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Artem G. Evdokimov,[a] D. Eric Anderson,[b]† Karen M. Routzahn[b] and David S. Waugh[a,b]*

[a]Protein Engineering Section, National Cancer Institute – Frederick Cancer Research and Development Center, PO Box B, Frederick, MD 21702, USA, and [b]Structural Biology Core Facility, Program in Structural Biology, National Cancer Institute – Frederick Cancer Research and Development Center, PO Box B, Frederick, MD 21702, USA

† Current address: Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892-0580, USA.

Correspondence e-mail: waughd@ncifcrf.gov

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A recombinant form of *Yersinia pestis* YopM with a C-terminal polyhistidine affinity tag has been overproduced in *Escherichia coli*, purified to homogeneity and crystallized using the hanging-drop vapor-diffusion technique. Several different crystal forms were obtained. The most suitable crystals for X-ray diffraction belonged to space groups $P4_222$ (unit-cell parameters a = 109.36, b = 109.36, c = 101.50 Å) and $C222_1$ (unit-cell parameters a = 71.73, b = 121.85, c = 189.79 Å). With a synchrotron-radiation source, these crystals diffracted to 2.4 and 1.9 Å resolution, respectively.

1. Introduction

Y. pestis, the causative agent of plague, is arguably the deadliest pathogen in history, having been credited with at least 200 million deaths in modern times (Perry & Fetherston, 1997). The hallmark of *Y. pestis* and related bacterial pathogens (*e.g.* enterohemorrhagic *E. coli*, *Salmonella* spp., *Shigella* spp., *Pseudomonas aeruginosa*, *Chlamydia psittaci* and *Bordetella* spp.) is the type III or contact-dependent secretion apparatus, which serves to direct the vectorial translocation of a small number of proteins, termed effectors, into the cytosol of eukaryotic cells (Finlay & Falkow, 1997; Galan & Collmer, 1999). Collectively, these effectors enable the bacteria to disarm the immune response of the infected organism, presumably by interfering with crucial signal transduction pathways.

Relatively little is known about the biochemical activities and targets of the seven *Yersinia* effectors YopE, YopJ, YopH, YopM, YopT, YpkA and LcrV. Except for YopJ and YopT, however, all of them are indispensable virulence factors (Cornelis *et al.*, 1998). YopH is the most extensively characterized effector. The structure of its C-terminal domain, a potent tyrosine phosphatase, has been determined by X-ray crystallography (Stuckey *et al.*, 1994). A distinct phosphotyrosine-dependent protein-binding activity has been ascribed to the N-terminal domain of YopH (residues 1–130), but this region of the protein bears no recognizable similarity to the PTB or SH2 domains (Black *et al.*, 1998). YopH is one of several effectors for which intracellular targets have been identified; it dephosphorylates focal adhesion kinase (FAK) and $p130^{Cas}$, thereby antagonizing bacterial phagocytosis and causing the disruption of focal adhesions at sites of cell attachment to the extracellular matrix (Persson *et al.*, 1997). YopE is a GTPase-activating protein (GAP) that regulates the activity of Rho family G-proteins (Rho, Rac and Cdc42), which control rearrangements of the actin cytoskeleton in eukaryotic cells (Pawel-Rammingen *et al.*, 2000). By causing actin microfilament stress fibers to be disrupted, YopE contributes to the bacterium's ability to resist phagocytosis. Although its amino-acid sequence is unrelated to that of YopE, a very similar effect is elicited by YopT (Iriarte & Cornelis, 1998), which induces an unknown chemical modification of RhoA (Zumbihl *et al.*, 1999). Another effector with a known target, YopJ, inhibits the activation (phosphorylation) of MAP kinase kinase (MEK) by an as yet undetermined mechanism (Orth *et al.*, 1999). The N-terminal half of the 80 kDa YpkA protein is a serine-threonine kinase (Galyov *et al.*, 1993). The function of the C-terminal half of this protein is unknown and the molecular target(s) of YpkA in eukaryotic cells have yet to be identified. In addition to its roles as an effector, which evidently include the suppression of proinflammatory cytokines (Nedialkov *et al.*, 1997) and tumor necrosis factor (Schmidt *et al.*, 1999) as well as the inhibition of neutrophil chemotaxis (Welkos *et al.*, 1998), LcrV (V-antigen) seems to be an essential structural or regulatory component of the type III delivery apparatus, as it is also required for the translocation of several other Yop proteins (Sarker *et al.*, 1998). So far, no biochemical activity or cellular phenotype has been attributed to YopM, which has been observed to accumulate in both the cytosol and the nucleus of mammalian cells (Skrzypek *et al.*, 1998).

