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YopB and YopD Constitute a Novel Class of *Yersinia* Yop Proteins

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Virulent *Yersinia* species harbor a common plasmid that encodes essential virulence determinants (*Yersinia* outer proteins [Yops]), which are regulated by the extracellular stimuli Ca^{2+} and temperature. The V-antigen-encoding operon has been shown to be involved in the Ca^{2+}-regulated negative pathway. The genetic organization of the V-antigen operon and the sequence of the *lcrGVH* genes were recently presented. The V-antigen operon was shown to be a polycistronic operon having the gene order *lcrGVH-yopBD* (T. Bergman, S. Håkansson, Å. Forsberg, L. Norlander, A. Macellaro, A. Bäckman, I. Bölin, and H. Wolf-Watz, J. Bacteriol. 173:1607–1616, 1991; S. B. Price, K. Y. Leung, S. S. Barve, and S. C. Straley, J. Bacteriol. 171:5646–5653, 1989). We present here the sequence of the distal part of the V-antigen operons of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. The sequence information encompasses the *yopB* and *yopD* genes and a downstream region in both species. We conclude that the V-antigen operon ends with the *yopD* gene. This conclusion is strengthened by the observation of an insertion-like element downstream of the *yopD* gene. The translational start codons of YopB and YopD have been identified by N-terminal amino acid sequencing. By computer analysis, the *yopB* and *yopD* gene products were found to be possible transmembrane proteins, and YopD was shown to contain an amphipathic α-helix in its carboxy terminus. These findings contrast with the general globular pattern observed for other Yops. Homology between *Yersinia* LcrH and *Shigella flexneri* IppI and between *Yersinia* YopB and *S. flexneri* IpaB was found, suggesting conservation of this locus between these two genera. YopB was also found to have a moderate level of homology, especially within the hydrophobic regions, to members of the RTX protein family of alpha-hemolysins and leukotoxins, indicating that YopB might exhibit a similar function.

The genus *Yersinia* consists of 10 species, of which 3 (*Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) are virulent. Common to all three virulent species is a plasmid of approximately 70 kb that is essential for virulence (23–25, 46, 69). This virulence plasmid encodes a number of secreted proteins called *Yersinia* outer proteins (Yops) (7, 14, 61). The *yop* genes are scattered around the virulence plasmid and have been shown to be contained in different operons (8, 15, 21, 34, 40, 42, 61). A number of these Yops have been shown to be virulence determinants, since specific *yop* mutants generally are avirulent (8, 21, 42, 61). The YopH and YopE proteins are involved in resistance of yersiniae to phagocytosis by macrophages. Therefore, these two proteins are implicated in the obstruction of the primary host defense (51, 52). The YopE protein has been shown to be a cytotoxic effector and to be involved in resistance to phagocytosis (52). YopH was found to exhibit a protein tyrosine phosphatase activity, suggesting that YopH interferes with regulatory or signal-transducing pathways of target cells (5, 26). The YopM protein exhibits homology to the α-chain of human platelet glycoprotein Ib, GPIbα, and was shown to inhibit human platelet aggregation (34, 35).

The virulence plasmid has also been shown to be associated with the unique low-Ca^{2+} response of pathogenic yersiniae (8, 14, 15, 20–22, 25, 30, 45, 46, 61). This low-Ca^{2+} response is manifested by the requirement of at least 2.5 mM Ca^{2+} for prolonged growth at 37°C. Upon incubation at 37°C in Ca^{2+}-depleted conditions, the cells are restricted for growth after approximately two generations (68). Concomitantly with the low-calcium response, the virulence plasmid-encoded Yop proteins are expressed and secreted in large amounts into the culture supernatant. If the pathogen is grown at 26°C or in the presence of 2.5 mM Ca^{2+}, synthesis of Yop proteins is repressed.

The expression of the *yop* genes is under the control of the extracellular stimuli temperature and Ca^{2+} (8, 13, 14, 20, 21, 61). This regulation of expression is controlled by two different regulatory loops, one positive and one negative (3, 8, 13, 21, 48). The positive loop involves one activator, VirF (LcrF), which exhibits sequence homology to AraC of *Escherichia coli* (13, 14) and interacts with the *yop* promoters (32). When the cells are shifted from 26 to 37°C, the level of VirF increases, even in the presence of 2.5 mM Ca^{2+}, suggesting that the positive loop involves only temperature as a stimulus (13). The Ca^{2+} concentration of the growth medium regulates the level of Yop expression at 37°C. This effect is likely mediated through the expression of a transcriptional repressor that interacts with the *yop* promoters (3, 8, 21, 51). At a Ca^{2+} concentration of at least 2.5 mM, the putative repressor concentration is high, resulting in repression of the *yop* genes. Growth in a calcium-depleted medium results in a low concentration of an active transcriptional repressor and, accordingly, derepression of the *yop* genes. The complex negative regulatory loop involves at least five different loci (20). One of these loci is the V-antigen-containing operon (3, 42, 61). Mutations in this operon give the bacteria a temperature-sensitive phenotype (i.e., the

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mutant shows a reduced plating frequency at 37°C irrespective of Ca^{2+} concentration), indicating the importance of this operon in the negative control of Yop expression (3, 14, 21, 42, 49). This operon has previously been shown to contain at least five genes: *lcrGVH-yopBD* (3, 42, 48). A regulatory role for both the LcrV and LcrH proteins has been demonstrated (3, 47, 49). The LcrV protein is suggested to be involved in secretory mechanisms, while the LcrH protein is proposed to be a negative control element of yersiniae (3, 48, 49).

Polar mutations of the *yopB* and *yopD* genes gave rise to temperature-sensitive mutants (3, 20). This was probably due to a destabilization of the transcript which negatively influences the expression of LcrH (3). Thus, in order to study the function of YopB and YopD, it was necessary to obtain the sequence information of this distal part of the V-antigen operon so that specific mutants could be constructed.

So far, little is known about YopB and YopD. Rosqvist has shown by immunostaining that the *yopB* gene product binds to the same cytoskeleton structure in the target cell as the cytotoxin YopE. This finding could indicate a role in virulence for the YopB protein (50). YopD, on the other hand, has been implicated in the cytotoxic response or in the process of translocation of the YopE cytotoxin across the target cell membrane, as deduced by the use of microinjection techniques (53).

