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Secretion of Yop Proteins by Yersiniae

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Upon incubation at 37°C in the absence of Ca^{2+} ions, pathogenic strains of the genus *Yersinia* cease growing and produce large amounts of a series of plasmid-encoded proteins involved in pathogenicity. These proteins, called Yops (for *Yersinia* outer membrane proteins), are detected in both the outer membrane fraction and the culture supernatant. We present here the nucleotide sequence of genes *yop20* and *yop25* from *Yersinia enterocolitica* O:9. Protein Yop25 is very similar to YopE, the corresponding protein from *Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* O:8 (Å. Forsberg and H. Wolf-Watz, J. Bacteriol. 172:1547–1555, 1990). This is the first report of a *yop20* sequence of yersiniae. We present evidences that Yops are not membrane proteins. Their detection in the membrane fraction results either from copurification of large aggregates of extracellular Yops with the membrane fraction or from the adsorption of released proteins to the cell surface. In contrast with Yops, protein P1 has characteristics of a true membrane protein. The release of Yops by *Y. enterocolitica* occurs by a novel secretion mechanism that does not involve the cleavage of a typical signal sequence or the recognition of a carboxy-terminal domain.

Human pathogenic yersiniae (*Yersinia enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*) harbor closely related 70-kilobase plasmids called pYV that are necessary for virulence (3, 4, 23, 27, 28, 51). Upon incubation at 37°C in the absence of Ca^{2+} ions, strains harboring the plasmid stop growing and produce large amounts of plasmid-encoded proteins involved in pathogenicity (8, 25, 38, 45, 46; for reviews, see references 12 and 14). These proteins are referred to as Yops (*Yersinia* outer membrane proteins) since they were originally detected in the outer membrane fraction of bacterial extracts (6, 39, 47). However, Heesemann and co-workers have shown that Yops are also released in the culture supernatant (31, 32). Yops are currently considered as partly released and partly membrane bound. In addition to the Yops, *Y. enterocolitica* and *Y. pseudotuberculosis* strains incubated at 37°C produce a high-molecular-weight protein forming a fibrillar matrix on the bacterial surface (33, 50). This protein, known as protein P1 or YopA, confers resistance to the bactericidal activity of human serum (2, 36) and promotes intestinal colonization in mice (33).

Ten Yops in culture supernatants of *Y. enterocolitica* O:9 have been described (17, 38). The *yop* genes are part of at least six individual transcription units scattered around the plasmid and coordinately regulated by a 20-kilobase region of the pYV plasmid called the calcium region (15, 16). Transcription of *yop* genes is enhanced more than 100-fold by a temperature shift from 28 to 37°C (17, 38). According to operon fusion analysis, the presence of Ca^{2+} ions has only a moderate (three- to sixfold) repressor effect on the transcription of *yop* genes. In this work, we sequenced the genes encoding Yop20 and Yop25 from *Y. enterocolitica* O:9, and we analyzed the status of Yops produced by this strain with respect to their cellular localization. We present evidence that Yops are not membrane bound but are true released proteins. This has implications for the understanding of both the export process and the mode of action of these proteins. Secretion of Yops occurs through a new mechanism that involves neither a typical signal sequence nor a carboxy-terminal recognition domain.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Y. enterocolitica* W22703 (nalidixic acid resistant) is a restriction mutant (Res^- Mod^+) isolated previously in our laboratory (13) from the wild-type strain W227 of serotype O:9. According to restriction analysis, the virulence plasmid from that strain, called pYVe227, is identical to pYVe439-80, the plasmid from *Y. enterocolitica* 439-80, another typical O:9 strain (4, 35).

Bacteria were generally grown in tryptic soy agar (TSA; GIBCO Laboratories, Grand Island, N.Y.) or in tryptic soy broth (TSB; GIBCO). Yersiniae were cultured in brain heart infusion (Difco Laboratories, Detroit, Mich.) containing the appropriate antibiotics and 0.4% glucose (BHI), eventually supplemented with 20 mM $MgCl_2$ and 20 mM sodium oxalate (BHI_{ox}). Selective agents were ampicillin (300 μg/ml), nalidixic acid (20 μg/ml), and kanamycin (50 μg/ml).

Induction of the *yop* regulon. Yersiniae grown overnight at room temperature (22 to 24°C) in BHI were inoculated to an optical density at 600 nm of 0.1 in a conical flask containing 10 ml of BHI_{ox}. Cultures were incubated with rotatory shaking (±150 rpm) for 2 h at room temperature and then shifted for 0 to 8 h at 37°C (4 h for most purposes). Control cultures were left at room temperature for the same time. Similar results were obtained when TSB was used instead of BHI.

Cell fractionation and protein extraction. Culture supernatant proteins and membrane proteins were prepared by methods adapted from those of Heesemann et al. (31) and Achtman et al. (1), respectively. Cells were collected by centrifugation at either 8,000 × *g* for 10 min or at 1,900 × *g* for 30 min. Proteins from the 10-ml supernatant were allowed to precipitate overnight at 4°C after the addition of 4.76 g of $(NH_4)_2SO_4$. Proteins were subsequently pelleted by a 30-min centrifugation at 1,900 × *g*. Pellets were washed with 150 μl of H_2O. Yop proteins were collected by a 15-min

centrifugation in microtubes and suspended in 150 μl of 1.5× sample buffer (1× sample buffer is 3% [wt/vol] sodium dodecyl sulfate [SDS], 5% [vol/vol] β-mercaptoethanol, 10% [vol/vol] glycerol, 0.005% [wt/vol] bromophenol blue, 0.06 M Tris hydrochloride [pH 6.8]) eventually supplemented with 3.5 M urea and 5% β-mercaptoethanol to facilitate dissolution. It is noteworthy that the soluble Yops (Yop48, Yop41, Yop20, and Yop17) are partly lost during the washing step.