The 46 kDa YopM protein has a modular architecture dominated by 15 tandem copies of a degenerate leucine-rich repeat (LRR) motif. Variations of the LRR are found in a large number of proteins with diverse functions (Kobe & Deisenhofer, 1995). The LRR motifs

in YopM, which comprise the central 306 residues in the polypeptide sequence, are bracketed by 74 and 24 residues on the N- and C-termini, respectively. The function of the C-terminal domain is unknown. However, as is presumed to be true of all the effectors, the N-terminal portion of the YopM coding sequence is involved in targeting the protein for secretion and translocation (Cornelis & Wolf-Watz, 1997; Cornelis *et al.*, 1998). The overproduction, purification, crystallization and preliminary X-ray analysis of recombinant *Y. pestis* YopM reported here represent the first steps toward determining the three-dimensional structure of this essential virulence factor, which may improve our understanding of its biochemical function and facilitate the design or discovery of novel anti-plague agents.

2. Overproduction and purification of YopM

The open reading frame (ORF) encoding YopM was amplified from total *Y. pestis* genomic DNA (strain 195/P) by the polymerase chain reaction (PCR) using the following oligonucleotide primers: 5′-GAGAACCTGTACTTCCAGGGTATGTTCATAAATCCAAGAAATGTATC-3′ and 5′-ATTAGTGATGATGGTGGTGATGCTCAAATACATCATCTTCAAGTTTG-3′. This PCR amplicon was subsequently used as the template for a second PCR with the following primers: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGGAGAACCTGTACTTCCAG-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTATTAGTGATGATGGTGGTGATG-3′. The amplicon from the second PCR was inserted by recombinational cloning into the entry vector pDONR201 (Life Technologies) to create pKM645 and the nucleotide sequence of the entire insert was then confirmed experimentally. Next, the YopM ORF, now bracketed by a hexahistidine tag on its C-terminus and a recognition site for tobacco etch virus (TEV) protease (ENLYFQG) on its N-terminus, was moved from pKM645 by recombinational cloning into the destination vector pKM596, a derivative of pMal-C2 (New England Biolabs) that is designed to produce recombinant proteins as in-frame fusions to the C-terminus of *E. coli* maltose-binding protein (MBP). Thus, the final construct (pKM756) directed the expression of YopM in the form of an affinity sandwich, with MBP and a polyhistidine tag fused to its N- and C-termini, respectively. The MBP moiety could be removed by cleaving the fusion protein with TEV protease at a designed site in the linker to yield a recombinant *Y. pestis* YopM protein with a non-native glycine residue at its N-terminus and a hexahistidine tag at its C-terminus. The MBP-YopM-His_6 fusion protein was expressed at a very high level and in a soluble form in *E. coli* BL21 cells (data not shown). Cells containing the expression vector were grown to mid-log phase (OD_{600} = 0.5) in LB broth (Miller, 1972) containing 100 µg ml^{-1} ampicillin at 310 K, at which time isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 m*M*. 4 h after induction, the cells were pelleted by centrifugation and stored at 193 K.