In this study, we demonstrated by sequence analysis that the V-antigen-containing operon ends with the *yopD* gene and that the YopB and YopD proteins are of the same size and are highly conserved in *Y. pseudotuberculosis* and *Y. enterocolitica*. We showed that YopB and YopD are possible transmembrane proteins and that YopD contains an amphipathic domain in its carboxy terminus. We also showed that LcrH and YopB exhibit homology with IppI and IpaB of shigellae, respectively (2, 56, 65, 67), and that YopB, in addition, exhibits moderate homology with the RTX protein family of alpha-hemolysins and leukotoxins (19). We analyzed the sequences of the *yopB* and *yopD* genes of *Y. enterocolitica* to investigate whether the genetic organization in this species is similar to that in *Y. pseudotuberculosis*. We also wanted to examine whether the sequence information obtained can explain the apparent size difference observed, as deduced by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), between the YopB and YopD proteins of *Y. pseudotuberculosis* and those of *Y. enterocolitica* (46).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Y. pseudotuberculosis* YPIII(pIB1) and YPIII(pIB15) (3, 6, 20, 21) and *Y. enterocolitica* W22703(pYVe227) (12, 33) were used. The *E. coli* strains used were DH5α (28) and LK111 (40). The liquid growth medium for *Yersinia* strains consisted of brain heart infusion broth (Difco) supplemented with either 5 mM EGTA [ethylene glyco-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] and 20 mM $MgCl_2$ or 2.5 mM $CaCl_2$. The solid medium was a blood agar base containing 20 mM Na oxalate, 20 mM $MgCl_2$, and 0.2% glucose (Ca^{2+} free) or blood agar base supplemented with only 2.5 mM Ca^{2+}. *E. coli* strains were grown in Luria broth or on Luria agar.

DNA methods. Preparation of plasmid DNA, restriction enzyme digests, ligations, and transformations of *E. coli* were performed essentially as described by Maniatis et al. (38).

DNA sequencing. DNA fragments containing the *yopB* and *yopD* genes and downstream sequences of *Y. pseudotuberculosis* were subcloned into the pBluescript vector (Stratagene, La Jolla, Calif.). Polymerase chain reaction (PCR) fragments derived from the *Y. enterocolitica* virulence plasmid pYVe227, which contains the *yopB* and *yopD* genes and a downstream region, were cloned into either the pBC18 vector (10) or the pBluescript vector. The subclonings of the PCR fragments from *Y. enterocolitica* were performed at two separate times. The DNA sequences of these parallel subclones of *Y. enterocolitica* and the subclones of *Y. pseudotuberculosis* were determined by the dideoxy-chain termination method of Sanger et al. (54) with [α-^{35}S]dATP (Amersham Corp., Little Chalfont, United Kingdom) and modified T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, Ohio).

DNA and protein sequences were analyzed by the GCG (Genetics Computer Group sequence analysis software package [University of Wisconsin, Madison]) and PC Gene (IntelliGenetics, Inc., Mountain View, Calif.) computer programs. The FASTA and TFASTA homology searches of GCG were performed with the algorithms of Lipman and Pearson (36) and Pearson and Lipman (44).

The mean α-helical hydrophobic moment and the mean hydrophobicity were calculated by the procedure of Eisenberg et al. (16, 17). A segment of 11 amino acids was moved through the protein sequence, and the mean hydrophobicity moments for each segment were calculated. The mean hydrophobicity was plotted against the mean α-helical hydrophobic moment for all possible segments. These two parameters were also plotted as a function of the midpoint of the amino acid segment along the sequence. In most calculations, we used the normalized consensus hydrophobic scale of Eisenberg et al., which is especially suitable for membrane-related proteins (16, 17).

Analysis of Yop expression. *Yersinia* strains were grown at 26°C to an optical density of 0.1 at 550 nm. The cultures were then shifted to 37°C and grown for an additional 3 h before being harvested. The secreted proteins were precipitated with 10% trichloroacetic acid and subjected to SDS-PAGE on 12% acrylamide gels as described earlier (20, 31). Proteins were visualized by Coomassie blue staining.

Two-dimensional gel analysis. Two-dimensional protein gel electrophoresis was performed according to the protocol of VanBogelen and Neidhardt (63).

Northern (RNA) blot analysis. Northern blot analysis was performed as described earlier (3).

Nucleotide sequence accession numbers. The GenBank-EMBL accession numbers of the *yopBD* sequences in Fig. 3 are L06215 (pIB1 of *Y. pseudotuberculosis* YPIII) and L06216 (pYVe227 of *Y. enterocolitica* W22703).

RESULTS

DNA sequence analysis of *yopB* and *yopD* of *Y. pseudotuberculosis* and *Y. enterocolitica*. The partial sequence of the *lcrGVH-yopBD* operon, ending with the *lcrH* gene (Fig. 1), has previously been presented (3, 48). Therefore, we sequenced the *yopB* and *yopD* genes of *Y. pseudotuberculosis* YPIII(pIB1) and a downstream region to obtain the genetic structure of the entire operon. Similarly, the V-antigen operon of *Y. enterocolitica* has the same genetic organization as that of *Y. pseudotuberculosis*, but the sequence of the *Y. enterocolitica* V-antigen operon has not been determined (42). Because of apparent size differences between the *Y. pseudotuberculosis* and *Y. enterocolitica* YopB proteins as well as their YopD proteins, we also sequenced the corre-