Total cell proteins were prepared by suspending a cell pellet from 1 ml of culture in 200 μl of sample buffer containing 3.5 M urea and 5% β-mercaptoethanol.

For cell fractionation, cells pelleted from a 10-ml culture at an optical density at 600 nm of about 2 were disrupted by sonication in 3 ml of sonication buffer (10 mM Tris hydrochloride, 5 mM $MgCl_2$ [pH 8]). Large fragments and undisrupted cells were removed by one or two low-speed centrifugation steps (20 min at 1,000 × *g*). Total membrane proteins were then pelleted by 60 min of centrifugation at 40,000 × *g*. Soluble proteins (cytoplasmic plus periplasmic proteins) were precipitated from the supernatant by the addition of 3 volumes of acetone. After at least 1 h of incubation at 4°C, these proteins were harvested by 10 min of centrifugation at 8,000 × *g*, dried, and suspended in 600 μl of 1.5× sample buffer. To dissociate inner and outer membrane proteins, total membranes were suspended in 400 μl of sonication buffer containing 2% (vol/vol) Triton X-100. Proteins from the outer membrane fraction were collected by 30 min of centrifugation in a microtube and were suspended in 150 μl of 1.5× sample buffer. One volume of 3× sample buffer was added to 2 volumes of the Triton X-100-soluble fraction to form the inner membrane fraction.

Sequence of the N terminus of secreted Yop51. Yop51 was overproduced by *Y. enterocolitica* W22703 containing both pBM15 and pTM160. pBM15 is a mutant of the pYVe plasmid that has an insertion of Tn*2507* in gene *yop48* (38). pTM160 is a mobilizable multicopy plasmid carrying the *yop51* gene expressed from its own promoter.

Large filamentous aggregates produced by *Y. enterocolitica* W22703(pBM15)(pTM160) were collected from the culture medium and dissolved in 7 M urea–10% β-mercaptoethanol. As estimated by SDS-polyacrylamide gel electrophoresis (PAGE) scanning, this method allowed us to obtain around 100 μg of about 90% pure Yop51 protein per ml of culture. The protein was dialyzed against 50 mM ammonium bicarbonate and lyophilized. The N-terminal sequence of the protein was kindly determined by C. Capiau and K. Conrath from SmithKline Biologicals (Rixensart, Belgium).

Xylene and hexadecane extraction. Samples of 200 μl of *p*-xylene or *n*-hexadecane were mixed for 5 min with 300 to 400 μl of culture in a microtube. After 10 min of centrifugation, the solvent was discarded. Supernatant proteins were precipitated together with proteins floating at the interface between the solvent and the culture supernatant by the addition of 4 volumes of acetone. After 1 h of incubation at −20°C, proteins were collected by centrifugation and dissolved in sample buffer containing 3.5 M urea and 5% β-mercaptoethanol. Cell-associated proteins were prepared by suspending the cell pellets in 1.5× sample buffer supplemented with 3.5 M urea and 5% β-mercaptoethanol.

Nucleotide sequence and sequence analysis. Genes *yop20* and *yop25* were sequenced from fragments *Eco*RI 4 and *Bam*HI 1 of the pYVe439-80 plasmid, cloned earlier in pACYC184 (35). Subfragments were cloned either into plasmids pTZ18R and pTZ19R (Pharmacia, Uppsala, Sweden) or into their mobilizable derivatives pBC18R and pBC19R (B. China, T. Michiels, and G. Cornelis, Mol. Microbiol., in press).

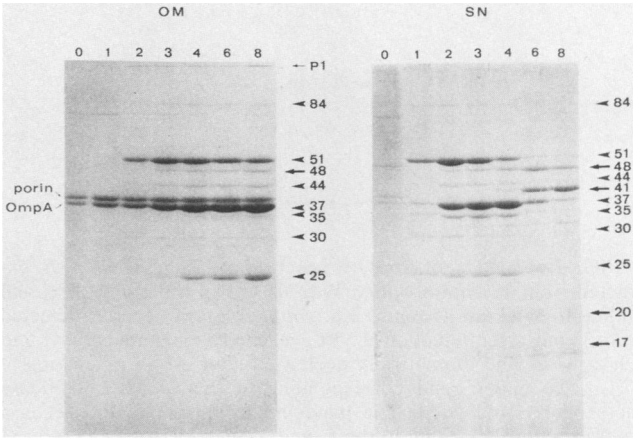

FIG. 1. Kinetics of Yop production. Outer membrane (OM) and supernatant (SN) proteins extracted from *Y. enterocolitica* W22703 (pGC565) grown in oxalated TSB after 0, 1, 2, 3, 4, 6, and 8 h of incubation at 37°C. Eleven Yops were detected and are identified by their apparent molecular weights in thousands. Among them, Yop17 had not been described. Small arrows identify the less soluble Yops, and long arrows point to the most soluble Yops. OmpA and a porin are shown as typical outer membrane proteins (51).

The nucleotide sequence was determined by the method of Sanger et al. (41) with T7 DNA polymerase (Sequenase; U.S. Biochemical Corp., New Haven, Conn.) for elongation. Single-stranded DNA was obtained from strain LK111 or JM101 (49) overinfected with the M13K07 helper phage.

RNA extractions and primer extensions were done exactly as described by Michiels and Cornelis (37).

DNA and protein sequences were analyzed on a Micro Vax Computer (Digital Equipment Corp.) with the program package of Claverie (11) and FASTA, FASTN, FASTP, ALIGN, and NAQ software from the Protein Identification Resource program package.

Hydrophobicity analysis. The mean α-helical hydrophobic moment and the mean hydrophobicity were calculated by the procedure of Eisenberg et al. (20, 21). A segment of 11 amino acids was moved through the protein sequence, and the mean hydrophobicity moments per segment were calculated. The mean hydrophobicity was plotted versus 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 (20, 21) of Eisenberg et al., because it is especially suitable for membrane-related proteins.

