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The *yopM* Gene of *Yersinia pestis* Encodes a Released Protein Having Homology with the Human Platelet Surface Protein GPIbα

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In *Yersinia pestis* KIM, there are 11 Yops (yersinial outer membrane proteins) encoded by the low-Ca^{2+} response virulence plasmid pCD1. Only Yops M and N are found in easily detectable amounts in the culture medium. In this study, we located and characterized the *yopM* gene to obtain clues about its role in the virulence of *Y. pestis*. Rabbit antibody was raised against Yops M and H, copurified from the supernatant of *Y. pseudotuberculosis* 43(pGW600, pCD1 *yopE*::Mu dI1[Ap^r *lac*]). This antiserum was adsorbed with an *Escherichia coli* clone that strongly expressed YopH. The resulting YopM-specific antibody was used to screen a *Hin*dIII library of pCD1. *Hin*dIII-F and several subclones from it expressed YopM in *E. coli* minicells. A DNA fragment of 1.39 kilobases from *Hin*dIII-F was sequenced and found to contain a 367-amino-acid open reading frame capable of encoding a protein with molecular mass (41,566 daltons) and isoelectric point (4.06) similar to those of YopM. The +1 site of the *yopM* gene was determined by primer extension. The DNA sequence contained repeating structures: 11 pairs of exact direct repeats, two exact inverted repeats, and three palindromes, ranging from 10 to 42 bases in size. One consensus 14-amino-acid sequence was repeated six times in the predicted protein sequence. The YopM sequence shares some significant homology with the von Willebrand factor- and thrombin-binding domain of the alpha chain of human platelet membrane glycoprotein Ib. These findings suggested a testable hypothesis for the function of YopM.

The Yops, yersinial plasmid-encoded outer membrane proteins, are a set of major proteins originally discovered in the outer membranes of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (9, 33, 43). These proteins are encoded by approximately 75-kilobase (kb) low-Ca^{2+} response (Lcr) plasmids and are expressed maximally during growth at 37°C in the absence of Ca^{2+} (2, 32, 33, 42). In *Yersinia pestis* grown in vitro, Yops are present only transiently in outer membranes (37, 43), due to rapid degradation by a proteolytic enzyme(s) encoded by the plasminogen activator/coagulase gene (*pla*) on the pPCP1 plasmid unique to *Y. pestis* (39, 40). Yops are seen in the outer membranes of *Y. pestis* that lack this plasmid (37, 39, 40), and when the Lcr plasmid of *Y. pestis* is transferred to *Escherichia coli* or *Y. pseudotuberculosis*, several Yops are expressed (34, 43, 48).

There is growing evidence that the Yops are important in pathogenesis by the three human-pathogenic yersiniae. These proteins evidently are expressed during infection, because anti-Yops antibodies are present in sera recovered from patients or mice infected with *Y. pseudotuberculosis*, *Y. pestis*, or *Y. enterocolitica* (34). In *Y. pestis*, insertions of Mu dI1(Ap^r *lac*) that abolished expression of Yops E, H, or K and L caused avirulence or strongly decreased virulence in mice (42). A mutant of *Y. pseudotuberculosis* deleted for the entire *yopH* gene was found to be avirulent when injected intraperitoneally in mice (3), and the bacteria also lost the inhibition of phagocytosis associated with this Yop (35). A YopE$^-$ insertion mutant of *Y. pseudotuberculosis* constructed by the same laboratory was found to be avirulent in mice when administered orally (12). Thus, it is evident that Yops are important surface antigens that may serve as virulence factors for yersiniae.

In *Y. pestis* KIM, there are 11 Yops (Yops B, C, D, E, F, H, J, K, L, M, and N). Only Yops M and N are released apparently intact into the culture medium along with the V antigen, LcrV (41); the other Yops are turned over, and their degradation products are found in the culture medium. YopM is a strongly acidic protein with a molecular mass of ca. 45 kilodaltons (kDa), which comigrates with YopH in one-dimensional electrophoresis (32, 41). YopN, a less strongly acidic protein with a molecular mass of ca. 35 kDa, is present in smaller amounts than YopM in culture supernatants. The presence of these two Yops both in outer membranes and in the culture supernatant raises the possibility that they have a special role in virulence, which may not be shared by other Yops in *Y. pestis*. We are studying these Yops to define their role in the pathogenesis of plague. In this paper, we report the location, sequencing, and some interesting properties of the *yopM* gene. These findings generated a testable hypothesis for the function of YopM.

MATERIALS AND METHODS

Bacteria and plasmids. *Escherichia coli* K-12 strains HB101 (4) and χ1849 [F$^-$ *tonA53 dapD8 minA1 purE41 supE42* Δ(*fal-uvrB*)*40* λ$^-$ *minB2 his-53 nalA25 metC65 oms-1* T3^r Δ(*bioH-asd*)*29 ilv-277 cycB2 cycA1 hsdR2*] were obtained from Roy Curtiss III (Washington University, St. Louis, Mo.). *E. coli* DH5α was obtained from Bethesda Research Laboratories (BRL), Gaithersburg, Md. The *E. coli* minicell-producing strain M2141 was obtained from Frederick Neidhardt (University of Michigan, Ann Arbor, Mich.). These strains and their cultivation have been described previously (15, 19, 42).

Y. pestis strains all lacked the pigmentation virulence determinant (Pgm$^-$), a property genetically and biochemically unrelated to the low-Ca^{2+} response. *Y. pestis* KIM5 contains pCD1 and is Lcr$^+$, and KIM8 is a derivative of KIM5 that lacks pPCP1, which encodes plasminogen activator, coagulase, and the bacteriocin pesticin. *Y. pseudotuberculosis* 43[pGW600, pCD1 *yopE*::Mu dI1(Ap^r *lac*)] contains

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TABLE 1. Plasmids used in this study

Plasmid and derivative(s)	Description	Reference
pGW600	Tc^r; encodes native Mu repressor	21
pPCP1	Plasmid of *Y. pestis* KIM encoding plasminogen activator, coagulase, and the bacteriocin pesticin	39
pCD1	Lcr plasmid of *Y. pestis* KIM	14
pCD1 *yopE*::Mu dI1 (Ap^r *lac*)	$YopE^-$ Ap^r	42
pBR322	Ap^r Tc^r	5
pHM15	*Pst*I-E, -F, and -I of pCD1	This study
pHM41	*Hin*dIII-E, -F, -O, and -Q of pCD1	This study
pJIT6	*Hin*dIII-F of pCD1	This study
pMP1	Left *Hin*dIII-*Pvu*II fragment of *Hin*dIII-F of pCD1	This study
pMP3, pMP4	Right *Hin*dIII-*Pvu*II fragment of *Hin*dIII-F of pCD1	This study
pIC20R	$lacZ^+$ Ap^r	26
pMB3, pMB8	Left *Hin*dIII-*Bgl*II fragment of *Hin*dIII-F of pCD1	This study
pMB2, pMB7	Right *Hin*dIII-*Bgl*II fragment of *Hin*dIII-F of pCD1	This study
pMC4, pMC9	Right *Hin*dIII-*Cla*I fragment of *Hin*dIII-F of pCD1	This study
pUC19	$lacZ^+$ Ap^r	49
pME15, pME17	*Eco*RV-*Eco*RV fragment of *Hin*dIII-F of pCD1	This study
pMH1, pMH8	*Hin*cII-*Hin*cII fragment of *Hin*dIII-F of pCD1	This study

pCD1 with transposon Mu dI1(Ap^r *lac*) inserted at 22 kb on the pCD1 map and is $YopE^-$ (42).