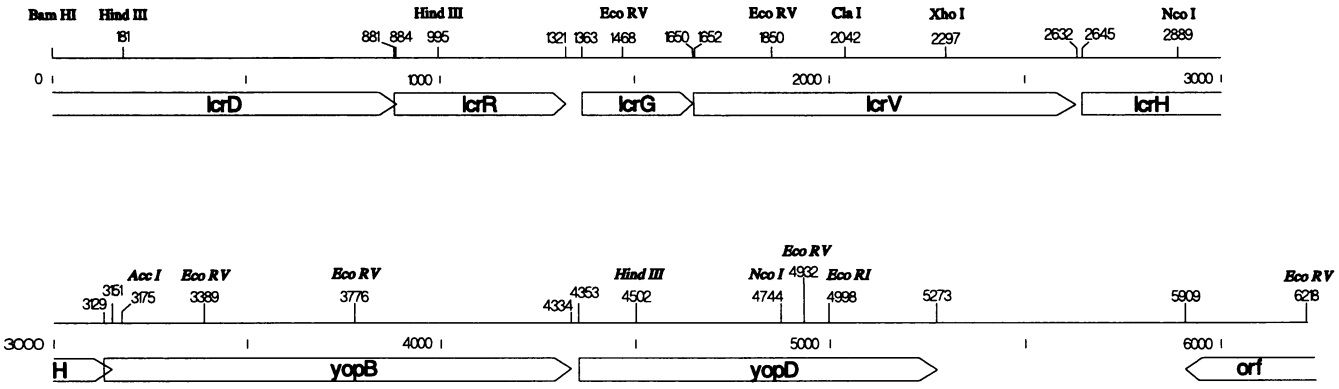

FIG. 1. Organization of the *lcrGVH-yopBD* operon of pIB1 of *Y. pseudotuberculosis* YPIII. The nucleotides are labeled starting with +1 at the BamHI restriction site of *Bam*HI fragment 1. The open reading frames *lcrD* and *lcrR* indicate the two genes of the upstream *lcrD* operon. orf, open reading frame.

sponding genes of *Y. enterocolitica* W22703(pYVe227). Two open reading frames corresponding to YopB (401 amino acids) and YopD (306 amino acids) were identified in both species (Fig. 2). Comparison of the proteins from the two species showed that the YopB proteins were 95.8% identical, with 17 amino acid differences (Fig. 2A), and that the YopD proteins were 98.0% identical, with only 6 amino acid differences (Fig. 2B).

For *Y. pseudotuberculosis*, the N-terminal amino acid sequence of YopB was previously determined, showing that the translational start of YopB overlaps the reading frame of *lcrH* by 23 nucleotides (3). The same putative translational start could be observed in the sequence of *Y. enterocolitica* (Fig. 3). No promoter motif could be identified in front of *yopB* or *yopD* in either species. This finding confirms the previous observation that when different polar mutations

```
YopB

pIB1      MSALITHDRSTPVTGSLLPYVETPAPAPLQTQQVAGELKDKNGGVSSQGVQLPAPLAVVA   60
pYVe227   -----------------V--I-------------------------------------

pIB1      SQVTEGQQQEVTKLLESVTRGAAGSQLISNYVSVLTKFTLASPDTFEIELGKLVSNLEEV   120
pYVe227   ----------I----------T--------------N----------------------

pIB1      RKDIKIADIQRLHEQNMKKIEENQEKIKETEENAKQVKKSGIASKIFGWLSAIASVIVGA   180
pYVe227   ----------------------------------------M--------I-----VI--

pIB1      IMVASGVGAVAGAMMVASGVIGMANMAVKQAAEDGLISQEAMKILGPILTAIEVALTVVS   240
pYVe227   ---------------I-------------------------QV----------------

pIB1      TVMTFGGSALKCLANIGAKLGANTASLAAKGAEFSAKVAQISTGISNTVGSAVTKLGGSF   300
pYVe227   --------------D-----------------------------------------------

pIB1      AGLTMSHAIRTGSQATQVAVGVGSGITQTINNKKQADLQHNNADLALNKADMAALQSIID   360
pYVe227   GS-----V-------------------------------------------------------

pIB1      RLKEELSHLSESHQQVMELIFQMINAKGDMLHNLAGRPHTV  401
pYVe227   ------------R--------------------------

YopD

pIB1      MTINIKTDSPIITTGSQLDAITTETVKQSGEIKKTEDTRHEAQAIKSSEASLSRSQVPEL   60
pYVe227   -------------------------G----V-----------------------------

pIB1      IKPSQGINVALLSKSQGDLNGTLSILLLLLELARKAREMGLQQRDIENKATITAQKEQVA   120
pYVe227   -------------------------------------------------S-------

pIB1      EMVSGAKLMIAMAVVSGIMAATSTVASAFSIAKEVKIVKQEQILNSNIAGREQLIDTKMQ   180
pYVe227   -----------------------------------------------D--------

pIB1      QMGNIGDKAVSREDIGRIWKPEQVADQNKLALLDKEFRMTDSKANAFNAATQPLGQMANS   240
pYVe227   --S-A-------------------------------------------------------

pIB1      AIQVHQGYSQAEVKEKEVNASIAANEKQKAEEAMNYNDNFMKDVLRLIEQYVSSHTHAMK   300
pYVe227   -------------------------------------------------------------

pIB1      AAFGVV  306
pYVe227   ------
```

FIG. 2. Deduced amino acid sequences of the YopB and YopD proteins of *Y. pseudotuberculosis*. The sequences are compared with the corresponding sequences of *Y. enterocolitica*. Dashes denote sequence identity. The *Y. enterocolitica* amino acids are indicated where the sequences differ.