RESULTS

Kinetics of Yop production. Cultures of *Y. enterocolitica* W22703 carrying either the wild-type pYVe227 plasmid or its kanamycin resistance-labeled derivative pGC565 (16) were incubated at 37°C in the absence of Ca^{2+} ions to induce Yop production. After 0 to 8 h of induction at 37°C, bacteria were collected and fractionated. Proteins from the culture supernatant (extracellular milieu), the outer membrane, the inner membrane, and the soluble fractions (cytoplasm plus periplasm) were analyzed by SDS-PAGE.

After 1 h of incubation at 37°C, Yops were only detected in the culture supernatant. After 3 to 4 h of heat induction,

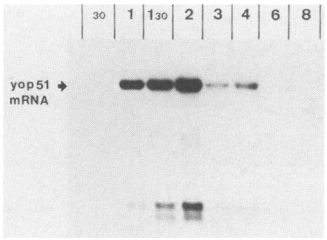

FIG. 2. Kinetics of *yop51* transcription. Total RNA was extracted from *Y. enterocolitica* W22703 grown for 2 h at 25°C and shifted to 37°C for 30 min to 8 h. *yop51* transcription was detected from 30 min after induction at 37°C, reached a maximum after 1.5 to 2 h at 37°C, and dramatically decreased after 3 h of incubation at 37°C. The major band corresponding to the *yop51* 1.6-kilobase messenger is indicated. The band in the bottom of the gel is a transcript corresponding to the 5′ end of the *yop51* gene (37).

some of the Yops progressively disappeared from the culture supernatant, whereas the same proteins increased in the outer membrane fraction (Fig. 1). The order of disappearance from the supernatant and appearance in the outer membrane fraction was as follows: (i) Yop51 and Yop25; (ii) Yop84, Yop44, Yop37, and Yop30; (iii) Yop35; (iv) Yop48 and Yop20; (v) Yop41 and Yop17. The Yop41 protein, also known as the V antigen (10, 38), was detected in both the supernatant and the soluble fractions but only scarcely in the outer membrane fraction. In contrast to Yops, protein P1 first appeared in the outer membrane fraction, whereas it was later recovered in low amounts from the culture supernatant.

To analyze the kinetics of *yop* gene transcription, total RNA was extracted after 0 to 8 h of incubation at 37°C and hybridized to an oligonucleotidic probe that was specific for the *yop51* transcript (Fig. 2). *yop51* transcription was detected from 30 min after the temperature shift, reached a maximum between 1.5 and 2 h, and dramatically decreased after 3 h, suggesting that Yops are mainly synthesized in the first 2 to 3 h after the temperature shift.

Solubility of the Yops. Large filaments appeared in the culture medium after 3 to 4 h of induction at 37°C (Fig. 3). These filaments could be collected directly from the culture with a glass rod and were run on SDS-PAGE together with supernatant fractions from wild-type or plasmidless strains. These filaments were nearly pure preparations of Yops, free of membrane or cytoplasmic proteins (Fig. 3). Yops found in such preparations were the same as those generally detected in the outer membrane preparations: i.e., Yop84, Yop51, Yop44, Yop37, Yop35, Yop30, and Yop25. These results strongly suggest that these Yops are poorly soluble and form large aggregates in the extracellular milieu. The appearance of these Yops in the outer membrane fraction could thus result from the copurification of insoluble Yops with membranes. Dissolution of the filaments required urea and β-

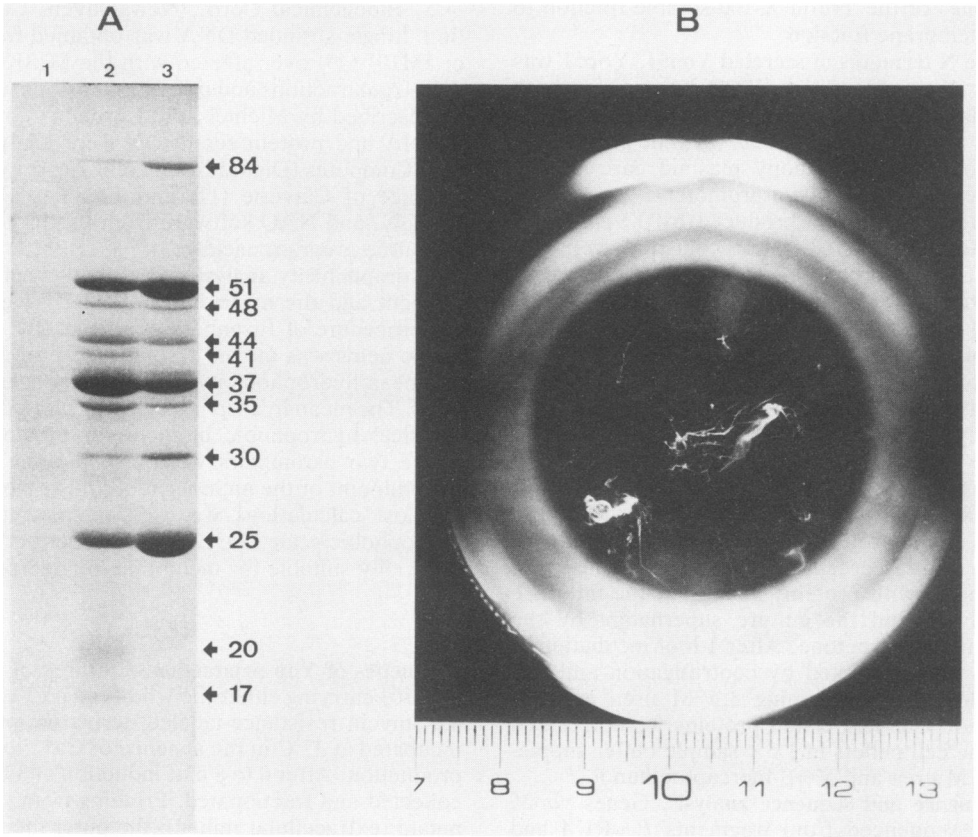

FIG. 3. Aggregation of Yops in cultures of *Y. enterocolitica* W22703. Filamentous aggregates became visible in cultures of *Y. enterocolitica* W22703 after 3 to 4 h of induction at 37°C. (B) Photograph of the culture in the conical flask, seen from above (scale in centimeters). These aggregates were collected with a glass rod and were run on SDS-PAGE (A, lane 3) together with supernatant proteins extracted from the same strain (lane 2) and from the plasmidless derivative (lane 1).

mercaptoethanol, suggesting that polar interactions and intermolecular disulfide bridges are involved in the aggregates.