Descriptions of plasmids pBR322, pUC19, pIC20R, pCD1, and pPCP1 have been reported earlier (5, 14, 26, 39, 49). The plasmids used and constructed in this study are listed in Table 1.

All bacteria carrying drug resistance markers were grown in the presence of the appropriate antibiotic(s) in concentrations of 25 μg/ml for tetracycline (Tc^r) or 50 μg/ml for ampicillin (Ap^r). When required, L-*meso*-diaminopimelic acid was added to the medium at a final concentration of 50 μg/ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside, purchased from Sigma Chemical Co., St. Louis, Mo., was used for screening pUC19 and pIC20R clones with inserts.

Cultivation of yersiniae and *E. coli* for production of Yops M and H. For the detection of YopM in yersiniae, strains were grown at 37°C in the defined medium TMH (43) with 20 mM $MgCl_2$ and no added Ca^{2+}. Yersiniae first were grown in exponential phase in TMH medium at 26°C for approximately seven generations. The cultures were shifted to 37°C when the A_{620} of the culture reached 0.4. Four hours later, the cultures were chilled on ice, and cell-free medium was recovered by centrifugation. Proteins in the culture supernatant were precipitated by adding trichloroacetic acid to a final concentration of 5% (vol/vol). The pelleted culture supernatant (extracellular proteins) and the culture pellet (cellular proteins) were boiled in electrophoresis sample buffer containing 1% (wt/vol) sodium dodecyl sulfate (SDS), 5% (vol/vol) β-mercaptoethanol, 25% (vol/vol) glycerol, and 0.06 M Tris hydrochloride (pH 6.8) to solubilize the proteins.

For the detection of YopM and/or YopH in *E. coli*, isolates containing different plasmids were inoculated in 50 ml of LB medium (27) in a 250-ml Erlenmeyer flask and allowed to grow for 9 h at 37°C with shaking at 250 rpm. The cells were harvested by centrifugation, and the cellular proteins were released by boiling in 0.5 ml of sample buffer.

For purification of Yops M and H, *Y. pseudotuberculosis* 43[pGW600, pCD1 *yopE*::Mu dI1(Ap^r *lac*)] was grown in 10 liters of 3% (wt/vol) NZ Amine A medium from which calcium was removed by chelation with oxalate (6). Following growth of the bacteria at 26°C for ca. 10 generations, the culture was diluted to an A_{620} of 0.4, and growth was allowed to continue until the culture turbidity reached an A_{620} of 4.0. Strong aeration (14 liters/min), agitation (400 rpm), and temperature control were provided by a New Brunswick Scientific Magnaferm fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.). The temperature was then shifted to 37°C, and the culture was allowed to grow for 4 h, to a final A_{620} of 10.0. The cells were collected with a Pellicon concentrator with a Durapore microporous membrane cassette (Millipore Corp., Bedford, Mass.) followed by centrifugation. The cells and supernatant medium were refrigerated overnight prior to further processing.

DNA techniques. The method of Kado and Liu (18) was used for routine screening of plasmid sizes. Plasmid DNA for transformation and restriction digests was isolated by the method of Birnboim and Doly (1). Restriction endonuclease digestion, cloning, and Southern analysis were accomplished by standard methods (25). In the Southern analysis, nick-translated *Pst*I fragments E, I, or E and F of pCD1 were used as probes to hybridize with the *Bam*HI, *Hin*dIII, and *Pst*I digests of pCD1.

Polyacrylamide gel electrophoresis. One-dimensional electrophoresis on SDS-polyacrylamide slab gels was carried out as described by Laemmli (22). Two-dimensional electrophoresis employing nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and SDS-polyacrylamide gel electrophoresis in the second dimension was performed as described previously (29). High- and low-molecular-weight protein standards were purchased from Bio-Rad Laboratories (Richmond, Calif.). Prestained protein molecular weight standards were purchased from BRL, and [^{14}C]methylated protein molecular weight standards were purchased from Amersham Corp. (Arlington, Ill.).

Minicell analysis. Minicells were isolated from *E. coli* M2141, labeled with [^{35}S]methionine (0.1 mCi/2.5 ml; New England Nuclear Corp., Boston, Mass.), and boiled in electrophoresis sample buffer as described by Clark-Curtiss and Curtiss (8) but with a modified labeling protocol. The minicells were preincubated for 90 min at 37°C in "Dapless death medium" (8) containing cycloserine at 40 μg/ml. At the time of labeling, a total of 2.5 ml of minicells (A_{620} of 0.5) was labeled for 60 min with a 5-min chase that followed the addition of 1 ml of LB medium with 2% (wt/vol) methionine. Volumes containing 160,000 cpm were analyzed on a 12% (wt/vol) polyacrylamide gel. The gels were impregnated with En^{3}Hance (New England Nuclear), dried, and exposed on

X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C for 24 h.

Preparation of outer membrane and extracellular products. Outer membranes of *Y. pseudotuberculosis* 43[pGW600, pCD1 *yopE*::Mu dI1(Ap^r *lac*)] from a 10-liter culture were isolated as described by Straley and Brubaker (43). Cells, converted to spheroplasts, were disrupted by sonication. The membranes were collected by centrifugation and then were fractionated into inner and outer membranes by isopycnic sucrose gradient centrifugation.

The 10 liters of supernatant was concentrated to 200 ml by the Minitan ultrafiltration system with a 10,000-molecular-weight cutoff microporous polysulfone membrane (Millipore Corp.). The concentrated supernatant was lyophilized, and the resulting powder was stored at −20°C.

Antiserum preparation. Lyophilized extracellular proteins were boiled in electrophoresis sample buffer. Yops M and H were separated from the rest of the extracellular products by electrophoresis into a 9% (wt/vol) SDS-polyacrylamide preparative gel. The gel portion containing Yops M and H was cut out and placed in glass tubes (9 mm inner diameter), each with a dialysis bag attached to one end. The tubes were immersed in electrophoresis running buffer without SDS, and the Yops were electroeluted into the dialysis bags at 10 mA per glass tube for 4 h at 4°C. The resulting solution containing Yops H and M was emulsified 1:1 in Freund complete adjuvant (Calbiochem-Behring, La Jolla, Calif.). Each of three New Zealand White rabbits was injected intramuscularly and subcutaneously with a total of 1 ml of the preparation (100 μg of protein). The injection regimen was repeated three to four times at 2-week intervals with Freund incomplete adjuvant (Calbiochem). Collected serum was tested by the Ouchterlony immunodiffusion reaction against the extracellular proteins containing Yops H and M.