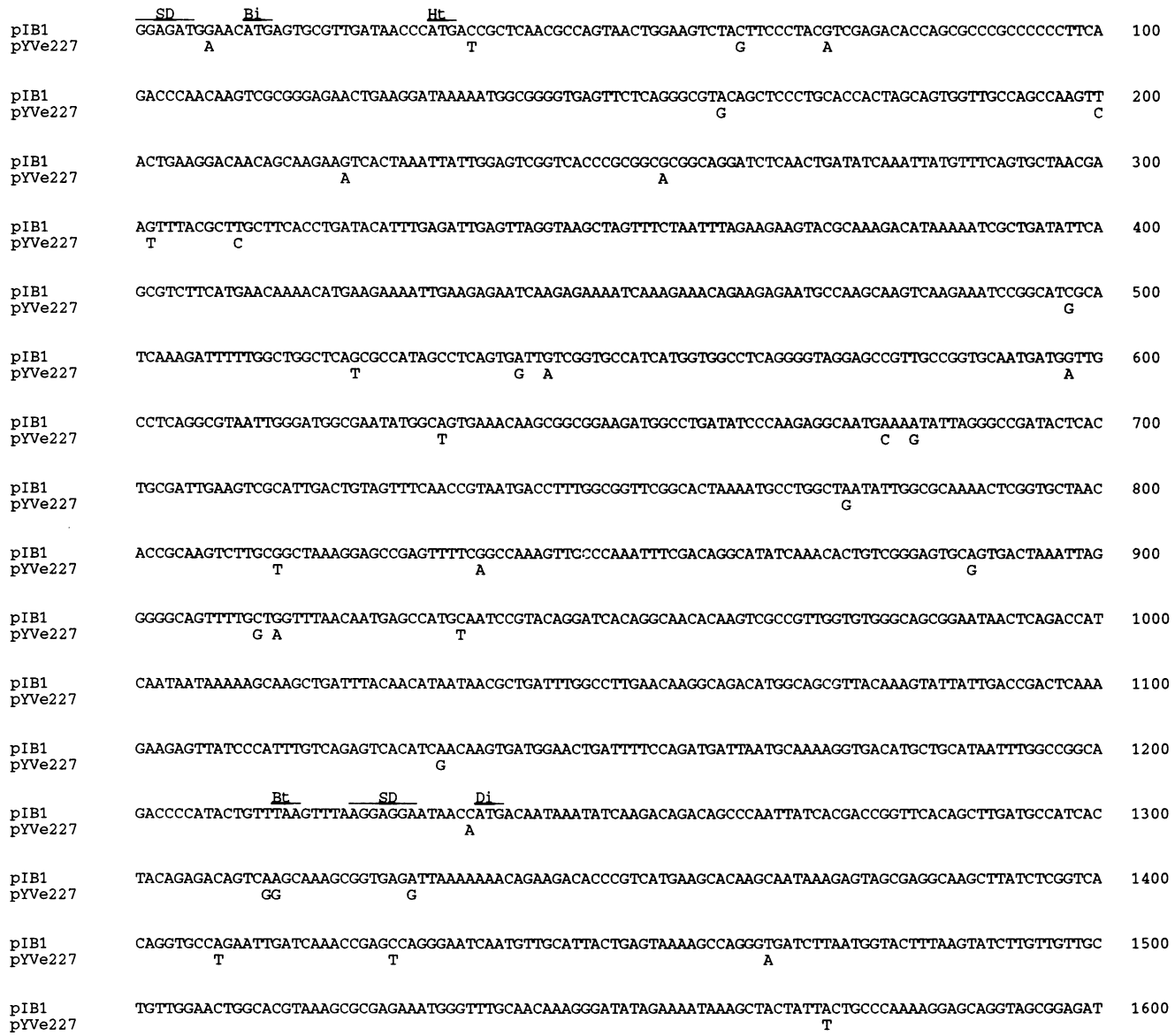

FIG. 3. DNA sequence of the *yopBD* regions of pIB1 of *Y. pseudotuberculosis* YPIII and pYVe227 of *Y. enterocolitica* W22703. Where the sequences differ, the pYVe227 nucleotides are indicated. SD denotes the consensus Shine-Dalgarno sequences found in front of *yopB* and *yopD*. Bi and Di denote the translational start codons for *yopB* and *yopD*, respectively. Ht, Bt, and Dt indicate the translational termination codons for *lcrH*, *yopB*, and *yopD*, respectively. The horizontal arrows indicate the palindrome of the putative transcription termination signal. The downward-pointing arrowhead indicates where the sequences of *Y. pseudotuberculosis* and *Y. enterocolitica* start to differ. The asterisk indicates the end of the open reading frame found in the opposite transcriptional direction.

were analyzed by Northern blotting, no internal promoter activity within the V-antigen-containing operon could be detected (3). Identical results were also obtained with *Y. enterocolitica* by using operon fusions (42).

A possible consensus ρ-independent termination signal (1) was found 13 bp downstream of the *yopD* stop codon in both species (Fig. 3). The termination signal consists of a 7-nucleotide imperfect palindrome with a 6-nucleotide spacing and a poly(U) tail. To determine whether the putative termination signal was functional, Northern blot analysis of the wild type, YPIII(pIB102), was performed with two DNA probes located on each side of the possible termination structure. The upstream probe (positions 1954 to 2174) (Fig. 3) yielded results identical to those yielded by a previously used *yopD*-specific DNA probe (3). However, the downstream probe (positions 2222 to 2416) (Fig. 3) gave a very weak signal, demonstrating that the ρ-independent termination signal is functional (data not shown).

Shine-Dalgarno consensus sequences (58) were found in front of both *yopB* and *yopD* (Fig. 3). YopD of *Y. pseudotuberculosis*, purified from the culture supernatant, revealed the same N-terminal amino acid sequence as the deduced amino acid sequence. Thus, like other Yops, YopB and YopD are not subject to N-terminal signal cleavage upon secretion into the culture supernatant.

