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Yersinia pestis YopM: Thrombin Binding and Overexpression

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In previous studies, *Yersinia pestis* YopM has been shown through mutational analysis to be necessary for virulence in mice and found to have homology with the thrombin-binding domain of the platelet receptor GPIbα. In this study, YopM was purified and shown by dot blot and chemical cross-linking tests to bind to human α-thrombin. No cross-linked product could be detected when human prothrombin was incubated with YopM. As a functional test of thrombin binding, it was shown that native but not boiled YopM inhibits thrombin-induced aggregation of human platelets. Control tests showed that YopM did not inactivate the platelets themselves, nor was its effect a nonspecific consequence of its very acidic isoelectric point. Microsequencing of YopM revealed an intact N terminus, indicating that functional YopM is not processed at the N terminus or secreted by a mechanism involving a cleavable signal sequence. Further characterization was made of an interesting effect on *yopM* expression that had been noticed in a previous study. A 1.5-kb *Hae*III subclone overexpressed YopM in both *Y. pestis* and *Escherichia coli* compared with a larger clone containing the 5.3-kb *Hin*dIII-F fragment. To search for a possible regulator of YopM expression, the *Hin*dIII-F fragment was sequenced, revealing several open reading frames and three large repeated sequences. Deletional analysis showed that these were not involved in regulation of *yopM*. The data implicated a DNA structure 5′ to *yopM* in moderating *yopM* expression.

The Yops are a set of secreted proteins encoded by the low-Ca^{2+} response (Lcr) virulence plasmids of the three yersiniae that are pathogenic in humans (*Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*). Expression and secretion of the Yops are carefully regulated in vitro, occurring maximally at 37°C in the absence of Ca^{2+} (3, 33, 35, 36, 48, 49).

The Yops appear to be secreted by a novel mechanism encoded by the Lcr plasmid (17, 30, 31). Studies with *Y. enterocolitica* and *Y. pseudotuberculosis* have shown that this mechanism does not involve a cleavable N-terminal signal required for traditional SecY-mediated secretion (8, 31) or recognition at the C terminus as found in the hemolysin-like secretion system (8, 28, 32). However, it was found that as few as 48 N-terminal amino acids were required for secretion of chimeric proteins, but no consensus sequence in this region has been found (29).

Y. pestis differs from the other yersiniae in that all Yops except M and N (47) are rapidly degraded by the pPCP1-encoded plasminogen activator which is not present in *Y. enterocolitica* and *Y. pseudotuberculosis* (45, 46). Because YopM is not degraded, it might interact with host defenses in a manner different from that of the other Yops.

Several of the Yops, including YopM, have been shown through mutational analysis to be essential for full virulence in mice (4, 11, 24, 32, 48). Several Yops have also been characterized as follows with regard to function: YopE is both antiphagocytic (39) and a cytotoxin that disrupts microfilament structure, with YopD being necessary for the entry of YopE into the target cell (40); YopH is antiphagocytic (38) and a phosphotyrosine phosphatase (2, 16), perhaps interfering with cellular signalling; YopN has been implicated in a novel first step of sensing Ca^{2+} (10).

Analysis of the YopM protein revealed significant homology with a region of the human platelet surface protein GPIbα (25). GPIbα binds thrombin and the von Willebrand factor, causing platelets to aggregate and initiate clot formation. In addition, thrombin activates platelets, causing them to release inflammatory mediators, including chemotactic factors, growth factors, vasoactive substances, mediators of vascular permeability, procoagulants, and bactericidal proteins.

The homology was in the general region of GPIbα known to bind thrombin, suggesting that YopM also might bind thrombin. This might prevent thrombin from binding to the platelet receptor, thereby preventing platelet aggregation and activation. In support of this idea, it was shown that a mixture of proteins from a YopM-expressing but not a $YopM^-$ strain of *Y. pestis* inhibited thrombin-induced platelet aggregation in vitro (24).

In the present study, YopM was purified and shown to interact directly with thrombin. Purified YopM was also able to inhibit thrombin-induced platelet aggregation but not aggregation mediated by other platelet agonists (ADP, epinephrine, and collagen).

In addition, the DNA flanking the *yopM* gene was further characterized, and a 5′ region that functions to control the level of *yopM* expression was identified.

MATERIALS AND METHODS

Bacteria and plasmids. *Escherichia coli* K-12 HB101 (5) and XL1-Blue {*endA1 hsdR17* [r_K^- m_K^+] *supE44 thi-1* λ^- *recA1 gyrA96 lac* [F′ *proAB lacI*^{q} *ZΔM15* Tn*10* (Tet^r)]} (Stratagene, La Jolla, Calif.) were used as hosts for the derivatives of plasmids pBR322 (6) and pBluescript SK^- (Stratagene). The plasmid derivatives used in this study are described in Table 1. pCD1 *yopH*::Mu dI1734 was derived from pCD1::Mu dI1 69.5 (48) by replacing the original Mu dI1 with Mu dI1734 as described previously (50) to stabilize the insert against further transposition.

All *Y. pestis* strains lacked the pigmentation virulence determinant (Pgm^-), rendering them avirulent except by an

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TABLE 1. Plasmids used in this study

Plasmid	Description	Reference or source
pCD1	Lcr plasmid of *Y. pestis* KIM	14
pCD1 *yopM*::*lacZYA*	YopM$^-$	24
pCD1 *yopH*::Mu dI1734	YopH$^-$ Km^r *lac*	This study
pBR322[a]	Ap^r Tc^r	6
pJIT6[b]	*Hin*dIII-F fragment of pCD1	25
pBS3[b]	*Hae*III fragment of *Hin*dIII-F of pCD1	24
pBS4[b]	*Eco*RV fragment of *Hin*dIII-F of pCD1	This study
pBS7[b]	*Hae*III-*Eco*RV fragment of *Hin*dIII-F of pCD1	This study
pBS11[b]	*Eco*RV-*Xma*III fragment of *Hin*dIII-F of pCD1	This study
pBS13[b]	*Acc*I-*Eco*RV fragment of *Hin*dIII-F of pCD1	This study
pBS15[b]	*Eco*RV-*Fsp*I fragment of *Hin*dIII-F of pCD1	This study
pBluescript SK$^-$	Ap^r	Stratagene
pBS10	*Hae*III-*Eco*RV fragment of *Hin*dIII-F of pCD1	This study

[a] For all derivatives of pBR322, fragments were cloned into the *Eco*RV site of the *tet* gene such that the *yopM* promoter is in the opposite direction of the plasmid *tet* promoter.
[b] The locations of restriction sites used in subcloning are shown in Fig. 5 and 6.

intravenous route of infection (51). *Y. pestis* KIM5 is Lcr$^+$ and contains the pPCP1 plasmid encoding the plasminogen activator implicated in degradation of the Yops (46) and the 110-kb plasmid encoding the F1 capsular protein and the murine toxin (37). *Y. pestis* KIM5-3233 is a YopM$^-$ mutant (*yopM*::*lacZYA*) described previously (24). *Y. pestis* KIM8 is a derivative of KIM5 missing pPCP1. *Y. pseudotuberculosis* 43 (48) lacks its native Lcr virulence plasmid. Antibiotics, when indicated, were used at 100 μg/ml for ampicillin and 25 μg/ml for kanamycin and streptomycin.