Yop48 and Yop20 were occasionally detected in aggregates, whereas Yop41 and Yop17 were not. These proteins are thus more soluble, in agreement with the fact that they were not or scarcely detected in membrane preparations.

Stability of the Yops. To confirm that the disappearance of the less soluble Yops from the culture supernatant fraction was not due to a degradation process, we looked at the stability of Yops produced by *Y. enterocolitica* W22703 (pGC565). After 2 to 4 h of incubation at 37°C, bacteria were harvested by centrifugation, and the supernatant was incubated at 37°C for 4 to 20 h. According to SDS-PAGE analysis, no significant decrease in the amount of Yops occurred upon such a prolonged incubation of the supernatant at 37°C, suggesting that Yops are rather stable.

Some Yops could be pelleted from the incubated supernatant by a single low-speed centrifugation step (8,000 × *g*, 5 min). The sedimented Yops were the same as those generally detected in membrane preparations or in filaments, confirming the observation that these proteins have a propensity to aggregate. Again, Yop48, Yop41, Yop20, and Yop17 remained in the supernatant, which confirmed their greater solubility.

Dissociation of Yops from membrane-anchored proteins. In an attempt to discriminate between aggregated and membrane-anchored proteins, we treated cells from induced cultures with hydrophobic agents such as *p*-xylene and *n*-hexadecane. Yop proteins were efficiently removed from the cell fraction by these agents, whereas protein P1 and the known membrane proteins such as OmpA were not (Fig. 4). Yops were recovered as aggregates at the interface between the culture supernatant and the organic solvent (Fig. 4B). Little cross-contamination of the two fractions was detected. This experiment confirmed that Yops are not anchored in the membrane and that Yops must be regarded as poorly soluble secreted proteins. However this does not exclude the possibility that some extracellular Yops are adsorbed to the cell surface via weak interactions.

In contrast with Yops, protein P1 appeared to be a true membrane-bound protein.

Hydrophobicity analysis. To confirm the difference in the status of Yops and protein P1, we looked for the existence of potential membrane domains in the sequences of proteins Yop20, Yop25, Yop51, and P1 of *Y. enterocolitica*. The sequence of P1 was from *Y. enterocolitica* O:3 (44). The sequence of Yop51 from *Y. enterocolitica* O:9 was from Michiels and Cornelis (37). The sequences of *yop25* and *yop20* from *Y. enterocolitica* O:9 are presented in Fig. 5 and 6. We investigated these protein sequences by the method of Eisenberg et al. (20, 21). A segment of 11 amino acids was moved through the protein sequence, and the mean hydrophobicity and the mean hydrophobic moment per segment were calculated. When these two parameters were plotted versus the amino acid sequence, all residues of Yop20, Yop25, and Yop51 were characterized by values described by Eisenberg et al. as characteristic of globular protein (Fig. 7). Only in the P1 protein a segment with a high hydrophobicity (>0.5) and a low hydrophobic moment (between 0.0 and 0.4), typical of membrane-associated structures, was observed (9, 20). This segment corresponds to residues 83 to 104 of the P1 (serotype O:3) protein sequence.

Estimation of the amount of secreted Yops. We took advantage of the dissociation of Yops from other proteins by solvent extraction to estimate the amount of Yops secreted by yersiniae after 4 h of incubation at 37°C. Yops isolated after *p*-xylene treatment were run in SDS-PAGE together with known quantities of a marker protein. The gels were scanned after Coomassie blue staining. As estimated by this procedure (data not shown), the total concentration of Yop in the culture medium reached about 200 to 300 μg/ml of culture containing about 3×10^9 CFU/ml. This corresponds roughly to 10 to 20% of the total cell proteins.

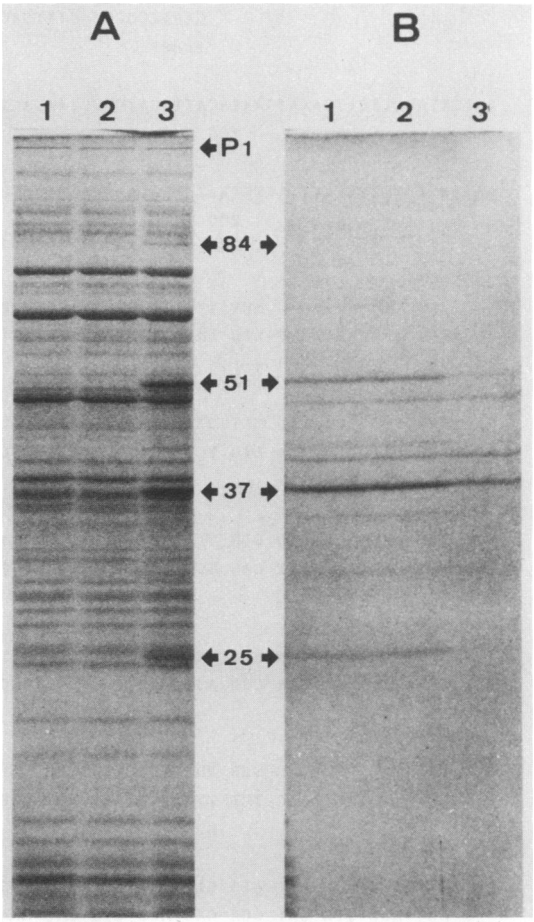

FIG. 4. Dissociation of Yops from cells by solvent extraction. Coomassie blue-stained SDS-PAGE of total cell proteins (A) and supernatant (plus interface) proteins (B) from induced cultures of *Y. enterocolitica* W22703. Lanes: 1, *n*-hexadecane treated; 2, *p*-xylene treated; 3, untreated control. Yops that can be identified among total cell proteins are indicated by their apparent molecular weights in thousands. Yops are removed from the cell fraction upon treatment with the solvent, whereas protein P1 remains.