No additional open reading frames downstream of *yopD*

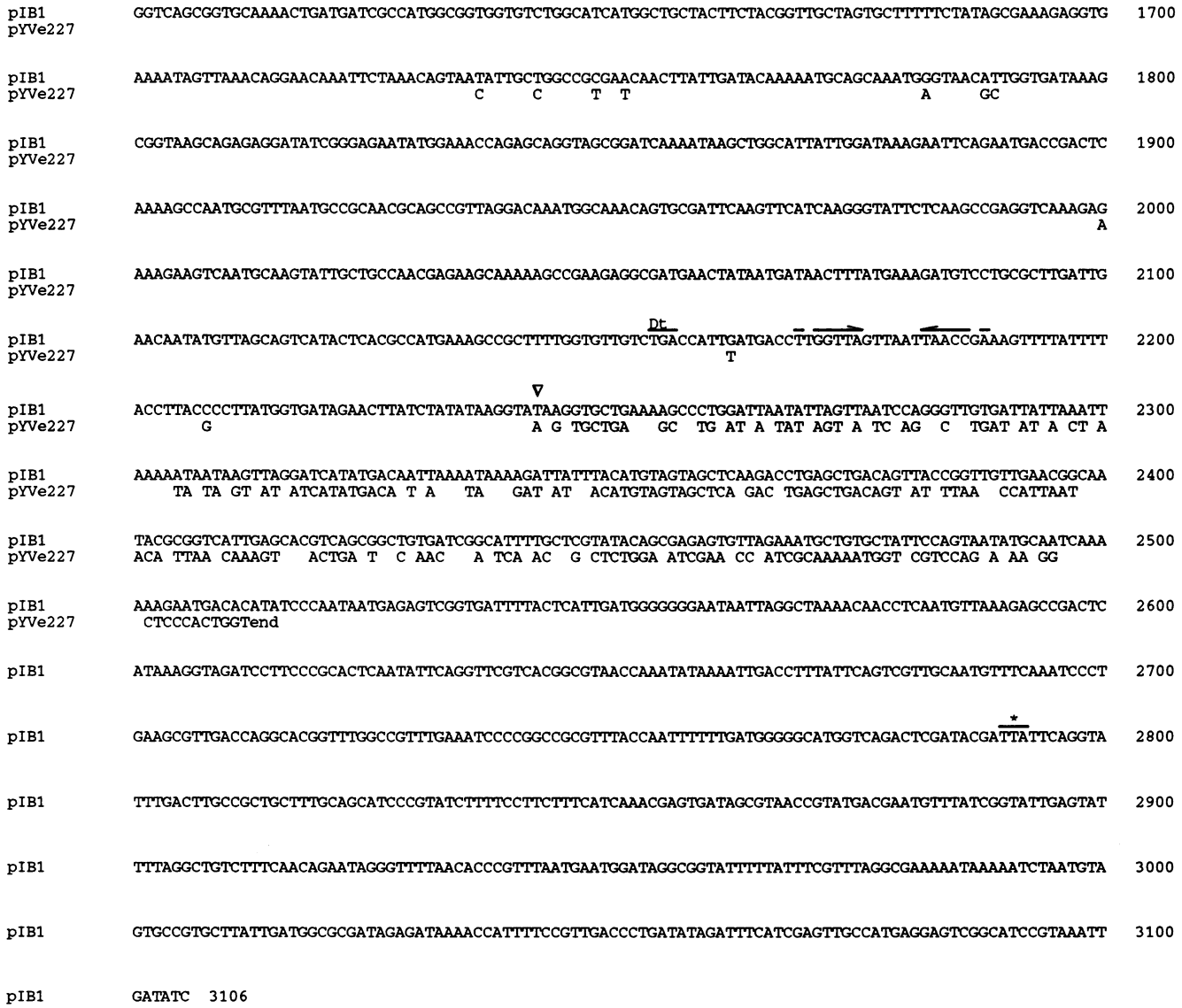

FIG. 3—*Continued.*

were found in either of the two species. In *Y. pseudotuberculosis*, one putative open reading frame, 637 bp downstream of the *yopD* translational stop codon, was identified in the opposite orientation from the *lcrGVH-yopBD* operon. The start of this open reading frame was located outside of the sequenced DNA region. The part that was sequenced constitutes an open reading frame encoding 104 amino acid residues. A GCG TFASTA computer homology search revealed a high level of homology (46% identity) to the transposase of the Tn*610* transposable element of *Mycobacterium fortuitum* (39). The sequence analysis for *Y. enterocolitica* was not extended this far downstream of the *yopD* stop codon. However, 86 bp downstream of the *yopD* gene in *Y. pseudotuberculosis* (Fig. 3), the sequences of *Y. pseudotuberculosis* and *Y. enterocolitica* start to diverge, suggesting that this insertion-like element is not present in *Y. enterocolitica*. Thus, these results imply that there are no additional open reading frames downstream of *yopD*, indicating that *yopD* is the last gene of the V-antigen-encoding operon.

Structural analysis of YopB and YopD. Computer analysis of YopB showed that the protein encompasses 401 amino acids in both species, resulting in molecular masses of 41,795 Da in *Y. pseudotuberculosis* and 41,942 Da in *Y. enterocolitica*. The isoelectric points (pIs) were deduced to be 7.29 and 6.73, respectively, by using GCG software. Analysis of the deduced amino acid sequences of the YopB proteins of both species revealed two hydrophobic regions, separated by 15 amino acids, located in the central part of the protein and consisting of 44 (positions 165 to 208) and 35 (positions 224 to 258) amino acid residues. The YopD protein encompasses 306 amino acids, with molecular masses of 33,357 Da in *Y. pseudotuberculosis* (pI 6.99) and 33,234 Da in *Y. enterocolitica* (pI 6.56). Hydropathy analysis predicted one hydrophobic region in the middle of the YopD protein of each species consisting of 31 amino acids (positions 122 to 152) and one putative amphipathic domain (18) in the carboxy terminus at positions 278 to 292 (Fig. 4). To further investigate the significance of the hydrophobic domains in YopB and YopD, we computed Eisenberg plots (16). By this method, it is

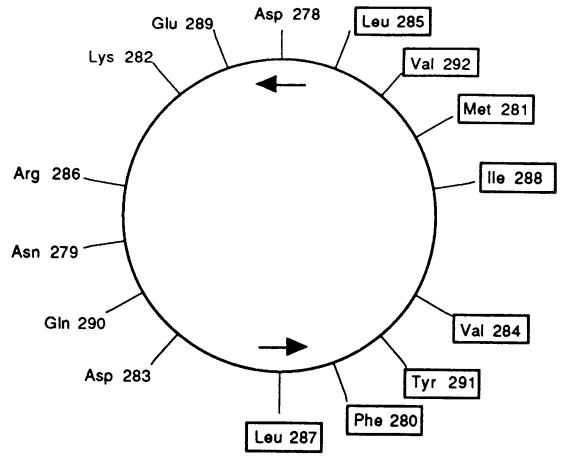

FIG. 4. Helical wheel projection of the amphipathic domain (18) of YopD (amino acid residues 278 to 292). The average hydrophobicity of this domain is −0.03. Hydrophobic residues are boxed.

possible to identify surface-located, globular, and possible transmembrane proteins. Both YopB and YopD fit the pattern of transmembrane proteins according to the Eisenberg model. Thus, YopB and YopD diverge from the globular pattern of YopE, YopH, and YopQ (41).