For the preparation of YopM-specific antibody, a strongly expressing YopH clone containing the *Pst*I-E, -F, and -I fragments of pCD1 (pHM15) in *E. coli* χ1849 was used. Four 500-ml cultures of *E. coli* χ1849 containing pHM15 were grown in 2-liter Erlenmeyer flasks for 9 h at 37°C with shaking at 250 rpm. The cultures were pooled and pelleted by centrifugation, and the cells were solubilized by boiling in 40 ml of electrophoresis sample buffer to release the cellular proteins including YopH. SDS in the whole-cell lysate was removed by two overnight extractions at −20°C with 80% acetone, followed by centrifugation to pellet the SDS-free whole-cell fraction. The final fraction was vacuum dried; dissolved in Western buffer containing 50 mM Tris hydrochloride, 150 mM NaCl, and 5 mM $MgCl_2$ (pH 7.5); mixed with 3 ml of anti-Yops M and H antiserum; and incubated at 37°C with gentle shaking for 6 h. The insoluble immunocomplex was separated from the supernatant by centrifugation. The supernatant was supplemented with 0.02% (wt/vol) sodium azide and stored at 4°C. This antibody preparation reacted with YopM but did not detect YopH in *E. coli* χ1849(pHM15) in a Western immunoblot analysis, indicating that it was YopM specific (data not shown).

Western analysis and immunological screening of colonies. Detection of YopM and YopH by Western blot was performed as described by the Bio-Rad Immun-blot assay kit (Bio-Rad Laboratories, Richmond, Calif.). Electrophoretic transfer of proteins separated on 9% (wt/vol) SDS-polyacrylamide gels to nitrocellulose membranes was performed essentially as described by Towbin et al. (45). Western buffer was used for subsequent steps. After the electrophoretic transfer, unbound sites on the immunoblot were blocked by gentle agitation in 0.5% casein for 30 min at room temperature. Primary antibody at 3 μg/ml was added, and the membrane was incubated for an additional 2 h. After 3 10-min washes in Western buffer, horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Sigma) was added at a 1:1,000 dilution, and incubation was continued for 2 h, followed by three washes in Western buffer. Development was carried out in Western buffer containing 0.015% (vol/vol) H_2O_2 and 0.05% (vol/vol) 4-chloro-1-naphthol and was stopped by washing in distilled water.

An immunological colony blot method (16) was used to detect the expression of YopM and YopH by colonies of *E. coli* χ1849 containing *Hin*dIII or *Pst*I pCD1 fragments cloned in vector pBR322. Bacterial colonies were first grown on petri plates until they reached 1 to 2 mm in diameter and then transferred to a nitrocellulose membrane by blotting. Bacteria on the membrane were allowed to lyse with chloroform vapor for 30 min. The membrane was washed for 30 min in Western buffer containing lysozyme at 40 μg/ml and DNase at 1 μg/ml. The membrane was washed three times in Western buffer and then was blocked and reacted with antibody as described above.

DNA sequencing strategy. DNA fragments were cloned into the bacteriophage M13 vectors M13mp18 and M13mp19 (28). Sequencing was performed by the dideoxy-chain-termination method of Sanger et al. (38) with Sequenase, modified T7 polymerase enzyme, and sequencing kit (United States Biochemical Corporation, Cleveland, Ohio) and the 17-base primer supplied with Sequenase or *yopM*-specific primers synthesized by the Macromolecular Structure Analysis Facility (University of Kentucky, Lexington, Ky.). The resulting DNA sequences were analyzed with the DNASIS (Hitachi Software Engineering Co., Ltd.), PC Gene (IntelliGenetics, Inc., Mountain View, Calif.), and Bionet (IntelliGenetics, Inc.) computer programs. Sequence homology between the α chain of the human platelet glycoprotein Ib and YopM of *Y. pestis* was found by using the searching algorithm of Pearson and Lipman (31) and the Bionet access to the NBRF-PIR database.

RNA isolation. RNA was isolated by a procedure developed by Shirish S. Barve (Ph.D. thesis, University of Kentucky, Lexington, 1989). *Y. pestis* KIM5 was cultured in TMH medium as described above. After 1.5 h of growth at 37°C, 20 ml of culture was rapidly mixed with 7 ml of ice-slush TMH. The culture was centrifuged at 13,800 × *g* at 4°C for 5 min to pellet the cells, and the supernatant was removed and discarded with a baked pipette. The cells were suspended in 6 ml of phenol-cresol denaturing solution containing 100 ml of distilled phenol, 14 ml of *m*-cresol, and 100 mg of 8-hydroxyquinoline; then 0.67 ml of 20% (wt/vol) SDS was added to give a final concentration of 2%, with vigorous mixing. RNA was then extracted from the solubilized whole-cell fraction with LiCl buffer as described previously (23). LiCl buffer (12 ml), containing 0.1 M LiCl, 3 mM Tris hydrochloride, and 3 mM EDTA, pH 7.8, was mixed continuously with the solubilized whole-cell fraction at 60°C for 5 min. The aqueous phase was separated from the phenol phase by centrifugation at 861 × *g* for 5 min at room temperature. The LiCl extraction was repeated, and phenol in the pooled aqueous phases was removed by ether extraction. The ether and lipid were removed from the aqueous phase by centrifugation at 861 × *g* for 5 min. RNA in the phenol-free aqueous phase was precipitated by the addition of 3 volumes of 95% ethanol and NaCl to a final concentration of 0.25 M, followed by overnight incubation at −70°C. The RNA was pelleted at 19,800 × *g* for 45 min at 4°C and washed once with 70% ethanol. The RNA pellet was par-

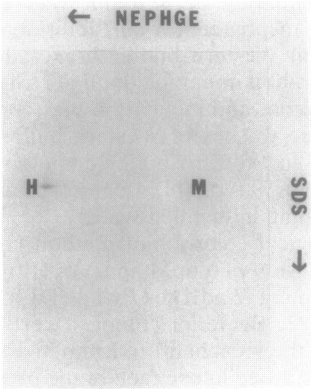

FIG. 1. Two-dimensional NEPHGE electrophoretic profiles of extracellular proteins from *Yersinia pseudotuberculosis* 43 [pGW600, pCD1 *yopE*::Mu dI1(Ap^r *lac*)] used as the source of YopM and YopH in this study. A 10-liter culture of *Y. pseudotuberculosis* was grown at 37°C in oxalated (low-Ca^{2+}) NZ Amine A medium. Proteins in the cell-free supernatant were analyzed by NEPHGE first-dimensional electrophoresis, followed by a second-dimension separation on a 12% (wt/vol) SDS-polyacrylamide gel. Spots due to Yops are indicated by letters.