DNA techniques. Plasmid DNA for restriction digests and transformations was obtained by the method of Birnboim and Doly (1). Restriction endonuclease digestions and cloning were accomplished by standard methods (27). Sequencing of the *Hin*dIII-F fragment was accomplished by the dideoxy-chain termination method (43), with the Sequenase 2.0 sequencing kit (U.S. Biochemical, Cleveland, Ohio). Double-stranded DNA templates were prepared by the alkaline lysis method (27) followed by removal of proteins with acidified phenol (pH 4.0) (Ameresco, Solon, Ohio). Oligonucleotides for sequencing were either the universal or reverse primer (United States Biochemical) specific for sequences flanking the multiple-cloning region of pBluescript or sequence-specific primers synthesized by the Macromolecular Structure Analysis Facility (University of Kentucky, Lexington).

Southern blot analysis. *Y. pestis* plasmid or genomic DNA was digested with the restriction enzyme *Hin*dIII or *Bam*HI and resolved on a 0.75% agarose gel (Sigma Chemical Co., St. Louis, Mo.). The DNA was vacuum blotted with a Trans-vac apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) onto GeneScreen membranes (New England Nuclear, Boston, Mass.). The blot was hybridized at 42°C overnight with an *Rsa*I-*Nco*I fragment of *Hin*dIII-F containing the repeated sequence R2 (see Fig. 5), labelled by nick translation (Promega, Madison, Wis.) under conditions allowing 20% mismatch. The stringency of hybridization was altered in some experiments by decreasing the formamide content in the hybridization buffer from 50 to 10% and hybridizing at room temperature, allowing up to 40% mismatch. In experiments with the *Shigella flexneri* virulence plasmid (a gift from Tony Maurelli, Uniformed Services University of the Health Sciences), the DNA was digested with *Sal*I. For these experiments, hybridization conditions which allowed up to 40% mismatch were used.

Preparation of cytoplasmic extracts, mixed membranes, and extracellular products for SDS-PAGE and Western analysis of YopM. *Y. pestis* and *Y. pseudotuberculosis* strains were grown in the defined medium TMH (48) without added Ca^{2+} at 26°C for approximately seven generations; the bacteria were then inoculated into fresh medium to an A_{620} of 0.2. After one doubling, the culture was shifted to 37°C and grown for 4 h before the bacteria were harvested by centrifugation. Extracellular proteins were recovered from the supernatants by the addition of ammonium sulfate to 70% saturation and precipitation overnight at 4°C. Washed bacteria were resuspended in 10 mM Tris-HCl (pH 8.0) and lysed by passage through a French press at 20,000 lb/in^2. Unlysed cells were removed by centrifugation at 12,200 × *g* for 10 min at 4°C. Membranes (inner plus outer) were separated from the cytoplasmic extract by centrifugation in a Beckman TL-100 ultracentrifuge at 263,800 × *g* for 30 min at 4°C. All fractions were stored in 10 mM Tris-HCl (pH 8.0) at −20°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with either 10 to 15% (wt/vol) acrylamide Phastsystem gradient gels (Pharmacia-LKB, Piscataway, N.J.) or standard 12.5% (wt/vol) acrylamide gels as described by Laemmli (22). Prestained and unstained molecular weight standards were from GIBCO-BRL (Gaithersburg, Md.). Silver staining of Phastsystem gels was carried out according to the manufacturer's protocol. Western blot analysis of SDS-PAGE gels was performed as previously described (34) with Immobilon P membranes (Millipore, Bedford, Mass.).

Preparation and use of antibodies. YopM-derived antipeptide antibodies were made according to previously reported methods (34). Briefly, peptides corresponding to the C-terminal amino acids 356 to 367 (NH_2-[C]ETTDKLEDDVFE-COOH) and to internal residues 185 to 196 (NH_2-[C]LEEL PELQNLPF-COOH) of the YopM protein were synthesized by the Macromolecular Structure Analysis Facility (University of Kentucky, Lexington). An additional amino-terminal cysteine residue ([C]) was added to each peptide to provide a site for hapten conjugation. The internal peptide was coupled to the protein carrier bovine serum albumin (BSA) with 0.25% (vol/vol) glutaraldehyde; the C-terminal

peptide was coupled to BSA by using the immunogen conjugation kit from Pierce Imject (Rockford, Ill.) with sulfo-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC)-activated BSA.

For each peptide, two female New Zealand White rabbits were given subcutaneous injections at multiple sites, with a total of 1 ml of a 1:1 emulsion of phosphate-buffered saline (PBS) (8% [wt/vol] NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4 [pH 7.2]) containing 1 mg of BSA-peptide conjugate with Freund's complete adjuvant. Antipeptide antibodies were detected by an enzyme-linked immunosorbent assay. Rabbits were given booster injections with 1 mg of BSA-peptide conjugate emulsified in Freund's incomplete adjuvant when antibody titers dropped.

Antibodies were purified from the serum on Sepharose 6B (Pharmacia-LKB) peptide affinity columns prepared and used according to the manufacturer's protocol. The affinity-purified antibody was stored in PBS at −20°C.

YopM purification. YopM was purified from *Y. pseudotuberculosis* 43 containing the *Y. pestis* Lcr plasmid carrying a mutation in *yopH* (pCD1 *yopH*::Mu dI1734) and the plasmid pBS10 (Table 1), which overexpresses YopM. This strain is designated *Y. pseudotuberculosis* 43(pCD1 *yopH*::Mu dI1734, pBS10). Cultures were grown to induce maximal expression of YopM in the defined medium TMH at 37°C without added Ca^{2+}, and proteins were recovered from the culture supernatant as described earlier. The supernatant proteins were suspended in and dialyzed against imidazole buffer (50 mM imidazole, 1 mM EDTA, 0.1% β-mercaptoethanol [pH 7.5]). This mixture was applied to a DEAE-Sephacel (Sigma) column equilibrated in imidazole buffer. The column was washed extensively with buffer until no further protein eluted from the column. Bound proteins were subsequently eluted with a NaCl step gradient from 100 to 400 mM in 50 mM steps. Each step was applied until no further protein eluted from the column. Column fractions were analyzed for protein content by SDS-PAGE. Fractions containing YopM were pooled, concentrated, and dialyzed with an Amicon stirred cell (Beverly, Mass.) with a YM10 Diaflo ultrafiltration membrane. The concentrate was stored in 1-ml aliquots at −70°C. This procedure for purifying YopM by DEAE chromatography was communicated to us by Jim Bliska of Stanley Falkow's laboratory (Stanford University).

N-terminal sequencing. A 65-μg sample of YopM was resolved by SDS-PAGE and electroblotted to Immobilon P membranes by using a Bio-Rad Transblot apparatus (Bio-Rad, Richmond, Calif.). The membrane was stained with Coomassie brilliant blue, and the YopM-containing band was cut out and subjected to 13 cycles of Edman degradation by the Macromolecular Structure Analysis Facility to determine the sequence of the N terminus.