Sequence homology analysis. No significant amino acid sequence homologies to YopD were found in the Swiss-Prot or GenBank-EMBL data banks. However, YopB and YopD were found to have a moderate level of homology to each other (Table 1). This could indicate that the two genes are products of a gene duplication that have diverged during evolution. The deduced protein sequence of the *lcrH* gene (3), which is located immediately upstream of *yopB* (Fig. 1), was found to have significant homology (Table 1) to the IppI protein of *Shigella flexneri* (Fig. 5). The *ippI* gene is encoded by an operon located on the virulence plasmid of *S. flexneri* (56). Therefore, we investigated whether there were homologies between other proteins encoded by the *Y. pseudotuberculosis* and *S. flexneri* operons. Interestingly, YopB was found to exhibit a moderate but significant level of amino acid homology (Table 1) to the IpaB protein (Fig. 6). The *ipaB* gene is located downstream of the *ippI* gene in the operon of shigellae (2, 56, 65, 67). Thus, the *ippI* and *ipaB* genes are in the same order as the *lcrH* and *yopB* genes of the V-antigen operon (Fig. 7).

Interestingly, the IpaB protein of *S. flexneri* was also found to contain two possible transmembrane regions, as deduced by computer hydropathy analysis. When the hydrophobic regions of the YopB and IpaB proteins were aligned, the degree of identity was elevated (Table 1). These observations suggest similar functions for YopB of yersiniae and IpaB of shigellae.

YopB was also found to exhibit homology to proteins of the RTX protein family of alpha-hemolysins and leukotoxins. However, the homology is not within the Ca^{2+}-binding RTX motif (Table 1) (19, 37, 62).

Two-dimensional gel analysis of the YopD protein. One-dimensional SDS-PAGE analysis shows apparent molecular masses of 34 kDa for YopD from *Y. pseudotuberculosis* and 37 kDa for YopD of *Y. enterocolitica* (46). To investigate whether this differential migration is due to differences in isoelectric points, two-dimensional gel analysis (63) was performed (data not shown). Only a small difference in pI that corresponded to the pI value given by the computer analysis was detected, and the migration of the proteins in the second dimension was not affected. To investigate whether the differential migration could be abolished by harsher denaturing conditions, Yop preparations were boiled for 20 min in sample buffer before being loaded onto gels. Yop preparations were also treated with 10 mM EDTA to chelate ions that might be involved in conformational changes of the proteins. None of these treatments altered the differential migration of the YopD proteins (data not shown).

DISCUSSION

In this study, we have demonstrated that the V-antigen-encoding operon ends after the *yopD* gene, showing that the operon encompasses the genes *lcrGVH-yopBD*, as previously suggested for *Y. pseudotuberculosis* (3) and *Y. enterocolitica* (42). This conclusion is based on the following observations: (i) a putative ρ-independent transcriptional termination signal was found 13 bp downstream of the *yopD* translational stop codon, and (ii) no additional open reading frames were found downstream of the *yopD* gene in the same orientation as the V-antigen operon. However, in *Y. pseudotuberculosis*, one putative open reading frame encoding 104 amino acid residues was found 637 bp downstream of the *yopD* gene in the opposite orientation from the V-antigen operon. The hypothetical peptide of this open reading frame, which has a putative translational start outside the sequenced area in *Y. pseudotuberculosis*, exhibited extensive homologies to transposases of a number of different insertion elements and transposons. The highest homology was to the transposase of Tn*610* of *M. fortuitum* (39). This observation indicates the presence of an insertion-like element downstream of the *yopD* gene in *Y. pseudotuberculosis*. Forsberg and Wolf-Watz reported that an insertion-like element is located immediately downstream of the *yopE* gene in *Y.*

TABLE 1. Homology scores[a]

Aligned proteins	Similarity (%)	Identity (%)	Length (amino acids)
LcrH and IppI	51.0	26.5	159
YopB and IpaB	47.0	23.6	400
Hydrophobic regions of YopB and IpaB	58.2	29.7	90
YopB and YopD	44.0	24.8	306
YopB and LktA (*Pasteurella haemolytica*)	47.8	23.5	400
YopB and HlyA (*Actinobacillus pleuropneumoniae*)	46.8	24.5	401
YopB and LktA (*A. actinomycetemcomitans*)	45.5	22.6	400
YopB and HlyA (*E. coli*)	44.0	18.3	401

[a] Deduced by the local homology algorithm of Smith and Waterman (60) (GCG software).

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LcrH   1 MQQETTDTQEYQLAMESFLKGGGTIAMLNEISSDTLEQLYSLAFNQYQSG 50
         |  :.|:.:..  |: . :.:|:|:  :|.|..| ::::||.|:: |..|
IppI   1 MSLNITENESISTAVIDAINSGATLKDINAIPDDMMDDIYSYAYDFYNKG 50

LcrH  51 KYEDAHKVFQALCVLDHYDSRFFLGLGACRQAMGQYDLAIHSYSYGAIMD 100
         :.|:|. .|. ||:.| |:  :::||:|. | .:|:: | . |. :  ::
IppI  51 RIEEAEVFFRFLCIYDFYNVDYIMGLAAIYQIKEQFQQAADLYAVAFALG 100

LcrH 101 IKEPRFPFHAAECLLQKGELAEAESGLFLAQELIADKPEFKELSTRVSSM 150
          .:   .||.::| |.  .  .|...:    ||: :.: ..|..:. |
IppI 101 KNDYTPVFHTGQCQLRLKAPLKAKECF....ELVIQHSNDEKLKIKAQSY 146

LcrH 151 LEAIKLKKE 159
         |:||.  ||
IppI 147 LDAIQDIKE 155
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FIG. 5. Alignment of *Y. pseudotuberculosis* LcrH and *S. flexneri* IppI. Identical residues are indicated by a vertical line, similar residues are indicated by two dots, and less similar residues are indicated by one dot.

enterocolitica but is not present in *Y. pseudotuberculosis* (22). After sequence comparison, these two insertion elements were shown not to be related.