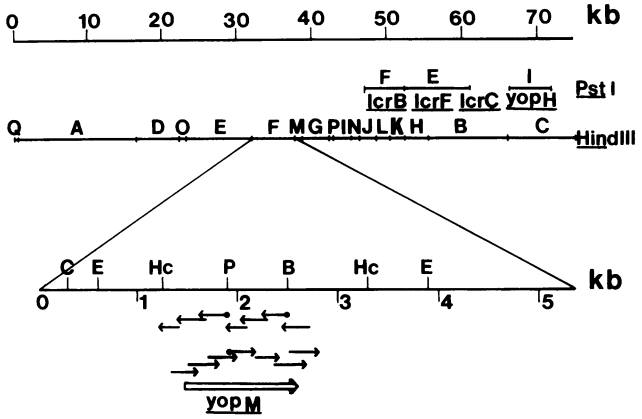

FIG. 2. Strategy for sequencing the *yopM* gene within the *Hin*dIII-F of pCD1. The open arrow shows the position and transcriptional direction of the *yopM* gene. Short solid arrows denote the sequenced templates. Arrows starting without a solid circle denote sequences obtained with synthetic oligonucleotides derived from *yopM* sequence data. Arrows with solid circles denote templates sequenced by using the primer supplied with the Sequenase kit. Sites for restriction endonucleases are *Cla*I (C), *Eco*RV (E), *Hin*cII (Hc), *Pvu*II (P), and *Bgl*II (B). The *Pst*I-E, -F, and -I fragments of pCD1 that contain *lcrC* and *lcrF*, *lcrB*, and *yopH*, respectively, are also indicated.

tially dried and then dissolved in 1 ml of distilled water treated with diethyl pyrocarbonate. The RNA sample was treated with 1 μl of RNase-free DNase I (12 μg/μl; BRL) at 37°C for 30 min and was then extracted three times with phenol-$CHCl_3$ to remove the DNase. The purified RNA sample was precipitated again with ethanol and dissolved in diethyl pyrocarbonate-treated water, and 20-μg portions were made from the stock based on its A_{260}. These were stored at −70°C.

Primer extension analysis. The oligonucleotide was end-labeled with [γ-^{32}P]ATP (New England Nuclear Corp.) by using T4 polynucleotide kinase as described by the BRL Manufacturing instructions. The primer extension experiment followed the protocol described previously (20) with some modifications. Labeled oligonucleotide (6×10^4 cpm) and 20 μg of RNA from *Y. pestis* KIM5 were used. A solution containing 80% deionized formamide, 40 mM PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid], pH 6.4), 400 mM NaCl, and 1 mM EDTA, pH 8, was used for hybridization of the oligonucleotide and the RNA at 30°C overnight. Moloney murine leukemia virus reverse transcriptase (1 μl) and 5 μl of Moloney murine leukemia virus reverse transcriptase reaction buffer (BRL), along with 3.5 μl containing 4 mM of each deoxynucleoside triphosphate (Sigma), 1.25 μl of RNAsin (Promega, Madison, Wis.), and 15.5 μl of diethyl pyrocarbonate-treated water were mixed with each oligonucleotide and RNA sample for the primer extension reaction. The final primer-extended products were suspended in 3 μl of Tris-EDTA buffer with 4 μl of stop solution supplied from the Sequenase kit. The sample was heated at 77°C for 3 min, and 1.5 μl was analyzed on a sequencing gel.

RESULTS

Copurification of YopM and YopH for antibody production. Because *Y. pseudotuberculosis* containing pCD1 was known to strongly express the *Y. pestis* Yops, this organism was used for the large-scale preparation of Yops M and H in this study. In the culture medium of *Y. pseudotuberculosis* 43[pGW600, pCD1 *yopE*::Mu dI1(Ap^r *lac*)], only Yops M and H were found as the dominant species along with a few high- and low-molecular-weight proteins in the background (Fig. 1). By running the extracellular proteins in a gel with a lower percentage of polyacrylamide (9%), Yops M and H were separated sufficiently from the background proteins to permit their copurification by cutting out the ca. 45-kDa region from the preparative gel. Following electroelution and removal of SDS, samples containing Yops M and H were then used to inject rabbits for antibody production.

Screening for YopM-positive clones by colony blot and localization of the *yopM* gene. A YopM-specific antibody preparation was used to screen a *Hin*dIII library of pCD1 in *E. coli* χ1849 to locate the *yopM* gene. One positive clone was found among 3,780 colonies. By restriction enzyme analysis, this isolate containing pHM41 was found to contain four *Hin*dIII DNA fragments, E, F, O, and Q (for location in the pCD1 map, see Fig. 2). Subclones that contained *Hin*dIII-F of pCD1 (pJIT6) also permitted weak expression of YopM, detectable by Western analysis, suggesting that the *yopM* gene was in the 5.35-kb *Hin*dIII-F. Subclones were made in high-copy-number plasmids (pUC19 or pIC20R) to increase the expression of YopM in *E. coli* (Fig. 3). Figure 4 indicates the strength of YopM expression by the subclones in the minicell-producing *E. coli* M2141.

Evidently, two unique restriction enzyme sites, *Pvu*II and *Bgl*II, were located inside the *yopM* gene, because subcloning of *Hin*dIII-F by using the above two sites abolished the expression of YopM (Fig. 3). *E. coli* containing *Hin*dIII-*Cla*I (pMC4 and pMC9), *Eco*RV-*Eco*RV (pME15 and pME17), and *Hin*cII-*Hin*cII (pMH1 and pMH8) fragments retained YopM expression, detected as a protein of ca. 46 kDa molecular mass. The smallest subclones permitting YopM expression were pMH1 and pMH8, which contained the 2-kb *Hin*cII-*Hin*cII fragment (Fig. 3). This localized the *yopM* gene to within the two *Hin*cII sites of *Hin*dIII-F of pCD1.