Dot blot analysis. A 0.1-μg sample of human α-thrombin (obtained from John W. Fenton II, Division of Laboratories and Research, New York State Department of Health, Albany, N.Y., and stored at −70°C in 0.7 M NaCl) was diluted in PBS, spotted onto dry nitrocellulose, and allowed to air dry. The membrane was blocked with TBS (50 mM Tris-HCl [pH 8.0], 150 mM NaCl) containing 5% (wt/vol) nonfat dry milk (Carnation Co., Los Angeles, Calif.) and incubated in a 0.4-mg/ml solution of YopM in TBS for 1 h. The blots were given three 10-min washes with TBS plus 0.05% (vol/vol) Tween 20 (TBST) and incubated with antibody against the C-terminal peptide diluted in TBST. After three washes, the nitrocellulose was incubated with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Sigma) diluted in TBST. Following three washes, the blots were developed by using the Protoblot Western blot alkaline phosphatase substrate system (Promega).

Cross-linking studies. A 50-μl volume of a PBS solution containing YopM and thrombin (or prothrombin in control experiments) at a concentration of 1 μM was incubated for 15 min at room temperature. The chemical cross-linker disuccinimidyl suburate (Pierce), diluted to 10 mM in dimethyl sulfoxide, was added to a final concentration of 0.1 mM, and cross-linking was carried out for 15 min on ice. The reaction was terminated by the addition of an equal volume of 2× SDS-PAGE sample buffer (20 mM Tris-HCl, 2 mM EDTA [pH 8.0], 5% [wt/vol] SDS, 10% [vol/vol] β-mercaptoethanol, 0.02% [wt/vol] bromphenol blue), and the samples were immediately analyzed by SDS-PAGE. To identify bands containing YopM or thrombin, the proteins separated on polyacrylamide gels were electroblotted to Immobilon P membranes and detected by using antibodies against the internal YopM peptide or against thrombin (American Diagnostica, Greenwich, Conn.). For some experiments, YopM was denatured by being boiled for 20 min. In experiments in which YopM was labelled with ^{125}I (as carrier-free $Na^{125}I$ [100 mCi/ml] from ICN, Cleveland, Ohio), iodination was carried out with Enzymobeads (Bio-Rad).

Preparation of platelets. Blood from healthy volunteers was collected in plastic syringes containing 3.8% (wt/vol) sodium citrate as the anticoagulant (final concentration, 0.42% [wt/vol]). The platelet-rich plasma was separated from the erythrocytes by low-speed centrifugation at 300 × *g* for 10 min. Platelet-poor plasma was harvested by centrifuging the remaining blood sample at 2,000 × *g* for 10 min. The latter was used to calibrate the Aggregometer. Plastic equipment was used for the preparation of platelets, and all manipulations were done at room temperature. To ensure reproducible results, platelets were processed and used within 3 h of blood collection.

Platelet aggregation studies. The platelet aggregation experiments were carried out in a dual-channel Chronolog Aggregometer (Chronolog Corp., Havertown, Pa.) with 0.312-in. (ca. 0.792-cm)-optical-path-length cuvettes incubated at 37°C. Twenty microliters of PBS containing 1.6 nmol of purified YopM or the control protein glucose oxidase (Sigma) was added to 450 μl of undiluted platelet-rich plasma containing approximately 3×10^8 platelets per ml, and the mixture was held for 1 min before the addition of 0.18 U of thrombin (2,686 U/mg) diluted in 20 μl of PBS containing 1% (wt/vol) BSA. The aggregation response was measured by the change in light transmission over time. In control experiments, the following additional platelet agonists and final concentrations were used: epinephrine, 5×10^{-6} M; ADP, 4×10^{-4} M; and collagen (Baxter Healthcare Corp., Miami, Fla.), 0.1 mg/ml.

Adenylate cyclase assay. YopM was assayed for adenylate cyclase activity under conditions used to measure adenylate cyclase activity in *Bordetella pertussis* (13). The assay was performed at 37°C for 10 min in the presence and absence of calmodulin and the reducing agent dithiothreitol. Cyclic AMP (cAMP) production was quantitated by monitoring the ability of the sample to compete with the binding of radioactive cAMP to antibody immobilized on a microtiter dish in a radioimmunoassay compared with a known quantity of cAMP (7). Additional tests for adenylate cyclase activity were kindly performed by Erik Hewlett, University of Virginia Health Sciences Center. In these, YopM was tested for adenylate cyclase enzyme activity in the presence and

absence of calmodulin (15), with *B. pertussis* adenylate cyclase toxin as a positive control.

Nucleotide sequence accession numbers. The GenBank accession numbers for sequences determined in this work are M91230 for PreyopM (sequence upstream of *yopM*) (1,300 bp) and M91231 for PostyopM (sequence downstream of *yopM*) (2,700 bp).

RESULTS

Construction of an overexpressing strain for purification of YopM. Subcloning of the *Y. pestis* pCD1 *Hin*dIII-F fragment had revealed a *Hae*III fragment cloned into pBR322 (pBS3; Table 1) that overexpressed *yopM* compared with a larger fragment cloned into the same vector (24). Experiments designed to address why this clone overexpresses the gene will be described later. For the purpose of purifying YopM, however, a slightly larger *Hae*III-*Eco*RV fragment, which also overexpressed YopM, was moved from pBS7 (Table 1) into the high-copy-number vector pBluescript SK$^-$ (pBS10; Table 1) to further increase YopM expression. This was electroporated into *Y. pseudotuberculosis* 43 containing the *Y. pestis* Lcr plasmid pCD1 *yopH*::Mu dI1734.

Y. pseudotuberculosis was chosen for the purification procedure rather than *Y. pestis* because of its greater resistance to lysis upon handling, minimizing contamination of secreted proteins (the starting material for purification) with cytoplasmic proteins. In addition, it lacks the pPCP1-encoded plasminogen activator protease that might otherwise partially degrade some of the YopM.

The virulence plasmid pCD1 was necessary to provide the genes needed for strong expression and secretion of the Yops. The *yopH* derivative of pCD1 was chosen to eliminate expression of YopH, which, in addition to being one of the major proteins found in culture supernatants, has a molecular weight similar to that of YopM and would interfere with analysis of purified proteins by one-dimensional SDS-PAGE.

A comparison of the amount of YopM produced by the resulting strain, *Y. pseudotuberculosis* 43(pCD1 *yopH*::Mu dI1734, pBS10), with that produced by *Y. pestis* KIM8 is shown Fig. 1A.

For purification of YopM, *Y. pseudotuberculosis* 43 (pCD1 *yopH*::Mu dI1734, pBS10) was grown in the defined medium TMH under conditions inducing strong secretion of the Yops. Secreted proteins were precipitated from the supernatant, and YopM was purified from the precipitate by DEAE chromatography. A silver-stained gel containing YopM obtained by this procedure is shown in Fig. 1B. In this experiment, 35 mg of YopM was obtained from a 4-liter culture grown to an A_{620} of 1.0.

