related characterized colicin type was pore-forming colicin Ib (Fig. 4), although only a cytotoxic domain had a similar sequence. In fact, *cfyI*-positive *E. coli* strains were partially immune to colicin Ib (data not shown), suggesting common ancestry for colicin F_Y- and Ib-encoding regions.

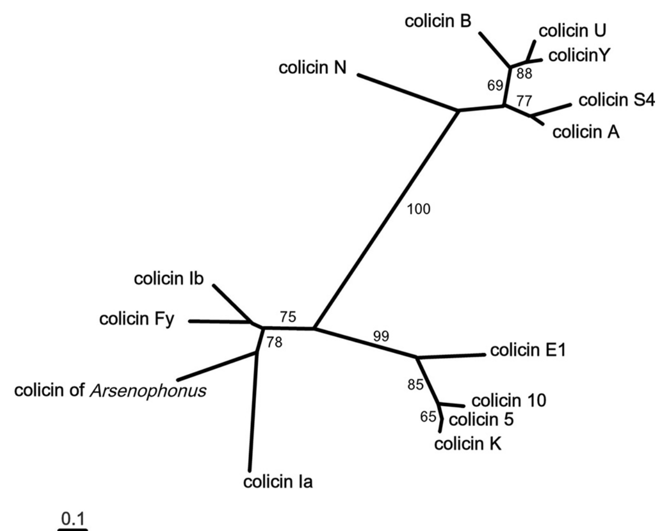

FIG 4 Unrooted tree of colicin pore-forming domains. The tree was constructed from sequence data of colicin genes at the 3′ end. The bar scale represents 0.1 nucleotide substitution per site. Bootstrap values based on 1,000 replications are shown next to branches.

Since none of the standard colicin *E. coli* indicators were susceptible to colicin F_Y, the *Yersinia*-specific receptor and/or translocation system required by colicin F_Y was expected. Transposon mutagenesis revealed *yiuR* as the gene encoding the putative colicin F_Y receptor. Although YiuABC proteins were shown to be involved in the iron acquisition system in *Y. pestis*, YiuR was not required for iron uptake (35); as a result, the function of the predicted outer membrane receptor YiuR protein remains unknown. YiuR displayed 37% identity with the Cir protein of *E. coli* involved in iron acquisition and in colicin and microcin uptake (9, 11, 33, 37, 50). Interestingly, introduction of the *yiuR* gene into *E. coli* strain TOP10F′ did not restore susceptibility to colicin F_Y. However, introduction of both the *yiuR* and *tonB* genes from *Y. kristensenii* restored the susceptibility of *E. coli* strain TOP10F′ to colicin F_Y. The fact that the *tonB* gene of *Y. kristensenii* Y276 was not found by screening of colicin F_Y-unsusceptible insertion mutants probably reflects a decreased viability of *Y. kristensenii tonB* mutants similar to that of *Salmonella enterica* serovar Typhi (23) and *Escherichia coli* strain C6 (Φ) *tonB* mutants (17). The *tonB* gene of *Y. kristensenii* ($tonB_{YK}$) and the *tonB* gene from *E. coli* DH10B are identical at only 46% of their amino acid residues. Lack of cross-complementation in *E. coli* was also described for TonB of *Y. enterocolitica* ($TonB_{YE}$) and TonB of *S. marcescens* ($TonB_{SM}$) (20, 36). The $TonB_{YE}$ protein failed to interact with colicins (completely with D, Ia, and Ib and partially with B and M) and also with *E. coli* receptors (36). The fact that the TonB protein of *E. coli* was not able to mediate the translocation of colicin F_Y through the bacterial envelope of an *E. coli* $yiuR^+$ strain could result from the inefficient energizing of the YiuR protein by TonB of *E. coli* ($TonB_{EC}$) and/or from inefficient interaction between colicin F_Y and $TonB_{EC}$. The lowest sequence identity was found in the middle part of the TonB proteins of *E. coli* and *Y. kristensenii*, which was previously described as an important region for interaction with TonB boxes of colicins or receptors (7, 20). Although deletion of Q160 from the TonB protein resulted in colicin-specific decreased susceptibility (78), deletion of seven amino acids (157S to Y163) from TonB caused complete resistance to all of the colicins tested (78). The authors hypothesized that the Q160 region may be a part of a larger region that is required for contact with the outer membrane receptor. $TonB_{EC}$ and $TonB_{YK}$ differ at position 160 (Q and K, respectively) and only 3 out of 7 amino acids residues were identical in the 157-to-163 region.

The TonB box sequence of YiuR was identical in all four species *Y. enterocolitica*, *Y. kristensenii*, *Y. pestis*, and *Y. pseudotuberculosis* (DTMVVTA). A very similar TonB box was found in the N terminus of colicin F_Y (DTMTVTG). In contrast, the TonB box sequences of the Cir protein and colicin Ia/Ib are ETMVVTA and EIMAVDI, respectively. Since it is known that TonB box substitutions leading to inactive mutants can be suppressed by mutations in TonB around position 160 (6), it is likely that TonB boxes evolved together with TonB proteins. In fact, evolution of colicin TonB boxes together with TonB could result in novel colicin types with different spectra of susceptible bacterial strains. The observed similarity between the Cir and YiuR proteins, together with the partial cross-immunity of colicin F_Y and colicin Ib producers, suggests a common evolutionary origin of both the colicin F_Y-YiuR and colicin Ib-Cir systems.

The above-described colicin F_Y is a novel colicin type similar to previously described colicins with susceptible strains in the genus *Yersinia* and is especially active against strains of *Y. enterocolitica*. The susceptible yersiniae are killed via YiuR-mediated reception, TonB-mediated translocation through the cell envelope, and the pore-forming lethal effect on the cell membrane.

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