Two forms of YopM. Only one form of YopM was detected by autoradiography in *E. coli* minicells (Fig. 4). However, when Western analysis was used to examine the same samples, two forms of YopM were observed: the 46-kDa

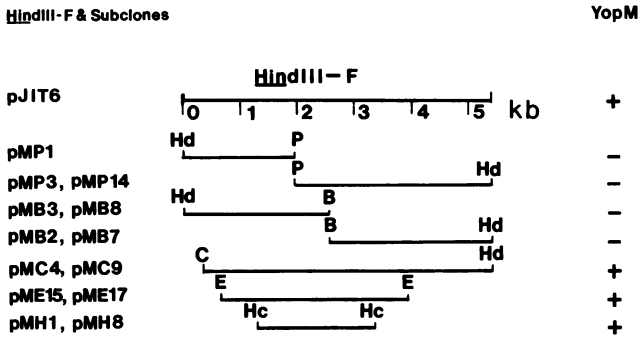

FIG. 3. Subclones of *Hin*dIII-F and their expression of YopM in *Escherichia coli* DH5α. The expression of *yopM* was assessed from Western blots. Whole-cell lysates of the *E. coli* strains containing different plasmids were electrophoresed in a 9% (wt/vol) SDS-polyacrylamide gel. The protein bands were transferred onto a nitrocellulose membrane. Expression of YopM was detected by YopM-specific antiserum. Sites for restriction endonucleases are *Cla*I (C), *Eco*RV (E), *Hin*cII (Hc), *Hin*dIII (Hd), *Pvu*II (P), and *Bgl*II (B).

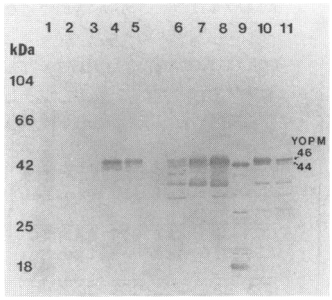

FIG. 5. Western analysis of two forms of YopM produced in *Escherichia coli* and *Yersinia* spp. The production of YopM was examined in soluble whole-cell lysates of *E. coli* DH5α containing pUC19 (lane 2) or the *yopM*-encoding pMH1 (lane 4) and *E. coli* M2141 minicells containing pUC19 (lane 3) or pMH1 (lane 5). Soluble whole-cell lysates and supernatant from *Y. pestis* KIM5 (lanes 6 and 9, respectively), *Y. pestis* KIM8 (lanes 7 and 10, respectively), and *Y. pseudotuberculosis* 43[pGW600, pCD1 *yopE*::Mu dI1(Ap^r *lac*)] (lanes 8 and 11, respectively) were also examined. Lane 1 contains prestained molecular mass markers with sizes indicated in kilodaltons.

form seen in the autoradiogram, and a less prominent lower-molecular-mass form (ca. 44 kDa) (Fig. 5). The high-molecular-mass form was also the dominant species in *E. coli* DH5α. In *Y. pestis* KIM5, two forms of YopM were present in the solubilized whole cells (lane 6), but the two species were present in similar amounts. Only the low-molecular-mass form was found in the supernatant. In contrast, more of the high- than the low-molecular-mass form of YopM was found in both the whole cells and the supernatant from both *Y. pestis* KIM8 and *Y. pseudotuberculosis* 43[pGW600, pCD1 *yopE*::Mu dI1(Ap^r *lac*)]. These latter yersiniae lack pPCP1, which encodes the plasminogen activator responsible for the rapid turnover of Yops in *Y. pestis* KIM5. In all of the yersiniae, some further degradation of YopM was evident in both whole-cell lysates and supernatant preparations. This was not seen in *E. coli*; the protease(s) responsible has not been identified.

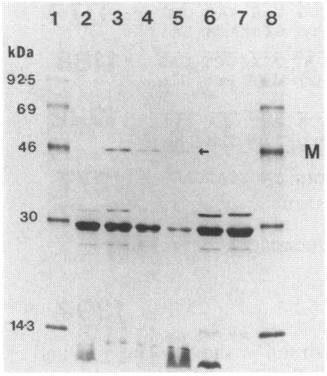

FIG. 4. Expression of *Hin*dIII-F and subclones in *Escherichia coli* minicells. ^{35}S-labeled minicells were prepared from *Escherichia coli* M2141 containing either the cloned *Hin*dIII-F of pCD1 in pBR322 (pJIT6, lane 2) or subcloned fragments of *Hin*dIII-F. Subclones included: *Hin*cII-*Hin*cII in pUC19 (pMH1, lane 3), *Eco*RV-*Eco*RV in pUC19 (pME15, lane 4), and *Cla*I-*Hin*dIII in pIC20R (pMC4, lane 5). Lanes 6 and 7 contain minicells having only the vector plasmids pBR322 and pUC19, respectively. Lanes 1 and 8 contain molecular mass markers, for which sizes are indicated in kilodaltons. The YopM band (M) migrated the same distance as the 46-kDa molecular mass marker. YopM was only weakly expressed in the minicells that contained *Hin*dIII-F (lane 2). A total of 1.6 × 10^5 cpm was loaded into each lane.

DNA sequence of the *yopM* gene and mapping of the transcriptional start point. Figures 2 and 6 show the sequencing strategy and DNA sequence, respectively, for 1.397 kb within the 2-kb *Hin*cII fragment of pCD1. There was a single large open reading frame of 1,104 bases, capable of translation into a 367-amino-acid, 41,556-Da polypeptide (Fig. 6). Apparently, YopM migrates abnormally in SDS-polyacrylamide gels, because this molecular mass is different from the 46 kDa estimated for YopM by electrophoresis. No signal peptide (30) was apparent at the amino terminus of the predicted protein or adjacent to the other two methionines at positions 239 and 341. We have not determined whether the ATG at position 164 (Fig. 6) is the actual translational initiation codon; it defines the start of the largest open reading frame consistent with the primer extension data. The isoelectric point of YopM was calculated to be 4.06 by the CHARGPRO computer program in PC Gene (IntelliGenetics, Inc.), which determines the net charge of a sequence as a function of pH. This highly acidic value is consistent with the location of YopM in two-dimensional electrophoresis. The 5′ end of the mRNA was mapped by primer extension of RNA isolated from *Y. pestis* KIM5 cells cultured at 37°C in TMH containing no Ca^{2+} (Fig. 7). There apparently were two transcription start points, at T and G, respectively 48 and 47 bases upstream from the potential ATG start codon (Fig. 6). Little primer-extended product was found when the mRNA came from cells grown in the presence of Ca^{2+}, consistent with previous findings that the expression of other Yops is regulated by Ca^{2+} at the transcriptional level (42).

Sequence analysis. The YopM sequence is A+T rich, with A and T residues constituting 62% of the total base composition. This is similar to the percent A+T in LcrV (62.1%) (S. B. Price, K. Y. Leung, S. S. Barve, and S. C. Straley, submitted for publication). Both *yopM* and *lcrV* have a greater percentage of A and T residues than do *lcrG* and *lcrH* of *Y. pestis* or *yopH* and *yopE* of *Y. pseudotuberculosis*, in which A and T residues constitute 53.1, 55.8, 53.6, and 55.4% of the total base composition, respectively (3, 12; Price et al., submitted for publication). A short, A+T-rich hairpin loop was located both upstream and downstream from the *yopM* gene; their function is not presently known.