N-terminal and C-terminal analysis of YopM. The purified protein was subjected to microsequencing of the N terminus. The resulting sequence, MFINPRNVSNTFL, agreed with that deduced from the previously reported DNA sequence (25), showing that YopM, like other Yops, is not secreted by a mechanism involving cleavage of an N-terminal signal sequence (31). Other experiments employing Western analysis showed that the C terminus of this protein is also intact; the antibody made against the last 12 amino acids of the protein recognized the secreted form of YopM.

YopM binds to thrombin. Because of the homology with the alpha chain of the platelet thrombin receptor GPIb, the ability of purified YopM to bind thrombin was tested in a dot blot assay. YopM was incubated with thrombin or control proteins immobilized on nitrocellulose, and bound YopM was detected with antibody against the C-terminal 12 amino acids of YopM. YopM was found to bind to thrombin but not control proteins such as BSA (Fig. 2) or gelatin (data not shown).

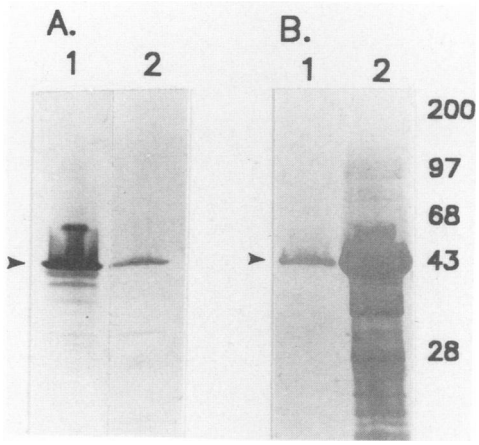


FIG. 1. YopM expression and purification. (A) Comparison of YopM in extracts of *Y. pseudotuberculosis* 43(pCD1 *yopH*::Mu dI1734, pBS10) and *Y. pestis* KIM8 cells. Cultures were grown under conditions to induce maximal Yops expression. Equivalent numbers of cells were solubilized in 1× SDS-PAGE sample buffer, separated on 10 to 15% (wt/vol) acrylamide denaturing Phastsystem gels, electroblotted to Immobilon, and detected with antipeptide antibody (against the C-terminal 12 residues of YopM). Lanes: 1, total cellular proteins from *Y. pseudotuberculosis* 43(pCD1 *yopH*::Mu dI1734, pBS10); 2, total cellular proteins from *Y. pestis* KIM8. (B) Comparison of purified YopM with starting material from culture supernatants. *Y. pseudotuberculosis* 43(pCD1 *yopH*::Mu dI1734, pBS10) was grown under conditions inducing strong secretion of Yops. A sample of the proteins precipitated from the culture supernatant (starting material) and a sample of the protein eluting from the column at 300 mM NaCl (purified YopM) were separated by SDS-PAGE and detected with a silver stain. Lanes: 1, purified YopM; 2, starting material.

In addition, the ability of the two proteins to interact in solution was examined by cross-linking analysis in which 1.0 μM concentrations of YopM and thrombin were incubated in the presence of the homobifunctional chemical cross-linker disuccinimidyl suburate and the products were analyzed by

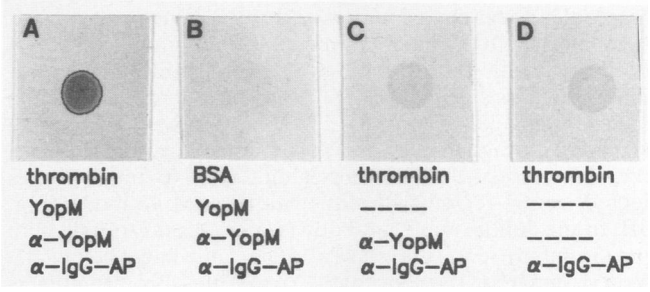


FIG. 2. Binding of YopM to thrombin immobilized on nitrocellulose. Thrombin or the control protein BSA was spotted onto nitrocellulose and incubated with a solution of YopM. Bound YopM was detected with a primary antipeptide antibody against the C-terminal 12 amino acids of YopM followed by the secondary antibody anti-immunoglobulin G (IgG) coupled with the enzyme alkaline phosphatase (AP). (A) Complete reaction sequence; (B) BSA substituted for thrombin; (C) immobilized thrombin incubated with primary and secondary antibody only; (D) immobilized thrombin incubated with secondary antibody only.

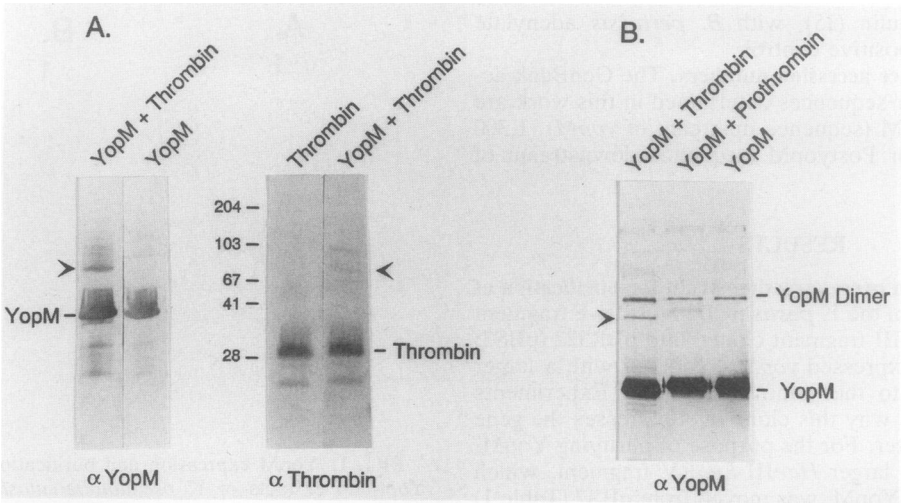


FIG. 3. Cross-linking of YopM to thrombin. YopM was incubated with thrombin or prothrombin at a concentration of 1.0 μM in PBS for 15 min. The cross-linker disuccinimidyl suburate was added to a concentration of 0.1 mM, and the mixture was incubated for 15 min on ice. The reaction was stopped by the addition of 2× SDS-PAGE sample buffer, and the proteins were analyzed in Western blots probed with an anti-YopM antibody against an internal amino acid sequence in YopM (see Materials and Methods) or antithrombin. The proteins present in the reaction mixtures are indicated above the lanes. The antibody used to detect the proteins is indicated below each Western blot. A YopM-thrombin cross-linked product is indicated by an arrowhead. (A) Products from cross-linked mixtures of thrombin and YopM (or the individual proteins) visualized with anti-YopM antibody or antithrombin antibody. The numbers give the sizes of molecular mass markers in kilodaltons. (B) Comparison of the products obtained by cross-linking YopM and thrombin with those from cross-linking YopM and prothrombin.

SDS-PAGE. In this experiment, a complex that had a mass approximately equal to the combined molecular masses of YopM (41.5 kDa) and thrombin (34 kDa) was generated. Importantly, this product was recognized by both anti-YopM and antithrombin antibodies (arrowheads in Fig. 3). In other experiments in which radioiodinated YopM was used to increase the limit of detection, a single ca. 75-kDa cross-linked product was obtained when YopM and thrombin were present at 20 nM (data not shown). When YopM was boiled prior to cross-linking, very little cross-linked product was obtained; instead, it appeared that boiled YopM was degraded by the proteolytic action of thrombin, as might be expected for a denatured protein (data not shown). In some cross-linking experiments, a second higher-molecular-weight product was generated that was also recognized by both antibodies. The molecular weight of this second product was consistent with its being either a complex of two YopMs and one thrombin or two thrombins and one YopM.