Mechanism of secretion of the Yops. To define whether a leader peptide is cleaved off during the process of Yop secretion, we purified Yop51 from the supernatant of *Y. enterocolitica* W22703(pBM15)(pTM160), a Yop51-overproducing strain, and sequenced the amino end of the protein. The N terminus of the secreted protein matched the N-terminal part of Yop51 deduced from the nucleotide sequence (Fig. 8) (37). Thus, no removal of an N-terminal signal sequence occurred during the process of Yop51 secretion.

DISCUSSION

The data presented in this paper show that Yops have different properties: some of the Yops appear to be soluble

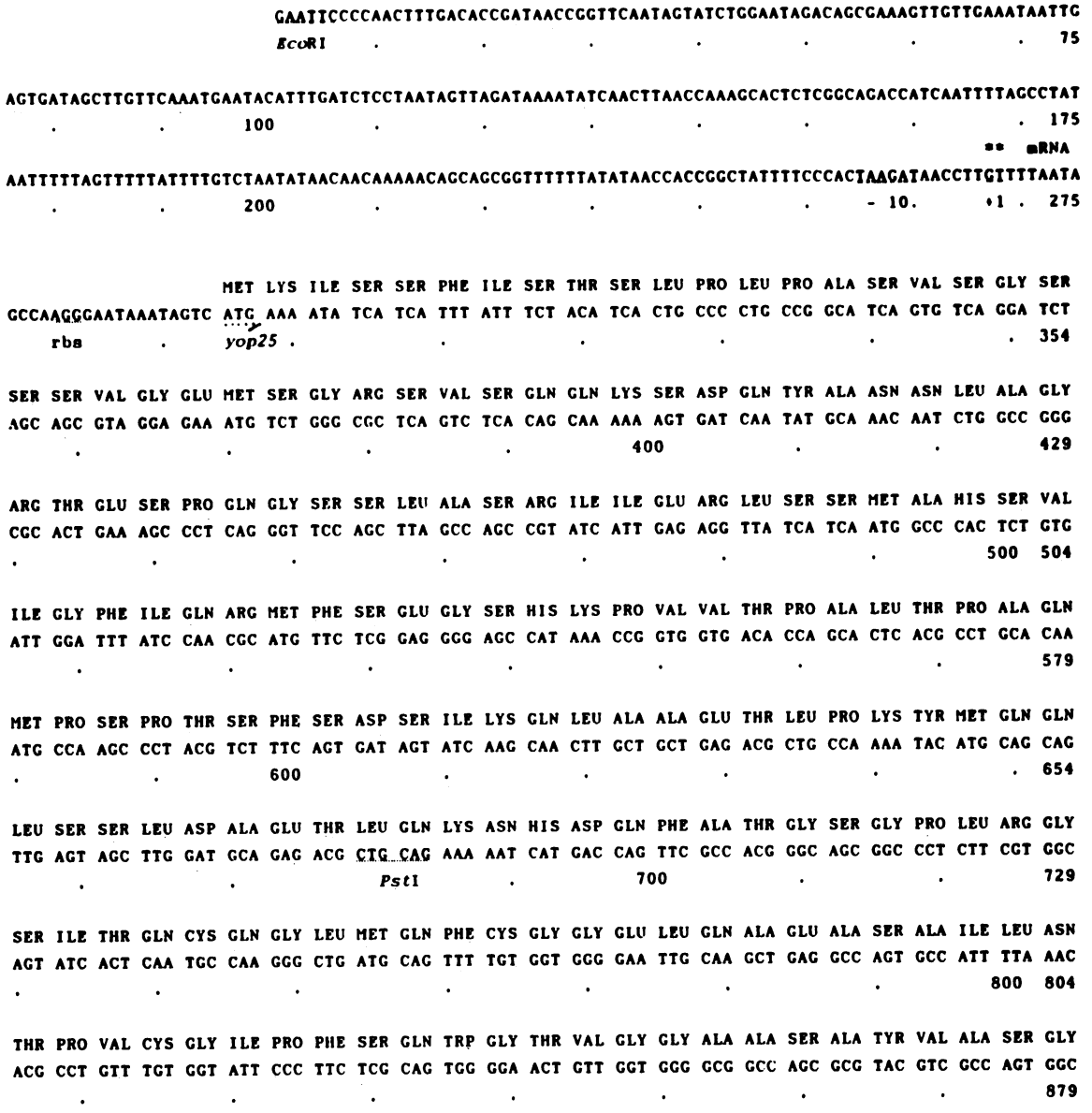

FIG. 5. Nucleotide sequence of gene *yop25* from *Y. enterocolitica* O:9. We sequenced a 1,152-base-pair *Eco*RI-*Xho*I fragment from pYVe439-80 from *Y. enterocolitica* O:9 encoding Yop25 (coordinates, 46.7 to 47.8 kilobases on the pYVe O:9 plasmid map [38]). The open reading frame corresponding to gene *yop25* starts at nucleotide 295 and ends at nucleotide 951. According to the nucleotide sequence, Yop25 would be a 219-amino-acid protein with a calculated molecular weight of 22,921. The transcriptional start (+1) of *yop25* was localized by primer extension at either nucleotide G267 or T268. A potential −10 box (TAAGAT) extends from nucleotides 255 to nucleotide 260. A potential ribosome-binding site (rbs) is indicated. This sequence has been submitted to GenBank under the accession number M33838.