We have also presented the sequences of *yopB*, *yopD*, and a downstream region in *Y. enterocolitica* W22703(pYVe227). When the deduced amino acid sequences of the proteins from the two species were compared, the YopB proteins were 95.8% identical, with 17 amino acid replacements, and the YopD proteins were 98.0% identical, with only 6 amino acid replacements. Thus, the YopB and YopD proteins show

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YopB   2 SALITHDRSTPVTGSLLPYVETPAPAPL.............QTQQVAGELK 39
         ..| . .. | :.|.|:..::... .:|            . || . |:.
IpaB  71 KSLNASSQLTLLIGNLIQILGEKSLTALTNKITAWKSQQQARQQKNLEFS 120

YopB  40 DKNGGVSSQGV............................QLPAPLAVVAS 61
         || ..: |:.                            |:...|. :..
IpaB 121 DKINTLLSETEGLTRDYEKQINKLKNADSKIKDLENKINQIQTRLSELDP 170

YopB  62 QVTEGQQQEVTKLLESVTRGAAGSQ...LISNYVSVLTKFT......... 99
         : .| .. . ..:  .:.::||..:   :  . :|: .|:|
IpaB 171 ESPEKKKLSREEIQLTIKKDAAVKDRTLIEQKTLSIHSKLTDKSMQLEKE 220

YopB 100 ........................LASPDTFEIELGKLVSNLEEVRKDI 124
                                 |||.. :  .: .||:. :| . .
IpaB 221 IDSFSAFSNTASAEQLSTQQKSLTGLASVTQLMATFIQLVGKNNEESLKN 270

YopB 125 KIADIQRLHEQNMKKIEENQEKIKETEENAKQVKKS.GIASKIFGWLSAI 173
         .:| :|.|:| ....:| . :.. ..  .|.:::.:  |..:|||:| | .|
IpaB 271 DLALFQSLQESRKTEMERKSDEYAAEVRKAEELNRVMGCVGKILGALLTI 320

YopB 174 ASVIVGAIMVAS..GVGAVAGAMMVASGVIGMAN.........MAVKQAA 212
         .||:.:|: .:.  :::||: |:||..:::. |.          ::..|.
IpaB 321 VSVVAAAFSGGASLALAAVGLALMVTDAIVQAATGNSFMEQALNPIMKAV 370

YopB 213 EDGLIS.........QEAM.....KILGPILTAIEVALTVVSTVMTFG 246
          :.||.          |::       |::|.||.||..||.:|..|: .:
IpaB 371 IEPLIKLLSDAFTKMLEGLGVDSKKAKMIGSILGAIAGALVLVAAVVLVA 420

YopB 247 GSALKCLANIGAKLGANTASLAAKG.AEFSAK.VAQISTGISNTVGSAVT 294
         . :     . :|||:.|.:.: :|. .:: :| : .:|..:.: :..||.
IpaB 421 TVGG....KQAAAKLAENIGKIIGKTLTDLIPKFLKNFSSQLDDLITNAVA 466

YopB 295 KLG...GSFAGLTMSHAIRTG..SQATQVAVGVGSGITQTINNKKQADLQ 339
         :|.   |. :: .:|..| ..  .||. :: :|.|: ||. ..   | :|
IpaB 467 RLNKFLGAAGDEVISKQIISTHLNQAVLLGESVNSA.TQAGGSVASAVFQ 515

YopB 340 H....NNADLALNKADMAALQSIIDRLKEELSHLSESHQQVMELIFQMIN 385
         :    | |||.|.| ::..| ..|.   |.:::| |  .::::. : .  .
IpaB 516 NSASTNLADLTLSKYQVEQLSKYISEAIEKFGQLQEVIADLLASMSNSQA 565