In contrast to the interaction with thrombin, no interaction of YopM with prothrombin was demonstrated by cross-linking analysis (Fig. 3B). A cross-linked product would have a molecular mass of ca. 115 kDa, which would migrate significantly higher than the YopM dimer (ca. 83 kDa) in a one-dimensional SDS-polyacrylamide gel. No band was detected in this region with either anti-YopM antibody (Fig. 3B) or the antithrombin antibody (which reacts strongly with prothrombin) (data not shown). The faint band below the YopM dimer in the lane with YopM plus prothrombin is prothrombin, which has weak cross-reactivity with the anti-YopM antibody (data not shown). The YopM dimer band prominently seen in Fig. 3B was variably present in these cross-linking experiments and probably was an artifact; this band was weakly present in a control lane for the experiment depicted in Fig. 3B, which contained only YopM that had not been subjected to cross-linking.

Inhibition of platelet aggregation. Earlier studies had demonstrated that a mixture of proteins from a YopM$^+$ but not a YopM$^-$ strain of *Y. pestis* could inhibit thrombin-induced platelet aggregation (24). In the present study, purified protein was used in the aggregation assay to determine whether YopM itself was able to inhibit thrombin-induced platelet aggregation. In these experiments, YopM was able to inhibit aggregation when incubated with platelet-rich plasma prior to addition of thrombin (Fig. 4B). YopM was an effective inhibitor at YopM-to-thrombin molar ratios ranging from 34:1 to 1,256:1, depending on the platelet preparation. When denatured YopM was used in the aggregation assay, no inhibition was seen, indicating that secondary structure, possibly a binding domain, was important for this activity (Fig. 4C).

Since thrombin is reported to bind nonspecifically to negatively charged surfaces such as glass (53), it was important to know whether it was this type of interaction that was accounting for thrombin's interaction with the very acidic YopM protein. The experiment with boiled YopM argued against this possibility but was subject to the concern that the boiled YopM might have been present in microaggregates that shielded important charges. Accordingly, a control experiment was performed in which YopM was replaced in the aggregation assay with the molar equivalent of a similarly charged protein, glucose oxidase, to determine whether it could substitute as an inhibitor. In this experiment, no inhibition was seen in the presence of glucose oxidase (Fig. 4D).

In comparisons of the YopM sequence with those in the Protein Identification Resource data base, YopM was found to have 26% homology over 367 residues with *E. coli* and *Saccharomyces kluyveri* adenylate cyclase and 25% homology over 367 residues with *Schizosaccharomyces pombe* adenylate cyclase. Since cAMP is reported to inhibit platelet aggregation (19), two types of control experiments were performed to examine the possibility that YopM was inhibiting aggregation by anergizing the platelets. In the first experiment, aggregation was inhibited with YopM as before,

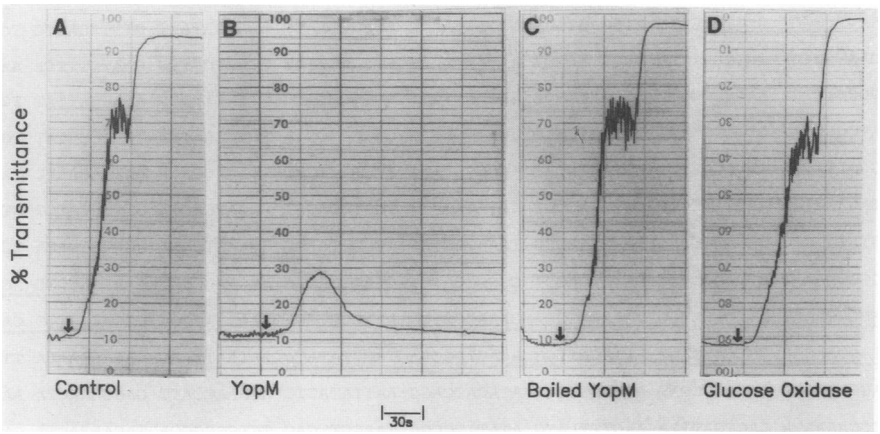


FIG. 4. Inhibition of thrombin-induced platelet aggregation by purified YopM. A 450-μl volume of platelet-rich plasma was continuously stirred in each cuvette of a dual-channel Aggregometer. Platelet aggregation after addition of thrombin (arrows) resulted in a biphasic increase in light transmission, corresponding to the formation of flocs followed by their removal from the solution as a clot accumulated around the stir bar in the cuvette. When YopM or glucose oxidase was added, the protein was incubated with platelet-rich plasma for 1 min before addition of thrombin. (A) Platelet-rich plasma incubated with 0.18 U of thrombin; (B) 1.6 nmol of YopM followed by 0.18 U of thrombin; (C) 1.6 nmol of boiled YopM followed by 0.18 U of thrombin; (D) 1.6 nmol of glucose oxidase followed by 0.18 U of thrombin.

and then an additional thrombin stimulus was given, resulting in normal aggregation (data not shown). This demonstrated that platelets retained their ability to respond to free thrombin. In the second experiment, platelets were induced to aggregate by other platelet agonists (ADP, epinephrine, or collagen) in the presence of inhibitory levels of YopM, and again normal aggregation was seen (data not shown). In separate experiments, YopM was assayed for adenylate cyclase activity, and no activity was found (data not shown).

YopM overexpression and sequencing of *Hin*dIII-F of pCD1. Previous work had shown (24) that a plasmid carrying the 1.5-kb *Hae*III fragment of *Hin*dIII-F (pBS3; Table 1) containing the YopM gene and only 150 bp of upstream and 350 bp of downstream DNA overexpressed YopM several-fold over levels expressed by a larger subclone containing the entire *Hin*dIII-F fragment (pJIT6; Table 1). In addition, overexpression was seen in both *Y. pestis* and *E. coli* (data not shown), suggesting that a regulatory element was contained within the *Hin*dIII-F fragment. To examine this possibility, the DNA upstream and downstream of *yopM* was sequenced, revealing several interesting elements which might mediate such regulation. The complete sequence is shown in Fig. 5, and a schematic diagram of the fragment is shown in Fig. 6A.