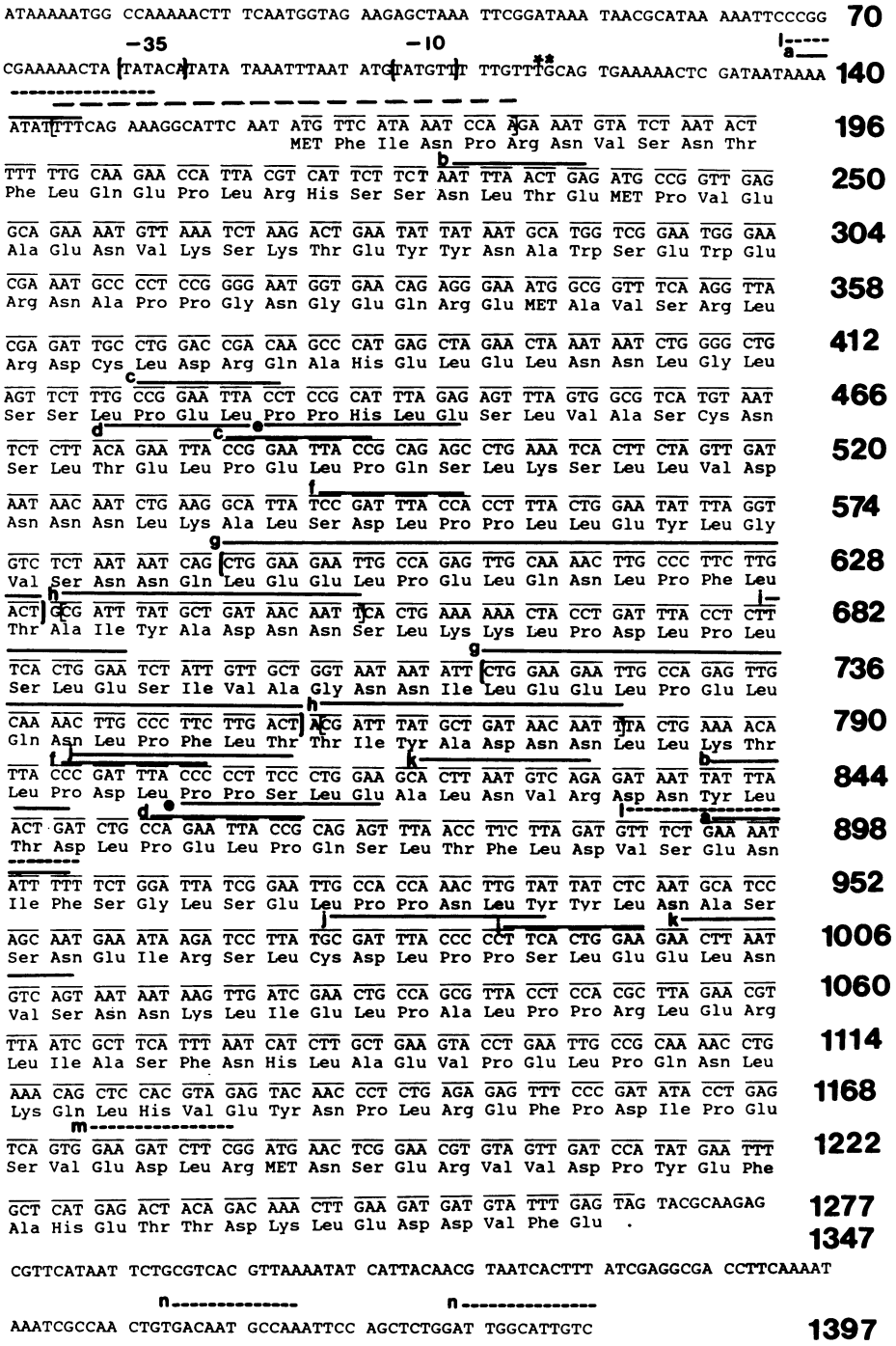

FIG. 6. Sequence of the *yopM* gene. The sequence of the nontranscribed (antisense) strand is shown. The −10 and −35 regions of the possible promoter are indicated, and the likely transcriptional start points are marked with asterisks. The dashed line shows the region homologous to the 35-base oligonucleotide used in the primer extension experiment. Solid lines, labeled a to k, show 11 pairs of exact direct repeats. Broken lines, labeled l to n, show the exact inverse complements. The two longest pairs of directly repeated sequences, g and h, are in the same reading frame and repeat at the amino acid level.

Eleven pairs of exact repeats and three inverse complements, ranging from 10 to 42 bases in size, were identified in the sequence (Fig. 6). Many of these repeated structures were clustered near the middle of the *yopM* gene. The largest and second largest directly repeated sequences were separated by only one base (Fig. 6). These two pairs of DNA repeats also repeated at the amino acid level, giving rise to 14- and 6-amino-acid repeats, respectively. In addition, there was homology at the amino acid level in the region immediately surrounding these exact repeats. There were 32 identical amino acids in a 37-amino-acid stretch containing each set of directly repeated sequences (Fig. 8B). In the hydropathy analysis (Fig. 8A), YopM had several hydrophilic domains, with a clustering of these near the N-terminus. The

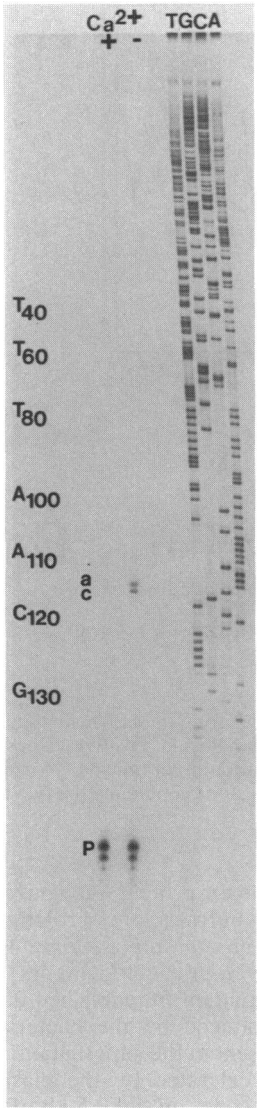

FIG. 7. Determination of the transcriptional start point of the *yopM* gene. Primer extension was carried out with RNA isolated from *Yersinia pestis* KIM5 grown at 37°C in defined medium lacking or containing 2.5 mM $CaCl_2$ (indicated by − and +, respectively). The sequencing reactions, indicated by A, C, G, and T, were primed with the same oligonucleotide used for the primer extension; accordingly, the sequence is the complement of the one shown in Fig. 6. Bands due to the oligonucleotide primer are indicated by a P. The two primer-extended products overlapped the A and C residues of the sequencing reactions and are indicated by a and c, respectively. Nucleotide positions are numbered in accordance with the numbering of the complementary sequence shown in Fig. 6.

carboxyl end of the protein at amino acid 359 was found to have the highest hydrophilic value. YopM also contains five major hydrophobic domains. The two most hydrophobic of these contained part of the two 14-amino-acid repeats. Each of these repeats started in the adjacent hydrophilic domain and contained the peak of hydrophilicity of that domain as well as about half of the hydrophobic domain (Fig. 8). The hydropathy profiles of these two 14-amino-acid repeats (long arrows in Fig. 8) were remarkably alike, and this characteristic might be significant in determining the configuration and antigenicity of the YopM molecule.