(Yop48, Yop41, Yop20, Yop17), whereas other Yops have the propensity to aggregate in the supernatant (Yop51, Yop25 > Yop 84, Yop44, Yop37, Yop30 > Yop35). As first proposed by Heesemann et al. (31, 32), our data show that Yops are true released proteins. Yops have been detected in the outer membrane of yersiniae prepared by either the Triton X-100 extraction method (39) or by the Osborne gradient method (46, 47). This detection of Yops in the

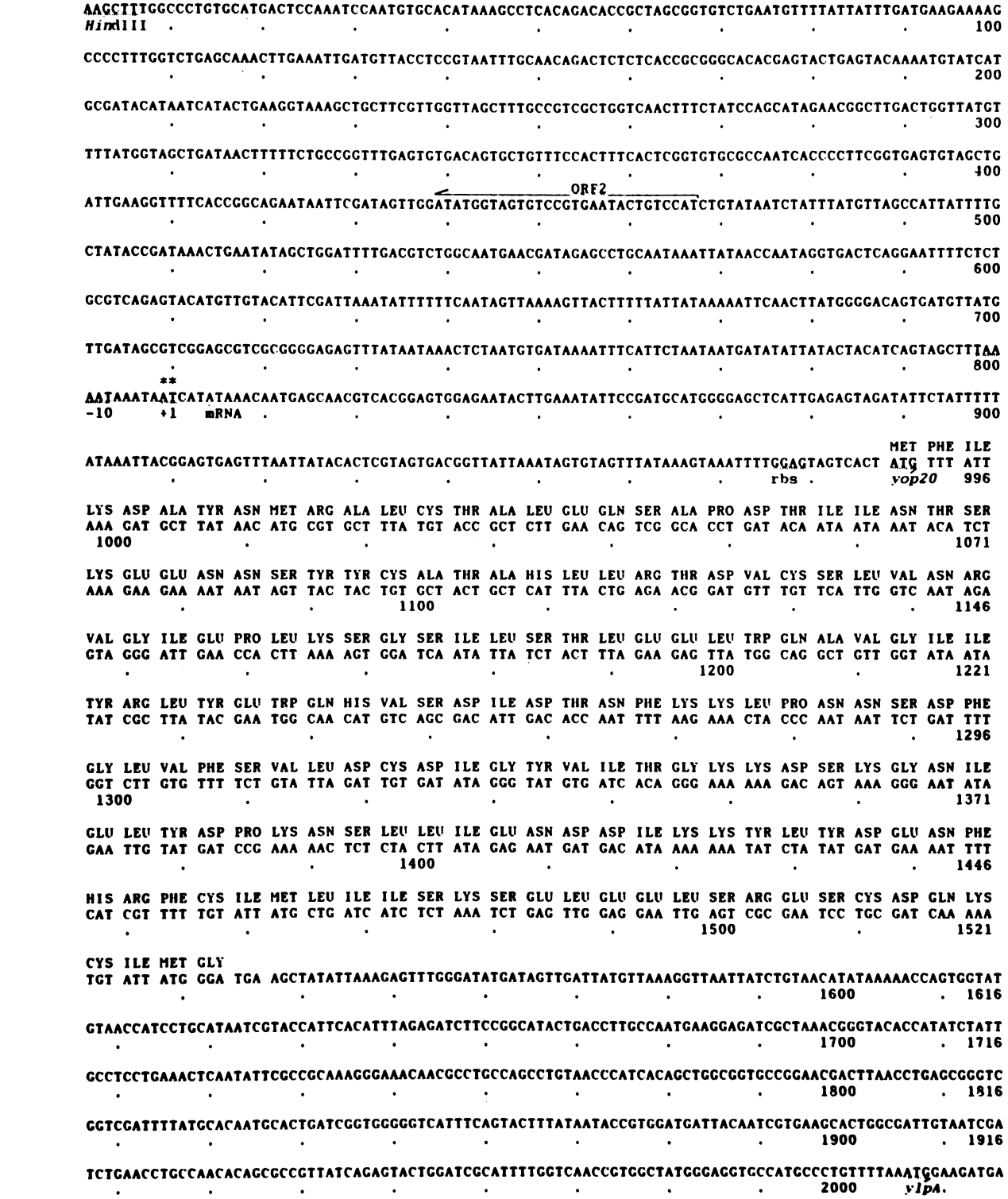

FIG. 6. Nucleotide sequence of gene *yop20*. Nucleotide sequence of a 2-kb fragment of the pYVe439-80 plasmid from *Y. enterocolitica* O:9 (coordinates, 9 to 7 kilobases on the pYVeO:9 plasmid map [38]). A 182-codon open reading frame (nucleotides 988 to 1533) was detected at the place predicted for *yop20* by transposon mutagenesis (38). That this open reading frame corresponds to *yop20* is evidenced by the fact that cloned fragments containing this open reading frame overexpressed Yop20 in a pYV-containing strain. According to the sequence, Yop20 is a 182-residue protein with a calculated molecular weight of 20,805. A potential ribosome-binding sequence preceeding *yop20* is indicated (rbs). The transcriptional start (+1) was determined by primer extension. Surprisingly, the promoter was found to be situated about 200 base pairs upstream of the translational start codon. This leader sequence has the capacity to encode a 24-residue peptide. Whether this peptide is expressed in vivo is not known. A large open reading frame starts backward from position 467 (ORF2) and reaches the beginning of the sequenced region without any stop codon. The gene upstream of *yop20* is thus transcribed in opposite orientation. At 480 base pairs downstream of *yop20* starts the gene encoding the lipoprotein YlpA (nucleotide 2013). This gene, described elsewhere, does not form an operon with gene *yop20* (China et al., in press). This sequence has been submitted to GenBank under the accession number M33786.