YopB 386 AKGDMLHNLAGRPHT 400
         .:.|: ..: ... .
IpaB 566 NRTDVAKAILQQTTA 580
```

FIG. 6. Alignment of *Y. pseudotuberculosis* YopB and *S. flexneri* IpaB. Identical residues are indicated by a vertical line, similar residues are indicated by two dots, and less similar residues are indicated by one dot. Hydrophobic regions predicted to form parts of membrane-spanning segments are boxed.

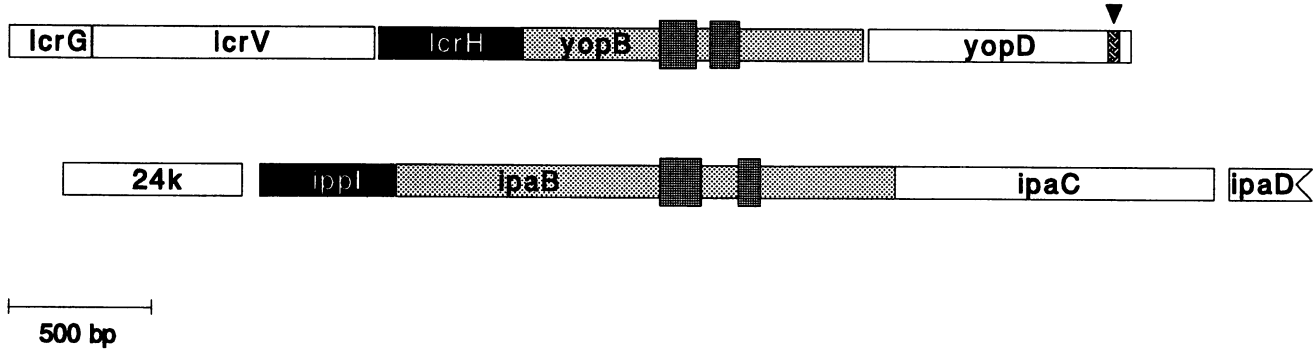

FIG. 7. Organization of the *lcrGVH-yopBD* operon of *Y. pseudotuberculosis* and the *ipa* operon of *S. flexneri*. The operons have been aligned to show the homologous regions. The hydrophobic membrane regions in *yopB* and *ipaB* are indicated by boxes extending above and below each map. The arrowhead points to the amphipathic region in *yopD*.

the same degree of conservation as the other Yop proteins characterized for the two species (8, 22, 40). The insertion-like element found downstream of the *lcrGVH-yopBD* operon in *Y. pseudotuberculosis* was not observed in *Y. enterocolitica* (Fig. 3). This could explain why the sequence homology between these two species starts to decrease 86 bp downstream of the *yopD* stop codon (Fig. 3), and it also explains why the restriction maps of the region downstream of the V-antigen operon differ between *Y. pseudotuberculosis* and *Y. enterocolitica* (unpublished results).

One-dimensional SDS-PAGE analysis showed an apparent molecular mass difference between the YopD proteins of *Y. pseudotuberculosis* (34 kDa) and *Y. enterocolitica* (37 kDa) (46). We could not find a rational explanation for this differential migration from the sequence information. Therefore, the YopD proteins of the two species were investigated by O'Farrell two-dimensional gel analysis (63). This analysis did not reveal any difference in the mobilities that could explain the differential migration in one-dimensional SDS-PAGE. Neither treatment with 10 mM EDTA nor extended boiling of the Yop preparations could alter the migration pattern of YopD in either species. We still have no explanation for the differential migration of the YopD proteins. One possibility is that the amino acid alterations lead to different three-dimensional configurations of the YopD proteins of the two species, resulting in different migrations in SDS-PAGE. The same type of differential migration, without significant differences at the DNA level, can be observed for the YopE proteins of *Y. pseudotuberculosis* and *Y. enterocolitica* (4, 22).

Both YopB and YopD are possible transmembrane proteins, as deduced by sequence analysis. YopB exhibits two hydrophobic regions in the central part of the protein, while YopD has only one large transmembrane region, also located in the central part of the protein. In addition, YopD has a putative amphipathic domain in the carboxy-terminal end. Previously characterized Yop proteins do not contain transmembrane regions (40), indicating that the YopB and YopD proteins form a special class within the Yop family. Although YopB and YopD contain possible transmembrane regions, they are exported to the culture supernatant during the low-calcium response, as are the other Yop proteins of *Y. pseudotuberculosis* and *Y. enterocolitica*. It is tempting to speculate that the transmembrane regions of YopB and YopD play important roles in the virulence of yersiniae. Interestingly, a YopD mutant of *Y. pseudotuberculosis* is attenuated in its cytotoxic effect on cultured HeLa cells. This could imply either that YopD amplifies the cytotoxic effect of YopE or that YopD is involved in the translocation of YopE through the eukaryotic cell membrane (53).

The amphipathic domain found in the carboxy-terminal end of the YopD implies interesting features of this protein. Amphipathic α-helices have been shown to be important structures of antibacterial peptides such as insect cecropins, *Xenopus* magainins, and the bee venom toxin melittin. The primary targets of cecropins and magainins are the inner and outer bacterial membranes. These peptides have also been shown to form channels in artificial membranes, and it is likely that this mechanism is involved in the killing of microorganisms by these peptides (for a review, see reference 9).

We suggest that YopD interacts with the target cell membrane to form channel structures involved either in translocation of the cytotoxic effector, YopE, into the cytoplasmic space of the target cell or in the cytotoxic effect of the YopD protein. We are currently investigating the role of the amphipathic domain of YopD.

An overall moderate level of amino acid sequence homology between YopB and proteins of the RTX protein family of alpha-hemolysins and leukotoxins was found (19, 62). The proteins of the RTX protein family have four hydrophobic regions that are believed to be involved in disrupting the target cell membrane (37). However, it was shown by Oropeza-Wekerle et al. that one peptide of 10 amino acids derived from transmembrane fragment V of HlyA of *E. coli*, was sufficient to induce hemolytic activity (43). Since the amino acid homologies between YopB and the RTX proteins are elevated in the hydrophobic regions, it is possible that the YopB protein exhibits a similar function. We have not been able to link any hemolytic activity to the YopB protein. However, the RTX proteins exhibit different host and cell specificities (57, 59, 62), which could indicate that YopB exhibits a similar activity with a cell specificity that is as yet unknown.

We have also shown that LcrH and YopB of yersiniae exhibit homology with the IppI and IpaB proteins, respectively, of shigellae. The IpaB protein has been shown to be necessary for the invasive phenotype of *S. flexneri* (for a review, see reference 27). Interestingly, IpaB harbors the same structural motif (two possible hydrophobic transmembrane domains) found in YopB and has been shown to be a contact hemolysin with a membrane-damaging activity (11, 29, 55). These findings could argue for the observed homology between YopB and the RTX proteins. We have also

found significant homology between LcrH of *Y. pseudotuberculosis* and IppI of *S. flexneri* (2, 56, 65). The IppI and IpaB proteins are encoded by one operon located on the virulence plasmid of *S. flexneri* in the same order as LcrH and YopB (Fig. 6). These observations suggest a conservation of this operon between yersiniae and shigellae, indicating a similar function of these proteins in the two genera. Moreover, Venkatesan et al. found striking similarities between repetitive sequences in the YopM protein of yersiniae and the IpaH protein of *S. flexneri* (66). These observations of similarities between proteins of yersiniae and shigellae could indicate a common ancestry of the virulence plasmids of these two genera. This hypothesis is consistent with the fact that the virulence plasmids of yersiniae and *S. flexneri* exhibit similar replicons (64).

In conclusion, we have shown that the V-antigen-containing operon ends with the *yopD* gene and that the YopB and YopD proteins are highly conserved between *Y. pseudotuberculosis* and *Y. enterocolitica*. Furthermore, we have shown that YopB and YopD are possible transmembrane proteins constituting a unique class of Yop proteins and that YopB has structural features in common with the RTX toxin family. Moreover, YopD is shown to contain an amphipathic domain in the carboxy-terminal end, which might suggest that YopD forms channel structures involved in the translocation of YopE. Additionally, we have found homology between LcrH and YopB of the V-antigen operon and IppI and IpaB of *S. flexneri*.

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