These two longest direct repeats were members of a family of six repeats that lay within the central two-thirds of the YopM sequence (from residues 77 to 321; Fig. 9). This family of sequences 19 residues in length contained 14 conserved amino acids. The consensus sequence was ASNN-L-EL PELPQ-L--L (Fig. 9).

Homology between YopM and human GPIbα. The thrombin- and von Willebrand factor-binding domain of the α chain of platelet membrane glycoprotein Ib (GPIbα [24, 44]) was found to be closely related to the predicted protein sequence of YopM (Fig. 10). They had 42 identical amino acids in a 125-amino-acid stretch with only four gaps, giving a 33.6% identity. Furthermore, 52 additional amino acids in this region belonged to the same amino acid group (i.e., hydrophilic or hydrophobic amino acids). If one takes these into consideration, the similarity in the same region goes up to 75.2%. The normalized alignment score (10, 11) was calculated as 250, suggesting that the homology between the two sequences was probably significant.

DISCUSSION

In this study, we located and sequenced the *yopM* gene of *Y. pestis* and presented evidence that it is regulated at the transcriptional level by Ca^{2+}. The *yopM* sequence yielded some important insights. The conserved upstream sequences in the promoter regions of *yopE* and *yopH* in *Y. pseudotuberculosis* are not present in *yopM* (3, 12), nor is there homology between the upstream region for *lcrGVH* and that for *yopM* (Price et al., submitted for publication). Yet all of these operons, *yopE*, *yopH*, *yopM*, and *lcrGVH*, are regulated at the transcriptional level by Ca^{2+} (42; Price et al., submitted for publication). This suggests that there is no highly conserved DNA sequence determinant for the Ca^{2+} regulatory effect within ca. 200 bases of the 5′ ends of these genes and raises the possibility that novel regulation may be revealed in future molecular studies of *yop* expression. No significant homology was found between *yopM* and *yopE* and *yopH* of *Y. pseudotuberculosis* with the DNASIS and PC Gene computer software for DNA and protein homology analysis. In the polycistronic V operon of *Y. pestis*, cistrons (*lcrG*, *lcrV*, and *lcrH*) were spaced less than 15 base pairs apart (Price et al., submitted for publication). No other open reading frames were found within 130 bases of the 3′ end of the *yopM* gene. In our laboratory, we have sequenced the *lcrGVH* region (Price et al., submitted for publication), and no homology was found when these sequences were compared with that of *yopM*. These nonhomologous virulence-related genes probably all serve distinct functions in pathogenesis, and their organization in separate operons provides for possible independent regulation as well as for coordinate regulation by *lcr* loci.

In the *yopM* DNA sequence, 11 pairs of exact direct repeats and five inverse complements, ranging from 10 to 42 bases in size, were found. The occurrence of these sequences was unique to *yopM*. Only one pair of exact direct repeats, 11 bases long, is found in *yopH* of *Y. pseudotuberculosis*, and this sequence bears no significant homology to the repeats found for *yopM*. No significant homology with any of the repeated sequences in *yopM* was found in *yopE* of *Y. pseudotuberculosis* or in *lcrG*, *lcrV*, and *lcrH* of *Y. pestis*. These structures in *yopM* probably relate to the distinct antigenicity, configuration, and function of this protein.

Two forms of YopM were found in *Y. pestis*: the dominant higher-molecular-weight form, probably the precursor form, found to be cell associated, and a lower-molecular-weight, probably processed form, found predominantly in the super-

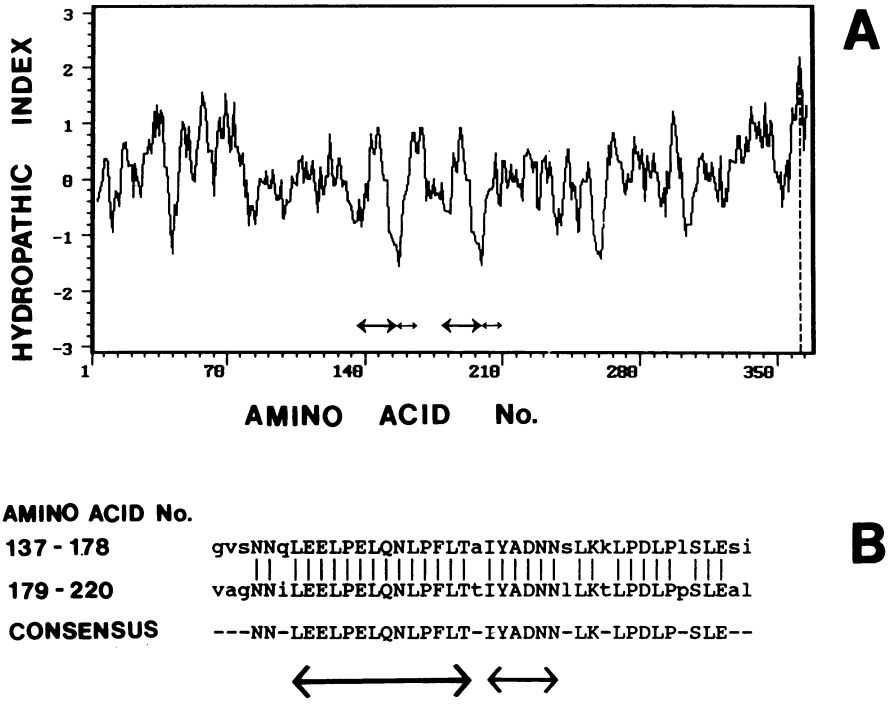

FIG. 8. Hydropathy analysis of the predicted YopM protein sequence. (A) Hydropathic index for YopM. The method of Hopp and Woods (17) was used to calculate the hydropathic index, employing an average group length of six amino acids. Positive values indicate hydrophilic groups, whereas the negative values indicate hydrophobic groups. The two longest pairs of exact direct repeats, 14 and 6 amino acids long, are indicated by long and short arrows, respectively. (B) Alignment of the two 42-amino-acid regions containing the two longest pairs of exact direct repeats. Identical residues are shown in capital letters.

natant (Fig. 5). In the NEPHGE analysis, both forms of YopM migrated to the same isoelectric point (data not shown). The YopM-specific antibody raised against the form of YopM in the culture medium of *Y. pseudotuberculosis* 43[pGW600, pCD1 *yopE*::Mu DI1(Ap^r *lac*)] (predominantly the higher-molecular-weight form) recognized the lower-molecular-weight form as well. This demonstrates their isoelectric and immunological relatedness. Two forms of YopM were also found in *E. coli* containing the smallest subclone, pMH1 (Fig. 5, lanes 4 and 5). We sequenced the entired 2 kb of yersinial DNA in pMH1 and found no other open reading frame that was big enough to encode the second protein band. Accordingly, the smaller form of YopM is not another protein recognized by the YopM-specific antibody. Furthermore, prolonged storage of the supernatant of *Y. pseudotuberculosis* 43[pGW600, pCD1 *yopE*::Mu DI1(Ap^r *lac*)] at 4°C favors the processing of YopM in the supernatant (unpublished data). This suggests that proteases produced by the bacteria can mature the YopM already present in the supernatant. The maturation of YopM may be accelerated by the plasminogen activator product of the *pla* gene, on the 9.5-kb pPCP1 (40).