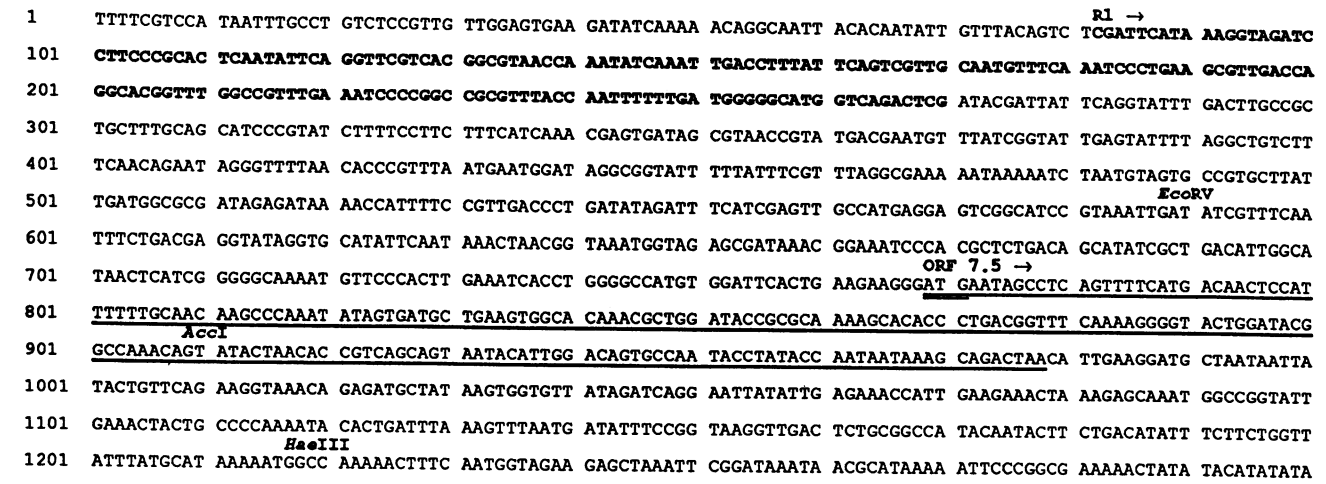
Unlike other regions of pCD1 sequenced thus far, which have a compact organization, encoding many closely spaced open reading frames (ORFs), the region of *Hin*dIII-F flanking *yopM* contained only five ORFs with the potential to encode proteins ranging in size from 7.5 to 15.9 kDa. Three very large repeated sequences were also found, one upstream and two downstream of the *yopM* gene. Alignment of the three repeats by using the CLUSTAL program of PC/GENE (Intelligenetics, Inc., Mountain View, Calif.) revealed 97% homology among 189 bp in each of the three sequences with an additional 87 bp of homology between R2 and R3 (Fig. 7).

To estimate the frequency of occurrence of these repeats in the *Y. pestis* genome, R2 was used as a probe in Southern analysis of *Hin*dIII digests of *Y. pestis* plasmid and chromosomal DNA. A strong signal from *Hin*dIII-F was seen, as expected for the presence of three copies of the sequence. A weaker but significant signal was obtained from the *Hin*dIII-N plus -O doublet on the gel, consistent with the possible presence of one copy of the repeat (data not shown). Linearized pPCP1 gave a signal comparable to that seen for *Hin*dIII-N and -O of pCD1. Because pPCP1 has a larger copy number than pCD1, this suggests that a copy (or copies) of the repeat present in this plasmid has only weak similarity to those in pCD1 (data not shown). No signal was obtained from the large capsular-antigen-encoding plasmid or from genomic DNA even when the stringency of the hybridization was decreased to allow up to 40% mismatch. Low-stringency Southern blot analysis of *Sal*I-digested plasmid DNA from *S. flexneri*, which has a YopM homolog, IpaH (18, 52), revealed only faint hybridization to four bands approximately 14 to 16, 4.8, 3.2, and 1.7 kb in size (data not shown).

To examine the contribution of the ORFs and repeats in the regulation of *yopM*, a series of subclones was constructed (Fig. 6B), eliminating one or all of these candidates, and the resulting plasmids were analyzed for YopM expression by Western blot analysis in *E. coli* (data not shown) and *Y. pestis* (Fig. 6B and 8). To eliminate differences due to vector promoters, all fragments were cloned into the same site within the *tet* gene in the opposite orientation to its promoter. The relative levels of YopM expression from these clones are summarized in Fig. 6B. These results showed no link between the expression of YopM and the ORFs or repeats.

DISCUSSION

In this study, we examined the expression and function of *Y. pestis* YopM. YopM was overexpressed from a subclone containing the *yopM* gene and very little flanking DNA in comparison with a larger clone containing the entire *Hin*dIII-F fragment. Since this overexpression was seen in both *E. coli* and *Y. pestis*, it seemed reasonable to hypothesize that the smaller subclone was missing a negative regulator contained within the larger *Hin*dIII-F fragment. This would make *yopM* similar to *yopE*, which has operon-specific regulation by the product of *yerA* located approximately 200



intervening sequence including the *yopM* gene published previously (24)