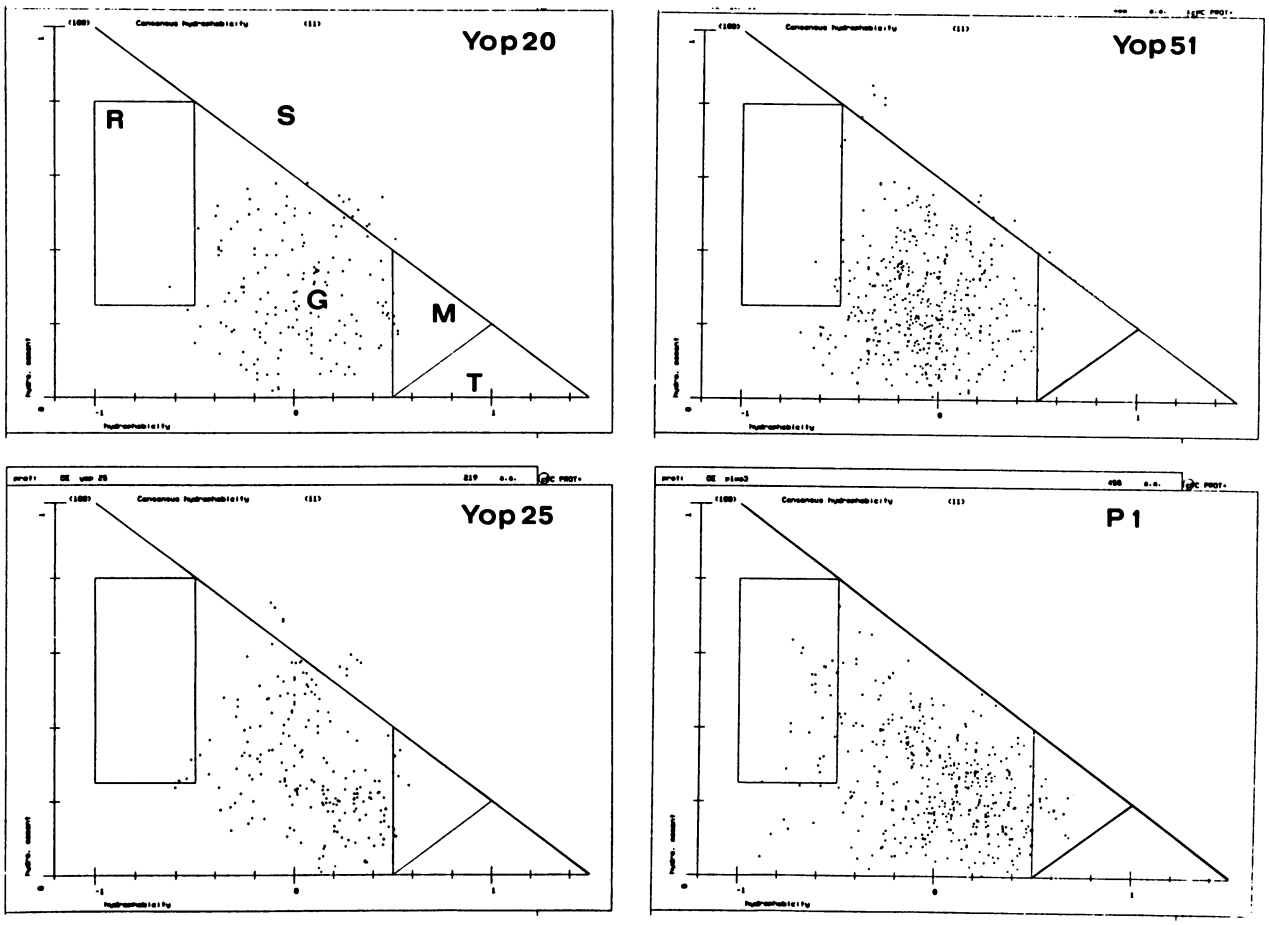

FIG. 7. Plot of mean hydrophobicity against the mean hydrophobic moment for all 11-residue segments of Yop20, Yop25, Yop51, and P1. M, Membrane domain; T, transmembrane domain; G, globular domain; R, receptor-binding domain. The sequence of protein P1 (*Y. enterocolitica* serogroup O:3) is from Skurnik and Wolf-Watz (44). The sequence of Yop51 (*Y. enterocolitica* O:9) is from Michiels and Cornelis (37). The sequences of Yop20 and Yop25 (*Y. enterocolitica* O:9) are presented in Fig. 5 and 6.

membrane fraction presumably results either from copurification of aggregated Yops with the membranes or from the adsorption of secreted Yops to the cell surface. Several observations support this view. (i) Yops are first detected in the supernatant and later in the membrane fraction. (ii) The appearance of Yops in the outer membrane fraction is concomitant with the decrease of the corresponding protein in the supernatant. (iii) Disappearance of the less-soluble Yops from the supernatant is not a consequence of degradation. (iv) There is a correlation between the propensity of a given Yop to aggregate in the supernatant and the presence of that Yop in the membrane fraction. (v) Yops still accumulate in the membrane fraction after 3 h of induction, whereas transcription of the *yop* genes at that time is dramatically reduced. (vi) Yops are separated from the cell fraction upon treament with hydrophobic agents such as xylene or hexadecane, whereas chromosome-encoded membrane proteins and protein P1 are not. (vii) According to the hydrophobic analysis of the sequences, Yops do not have the characteristics of membrane proteins.