For *Y. pestis* KIM8 and *Y. pseudotuberculosis*, neither of which has pPCP1, the high-molecular-weight form of YopM was present as the dominant species both in whole cells and in the culture medium. However, some low-molecular-weight form was present in Western analysis of these yersiniae, along with some still lower molecular weight species that also reacted with the anti-YopM antibodies (Fig. 5). These proteins presumably were generated by other prote-

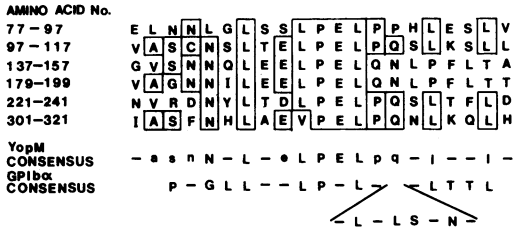

FIG. 9. Internal homologies in YopM. Six direct repeats found in the YopM sequence have been aligned with a minimum number of gaps. Highly conserved residues are enclosed in boxes. The consensus sequence is indicated below, where the highly conserved residues are shown in capital letters; other residues that occur in three or four repeats are shown in lowercase letters. A dash is used where there is no clear preference. The longest two direct amino acid repeats are in the regions of residues 143 to 156 and residues 185 to 198. The consensus sequence derived from the tandem leucine-rich repeats in the α chain of GPIb (24) is also shown.

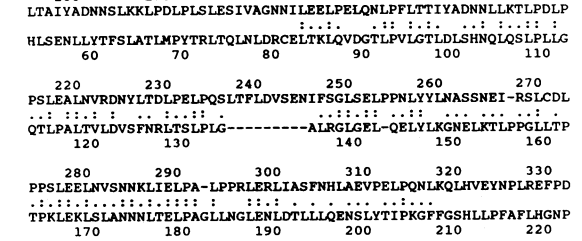

FIG. 10. Sequence homology between the α chain of the human platelet glycoprotein Ib (GPIbα) and YopM of *Yersinia pestis*. Identical residues are indicated by two dots. Single dots indicate amino acids belonging to the same hydrophilic or hydrophobic grouping.

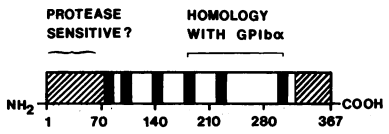

FIG. 11. Representation of YopM. The hydrophilic regions are indicated by hatched areas. Leucine-rich repeats are shown as solid black areas. The numbers indicate the length in amino acids.

ases present in the cells and might be break-down products of YopM. In the pulse-chase experiment of Sample and Brubaker (37), Yops such as B, C, D, E, F, J, H, and presumably M (YopM migrates with YopH in a one-dimensional gel) were rapidly degraded in the outer membranes of *Y. pestis* KIM, and low-molecular-weight degradation products gradually accumulated. These findings suggested that the degradation of membrane-associated Yops occurred at multiple possible sites, generating a variety of smaller species. A different picture evidently holds for YopM. Sample and Brubaker's data and our data show that YopM is degraded in outer membranes, following the same fate as other Yops. However, this protein can be released intact into the supernatant (*Y. pseudotuberculosis* or *Y. pestis* KIM8). There, it can be degraded exogenously, but one cleavage evidently is favored, generating the predominant low-molecular-weight processed form. This cleavage evidently also can take place slowly in whole cells of yersiniae or *E. coli*. It is significant that even in the pPCP1$^+$ *Y. pestis* KIM5, significant amounts of this predominant processed form of YopM escape degradation and are released into the supernatant. Furthermore, during infection, the proteolytic activity of the plasminogen activator may be occupied by host proteins. As a result, degradation of Yops may be limited in vivo, and YopM may be present in both full-length and processed forms on the bacterial surface as well as in blood and interstitial fluid. Both forms of the protein may be present and functional in vivo.

The elucidation of the role of YopM in the virulence of *Y. pestis* will require future experimentation, such as mutant analysis and virulence studies in mice. However, it is intriguing to find that the YopM sequence shares some significant homology with the α chain of human platelet membrane glycoprotein Ib (GPIbα). The homologous region of GPIbα is located at the N-terminal portion (Fig. 10), expressed on the surface of platelets, and contained within the von Willebrand factor- and thrombin-binding domain (24, 44). The α chain of GPIb includes seven tandem leucine-rich repeated sequences in the von Willebrand factor- and thrombin-binding domain (24), which share some homology with the six direct repeats in YopM, in which five leucines are present among 14 conserved amino acids (Fig. 9). Furthermore, there are 22 leucines among the 42 identical amino acids between the homologous region of YopM and the α chain of GPIb (Fig. 10). A diagram showing some of the predicted features of YopM is shown in Fig. 11. YopM is hydrophilic at both the amino and carboxyl termini, with the six leucine-rich repeats distributed in between. The region homologous with the α chain of GPIb includes three repeats located near the carboxyl terminus. If the processing of YopM occurs in the hydrophilic termini, both high- and low-molecular-weight forms of YopM would have the repeats and the homologous region with the α chain of GPIb. Accordingly, it is possible that the two forms of YopM are biologically functional and play the same role in virulence.

GPIbα serves as one of the two known platelet receptors for von Willebrand factor (36). The von Willebrand factor-GPIbα interaction is essential for normal hemostasis by mediating platelet adhesion to the exposed subendothelium of injured vessels and contributing to platelet-platelet aggregation (7, 46, 47). Von Willebrand factor binding to GPIbα may be one of the events mediating the development of thrombotic lesions (13). Thrombin converts fibrinogen to fibrin and also functions to activate platelets (47). It is possible that in human plague, as YopM is released, a competitive effect develops to prevent the normal GPIbα and von Willebrand factor interaction and/or to tie up thrombin in an ineffective complex. This may undermine the effectiveness of the blood-clotting mechanism as a means of entrapping bacteria and in turn further facilitate the spreading of *Y. pestis* in the host.

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ADDENDUM IN PROOF

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence data base and have been assigned the accession number M25810.

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