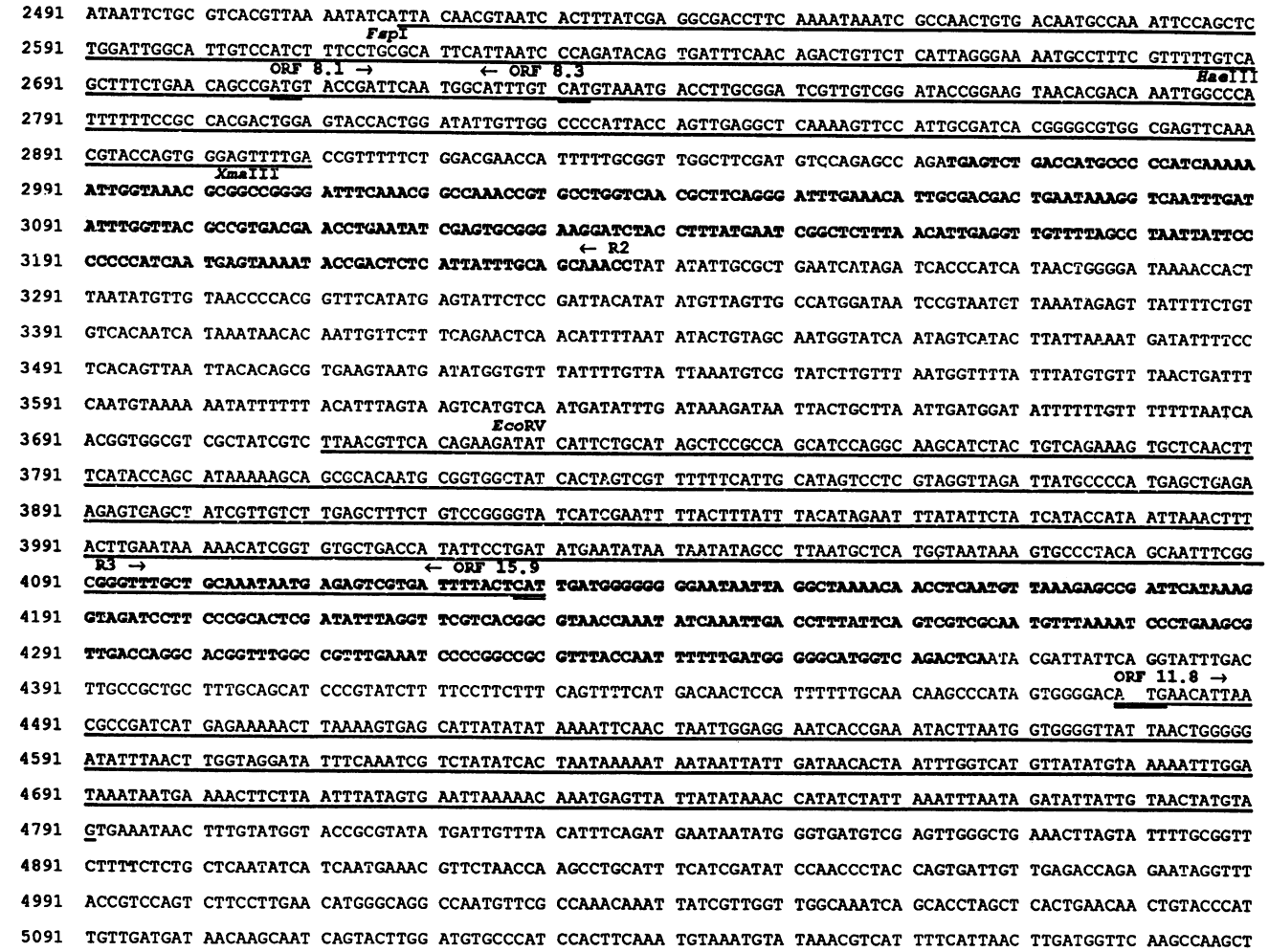


FIG. 5. Sequence of DNA upstream and downstream of *yopM* (GenBank accession numbers M91230 and M91231, respectively). Repeated sequences are in boldface type and are labelled R1, R2, and R3. ORFs are underlined and labelled according to the molecular mass (in kilodaltons) of each protein that they might encode. The start codon for each ORF is underlined twice. Restriction sites used for making subclones of *Hin*dIII-F are labelled with boldface type above the beginning of the recognition sequence. Bases 1209 to 1300 and 2491 to 2605 have been published previously but are shown to provide overlap with the published sequence and to show the end of ORF 8.3.

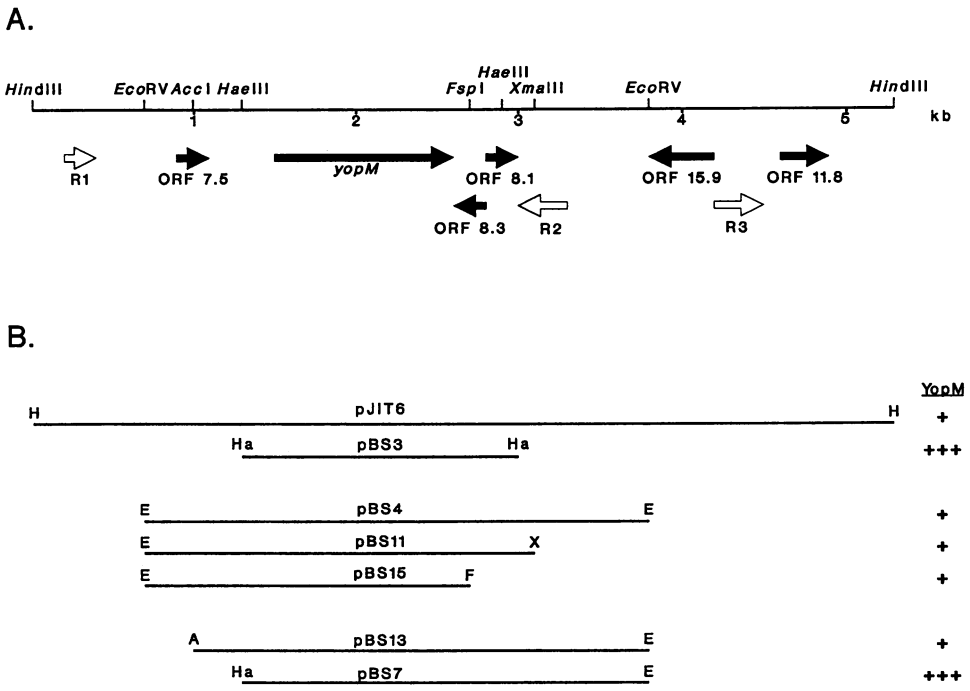


FIG. 6. (A) Physical map of the *Hin*dIII-F fragment of pCD1 and subclones containing the *yopM* gene. The *Hin*dIII-F fragment of pCD1 was found to contain five new ORFs in addition to *yopM*. These are indicated by filled arrows and labelled according to the molecular mass (in kilodaltons) of each protein that they might encode. Three large regions of nearly identical sequence are indicated by open arrows and labelled R1, R2, and R3. (B) Subclones of *Hin*dIII-F constructed to compare expression of *yopM* from DNA fragments with or without the various ORFs or repeated sequences. The relative levels of YopM expression determined by Western blot analysis are indicated (+, level expressed from pCD1 in *Y. pestis* KIM5).

bp upstream (12). Although sequencing of the *Hin*dIII-F fragment revealed several ORFs and three large repeated sequences that were potential candidates for imparting this regulation, deletional analysis showed that these elements were not involved in YopM expression.

```
R1  ----------------------------------------------------
R2  GG-TTTGCTGCAAATAATGAGAGTCGGTATTTTACTCATTGATGGGGGGG
R3  GGGTTTGCTGCAAATAATGAGAGTCGTGATTTTACTCATTGATGGGGGGG
    ..  ....................  ....................

R1  -----------------------------------CGATTCATAAAGG
R2  -AATAATTAGGCTAAAACAACCTCAATGTTAAAGAGCCGATTCATAAAGG
R3  GAATAATTAGGCTAAAACAACCTCAATGTTAAAGAGCCGATTCATAAAGG
     ..............................************

R1  TAGATCCTTCCCGCACTCAATATTCAGGTTCGTCACGGCGTAACCAAATA
R2  TAGATCCTTCCCGCACTCGATATTCAGGTTCGTCACGGCGTAACCAAATA
R3  TAGATCCTTCCCGCACTCGATATTTAGGTTCGTCACGGCGTAACCAAATA
    ******************.******.****************************

R1  TCAAATTGACCTTTATTCAGTCGTTGCAATGTTTCAAATCCCTGAAGCGT
R2  TCAAATTGACCTTTATTCAGTCGTCGCAATGTTTCAAATCCCTGAAGCGT
R3  TCAAATTGACCTTTATTCAGTCGTCGCAATGTTTAAAATCCCTGAAGCGT
    ************************.*********.****************

R1  TGACCAGGCACGGTTTGGCCGTTTGAAATCCCCGGCCGCGTTTACCAATT
R2  TGACCAGGCACGGTTTGGCCGTTTGAAATCCCCGGCCGCGTTTACCAATT
R3  TGACCAGGCACGGTTTGGCCGTTTGAAATCCCCGGCCGCGTTTACCAATT
    **************************************************

R1  TTTTGATGGGGGCATGGTCAGACTCG
R2  TTTTGATGGGGGCATGGTCAGACTCA
R3  TTTTGATGGGGGCATGGTCAGACTCA
    ************************.
```

FIG. 7. Alignments of the repeats R1, R2, and R3 found in DNA flanking *yopM*. The three repeated regions of DNA in *Hin*dIII-F were aligned by using the CLUSTAL program of PC/GENE. R1, 189 bases; R2, 274 bases; R3, 275 bases. Bases which are conserved between all three repeats are indicated by asterisks; bases conserved between two repeats are indicated by dots.