Thus, Yops should be regarded as poorly soluble proteins rather than membrane-anchored proteins. This does not

DNA	ATG	AAC	TTA	TCA	TTA	AGC	GAT	CTT	CAT	CGT	CAG	GTA	TCT	CGA	TTG	GTG	CAG	CAA	GAG	AGC
TRANSLATION	Met	Asn	Leu	Ser	Leu	Ser	Asp	Leu	His	Arg	Gln	Val	Ser	Arg	Leu	Val	Gln	Gln	Glu	Ser
EDMAN CYCLE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
RESIDUE	Met	Asn	Leu	Ser	Leu	Ser	Asp	Leu	Ser	Arg	Gln	Val	Ser	Arg	Arg	Val	Gln	Gln	Gln	Ser
picomoles	2369	875	2897	8501	1977	5723	1384	2094	1791	392	867	1028	3563	660	1302	857	601	670	1282	3241
RESIDUE									His						Leu				Glu	
picomoles									275						1185				1104	

FIG. 8. Comparison of the N-terminal amino acid sequences of Yop51 deduced from the nucleotide sequence (37) and determined by Edman degradation of released proteins. The major residues detected at cycles 9, 15, and 19 did not correspond to the sequence predicted from the nucleotide sequence. However, the expected residues were the second components detected in these cycles. This little discrepancy probably results from the low level of Yop51 purification.

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pYV439-80  MKISSFISTSLPLPASVSGSSSVGEMSGRSVSQQKSDQYANNLAGRTESPQGSSLASRII
pYV019     ----------T-------------------T------------------------  60
pYV8081    ----------T--------------------E----------------------T
pIB1       ----------T-------------------T------------------------

pYV439-80  ERLSSMAHSVIGFIQRMFSEGSHKPVVTPALTPAQMPSPTSFSDSIKQLAAETLPKYMQQ
pYV019     -----V----------------------P---------------------------- 120
pYV8081    -K-------A-E--K--------------P----------------------------
pIB1       -----V----------------------P----------------------------

pYV439-80  LSSLDAETLQKNHDQFATGSGPLRGSITQCQGLMQFCGGELQAEASAILNTPVCGIPFSQ
pYV019     -N-----M------------------------------------------------------ 180
pYV8081    ------------------------------------------------------------
pIB1       -N-----M-----A-------------------------------------------------

pYV439-80  WGTVGGAASAYVASGVDLTQAANEIKGLGQQMQQLLSLM*      identity
pYV019     ---I----------------------A----K-----*      210/219
pYV8081    ---I--------------------L---A---H------*    207/219
pIB1       ---I--V--------------------A----K-----*      208/219
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FIG. 9. Comparison of the YopE and Yop25 sequences deduced from the nucleotide sequences. The sequences of YopE from *Y. pseudotuberculosis*(pIB1) is from Forsberg and Wolf-Watz (25). The sequences of YopE from *Y. pestis*(pYV019) and *Y. enterocolitica* O:8(pYV8081) are from Forsberg and Wolf-Watz (26). All four sequences are 219 residues long and align without gaps. Residues identical to those of Yop25 from *Y. enterocolitica* O:9(pYVe439-80) are indicated by dashes. Diverging amino acids are indicated.

exclude the hypothesis that Yops are linked to the cell surface in a labile linkage, perhaps as lipoproteins. However, labeling experiments carried out with tritiated palmitic acid indicated that Yops are not lipoproteins (data not shown).

In contrast to Yops, protein P1 appears to be a true membrane-bound protein, since it was mainly detected in the membrane fraction and only appeared later and in low amounts in the supernatant fraction. This protein was not removed from the cell fraction by xylene or hexadecane treatment. Futhermore, analysis of the sequence of protein P1 revealed that this protein contains a potential membrane-associated domain. This status difference between Yops and protein P1 is corroborated by the fact that the regulation of P1 was also shown to differ from that of Yops (5, 7, 16, 43) and that the exportation of P1 probably follows a distinct pathway. Indeed, P1 was shown to contain a typical signal sequence that is cleaved during exportation (7, 40, 44).

The different status of Yops and P1 must be kept in mind, since this has implications in the understanding of both the exportation mechanism and the mode of interaction between these proteins and the host cells.

Yersiniae are equipped with a very efficient secretion mechanism: they can release as much as about 300 μg of proteins per ml of culture. Sequencing the amino terminus of the secreted Yop51 protein revealed that no signal sequence was cleaved at that end of the protein during the secretion process. A similar observation was previously made by Forsberg et al. (24) with protein YopE of *Y. pseudotuberculosis*. It is thus likely that all the Yops are exported by a common specific mechanism that does not involve a precursor carrying an N-terminal extension. Other examples of proteins exported by gram-negative bacteria without removal of a signal sequence have been extensively studied. These include the hemolysins produced by *Escherichia coli*, *Morganella morganii*, and *Proteus vulgaris* or the leukotoxin of *Pasteurella haemolytica* (22, 29, 30, 34, 48), as well as proteases secreted by strains of *Erwinia chrysanthemi* and *Serratia marcescens* (18, 48a). The secretion of all these proteins requires a carboxy-terminal signal region of about 50 amino acids that is not cleaved during secretion. Transposon insertion mutations have been obtained in the structural gene of Yop51, giving rise to truncated proteins (37). In those experiments, truncated Yop51 proteins lacking as many as 200 residues from the end of the molecule were still efficiently secreted, indicating that the C terminus of the protein is not involved in the secretion process (37). Similar observations were made with Yop84 (17) and with Yop48 and Yop37 (38). In conclusion, the release of Yop proteins by yersiniae involves a novel secretion mechanism that requires neither a typical signal sequence nor a C-terminal recognition region.

This investigation prompted us to determine the nucleotide sequences of *yop20* and *yop25* from *Y. enterocolitica* O:9. Gene *yop25* is almost identical to *yopE* from *Y. pseudotuberculosis* (25) and *Y. pestis* and *Y. enterocolitica* O:8 (26). The comparison of *yop25* and *yopE* is given in Fig. 9. As expected from the comparison of restriction maps, the insertion sequence found immediately downstream from *yopE* in *Y. enterocolitica* O:8 (26) was not present in the sequence of *Y. enterocolitica* O:9. The sequence of *yop20* is the first one to be reported for a yersinia. The protein of the PIR data base from NBRF (release 23, January 1990) that has the highest homology with Yop20 is AraH (42). These proteins have 27.4% identity in a stretch of 95 residues.

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