However, this analysis did show that the overexpression was the consequence of removing upstream DNA; deletion of 3′ sequences had no effect on expression, whereas removing 308 bp upstream of the *Hae*III site (pBS7 versus pBS13; Fig. 6) resulted in overexpression. It seems likely that in deleting this region, some DNA structure involved in mod-

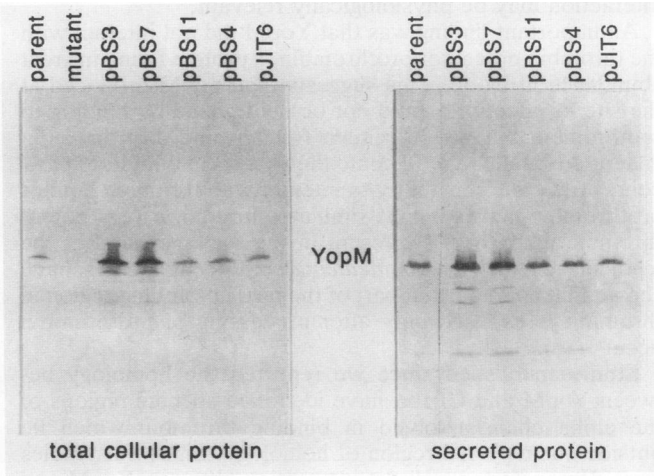


FIG. 8. YopM expression from representative *Hin*dIII-F subclones in *Y. pestis*. Parent *Y. pestis* KIM5 and mutant *Y. pestis* KIM5-3233 (YopM$^-$) cells containing the indicated plasmids were grown to induce Yop expression. Cells were harvested and solubilized in electrophoresis sample buffer. Protein from equal numbers of cells was separated by SDS-PAGE and transferred to Immobilon, and YopM was detected with an antipeptide antibody specific for YopM.

erating gene expression was destroyed. A candidate for imparting regulation by means of DNA structure might be a *Y. pestis* analog of the histone-like product of the *ymoA* gene characterized for *Y. enterocolitica* (9). This product has been found to work in conjunction with VirF in regulating the expression of other members of the *yop* regulon, such as *yopH* and *yadA* (23). The fact that overexpression is seen in *E. coli* as well as *Y. pestis* suggests that there might also be a *ymoA*-like gene product in *E. coli* able to regulate YopM gene expression.

The significance of the large repeats found flanking *yopM* is not clear. Since YopM has functional homology with eucaryotic proteins, an attractive hypothesis would be that these repeated sequences are remnants of some mobile genetic element that may have delivered the *yopM* gene to *Y. pestis*. If this were true, we might have expected to find strong homology with plasmid DNA from *S. flexneri* containing the YopM homolog IpaH, but this was not the case. Also, no homology was found between the repeats and known mobile elements or eucaryotic DNA sequences.

In order to further examine the function of YopM, the protein was purified to homogeneity from a strain engineered to overexpress it. Overexpression was achieved by truncating the upstream DNA flanking *yopM* while retaining the native *yopM* promoter. This construct was then moved into the commercial vector pBluescript to increase the copy number of the gene, further increasing YopM production.

Microsequencing of the purified protein showed that the secreted form of YopM has an intact N terminus, suggesting that like YopH (31), it is not processed at its N terminus during secretion. This is consistent with the observation that YopM has no obvious signal sequence (25). In addition, the C terminus also appears to be intact, since an antibody against the last 12 amino acids easily recognizes the secreted form of YopM.

In support of the hypothesis that YopM interacts with thrombin, YopM was shown to bind both to immobilized thrombin and to thrombin in solution. In the cross-linking analysis, a complex of the two proteins was seen at concentrations that were as low as 20 nM, suggesting that this interaction may be physiologically relevant.

An important finding was that YopM did not interact with the thrombin precursor prothrombin, a protein found in great abundance in blood. This suggests that YopM produced at the site of infection would not be neutralized by binding to prothrombin but would remain free to bind any thrombin generated locally. YopM could then act as an inhibitor at low concentrations. The ability to interact with thrombin but not prothrombin makes YopM similar to hirudin, a very potent thrombin inhibitor (21) present in the salivary gland of the medicinal leech *Hirudo medicinalis*. Recent studies have shown that an important part of the hirudin-binding region in thrombin is exposed only after processing of prothrombin occurs (26).

Studies published since we reported the homology between YopM and GP1bα have identified specific regions of the alpha chain involved in binding thrombin which lie outside our reported region of homology. Since our studies have demonstrated a direct interaction between YopM and thrombin, either the homologous region also plays an important role in GPIbα binding to thrombin or YopM interacts with thrombin in a different manner, independent of the GP1bα homology.

Because of the functional similarity between YopM and hirudin, we looked for YopM sequences having homology with hirudin's thrombin-binding domain (DFEEIPEE) (20, 42) and found several possible candidate sequences. For example, amino acids 142 to 148 (QLEELPE) and 329 to 335 (EFPDIPE) have four of the eight amino acids required for binding, with an additional two substituted by chemical equivalents. Perhaps it is such regions that account for YopM's interaction with thrombin.

In addition to demonstrating this interaction in the binding experiments, we showed in functional tests that YopM was able to inhibit one of the activities of thrombin, platelet aggregation. The results of this study appear to differ from those recently reported by Simonet et al. (44), in which *Y. pseudotuberculosis* was found to stimulate rather than inhibit platelet aggregation. These differing results might be explained by the fact that *Y. pestis* is thought not to produce the invasin protein (41, 54) believed to mediate the aggregation in *Y. pseudotuberculosis*. Moreover, Simonet et al. performed their studies with plasmid-free strains; therefore, their bacteria would not have produced YopM.

Although these experiments have not identified YopM's thrombin-binding domain, they have shown that an interaction between YopM and thrombin, having potential functional significance, does occur. An interaction such as this could have important consequences in vivo. One effect would be to prevent platelet aggregation and clot formation, which in concert with the pPCP1-encoded plasminogen activator could contribute to the dissemination of the bacterium throughout the body. This is analogous to the function of the streptococcal protein streptokinase, which promotes degradation of fibrin and is thought to aid in the dissemination of that bacterium. A second effect would be to inhibit the activation of platelets by thrombin, preventing the subsequent release of many inflammatory mediators in addition to substances that promote clotting, including chemotactic factors (platelet-activating factor), vasoactive substances (thromboxane A_2), factors affecting vascular permeability (serotonin), and even bactericidal proteins (β-lysins). Preventing the release of these factors would act to mute the local inflammatory response to the bacteria, allowing them to reach the high numbers seen in an infection. Consistent with this model, it has been shown through histopathologic analysis of infected livers and spleens that the initial acute inflammatory response at sites of infection wanes drastically and is not followed by further influxes of phagocytes (50). However, it is important to recall that this line of thinking arose from the finding, perhaps fortuitous, of a homology between YopM and GPIbα and the ensuing demonstration that YopM can bind thrombin and counteract an effect of thrombin on platelets. Thrombin can also affect cells other than platelets; it will be important to determine whether platelet function actually is the main target of YopM in vivo.

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