The cell pellet obtained from 6 l of medium (20 g) was thawed and resuspended in 200 ml of buffer *A* (20 m*M* $NaPO_4$ pH 7.4, 200 m*M* NaCl). Just prior to lysis, phenylmethylsulfonyl chloride (PMSF) and benzamidine were added to final concentrations of 1 and 2.5 m*M*, respectively. The cells were disrupted by three cycles of sonication (Sonics and Materials Vibracell, 1/2″ SM0896 horn) in 50 ml aliquots on ice for 45 s at a power level of 65%. Next, polyethylenimine (Sigma) was added to 0.1% and the crude lysate was clarified by centrifugation at 37 000*g* for 10 min. Solid ammonium sulfate was added to the supernatant to 35% saturation, after which the solution was clarified again by centrifugation as above. The supernatant was then adjusted to 75% saturation with solid ammonium sulfate and incubated on ice for 15 min. The precipitated material was pelleted by centrifugation as above, resuspended in 200 ml of buffer *A* and then filtered (0.45 µm) prior to chromatography. The sample was next applied to a column (XK-26/50, 50 ml) of Ni-NTA resin (Qiagen) equilibrated with buffer *A*. After loading, the column was washed with eight volumes of buffer *A* and the MBP-YopM-His_6 fusion protein was then eluted with a gradient from 0 to 500 m*M* imidazole in buffer *A*. The eluent was dialyzed against 20 volumes of buffer *B* (20 m*M* Tris–HCl pH 8, 2 m*M* EDTA, 200 m*M* NaCl, 1 m*M* DTT) and then concentrated by diafiltration to an absorbance of 5.0 at 280 nm. After concentration, the sample was adjusted to 0.1 m*M* in 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) and allowed to sit at room temperature for 30 min before MBP-TEV protease (Kapust & Waugh, 1999), expressed and purified in-house, was added at a ratio of 1:100 and the sample was dialyzed at room temperature against four changes (every 3 h) of 100 volumes of buffer *B* using dialysis cassettes (Pierce). The dialysis was necessary to remove methyl α-D-glucopyranoside introduced from the added protease.

Following dialysis, the sample was clarified by centrifugation and filtered (0.45 µm). It was then passed through an amylose column (100 ml, New England Biolabs, HR 1.6 × 50 cm) at 2 ml min^{-1} to absorb the free MBP, any residual uncleaved fusion protein and the MBP-TEV protease. The flowthrough fractions containing YopM-His_6 were pooled and dialyzed against 200 volumes of 20 m*M* Tris–HCl pH 7.6, 50 m*M* NaCl, 2 m*M* EDTA, 0.1 m*M* DTT. After dialysis, the sample was adjusted to 0.1 m*M* with tris-(carboxyethyl)-phosphine (TCEP) and allowed to sit at room temperature for 30 min. The sample was then applied to a MonoQ column (1.6 × 10 cm) and subjected to a 30-column gradient from 25 to 850 m*M* NaCl in 20 m*M* Tris–HCl pH 7.6. The fractions containing YopM-His_6 were pooled and adjusted to 0.8 *M* ammonium sulfate by the gradual addition of the solid salt. The solution was then applied to a column (8 ml, 1.6 × 4 cm) of Source PHE resin (Pharmacia) at a flow rate of 8 ml min^{-1}. Initially, the column was washed with five column volumes of 20 m*M* Tris–HCl pH 7.6, 0.4 *M* ammonium sulfate, after which the protein was eluted with a gradient from 0.4 to 0.2 *M* ammonium sulfate in 20 m*M* Tris–HCl pH 7.6 (15 column volumes). YopM-His_6 eluted toward the end of the gradient. Prior to crystallization, pure protein solutions were dialyzed extensively against 25 m*M* HEPES pH 7.5, 0.2% NaN_3, 5 m*M* DTT and were concentrated to the desired degree using a stirred cell (Amicon) with a nominal 10 kDa cutoff. Concentrated protein solutions were flash-frozen in liquid nitrogen and could be stored indefinitely at 193 K.

3. Crystallization

All the crystals reported in this article were grown by vapor diffusion in VDX 24-well plates containing 1 ml of precipitant solution per well. The first crystals of YopM (form 1, image not shown) were obtained using Crystal Screens I and II (Hampton Research). After refinement of the crystallization conditions, the precipitant solution contained 20% polyethylene glycol (PEG 6000), 200 m*M* NaOAc pH 8.5 (100 m*M* Tris–HCl). These crystals appeared after incubating a mixture of 3 µl of 12 mg ml^{-1} protein and 3 µl of the precipitant solution for two weeks. The crystals looked like clusters of irregular ‘spindles’ and reached

their maximum size of 0.4 × 0.2 × 0.15 mm in several weeks. Even though these crystals could be flash-frozen in a stream of cold nitrogen gas after being transferred into a 1:1 mixture of Paratone-N and light petroleum oil, better cryoprotection was achieved by gradually soaking the crystals in increasing concentrations of 2-methyl-2,4-pentanediol (0–20%) in the artificial mother liquor, followed by flash-freezing in liquid nitrogen.

The second crystal form (form 2), shaped like tetragonal bipyramids (Fig. 1*a*), was found using Wizard Screens I and II from Emerald Biotechnologies. The best precipitant solution consisted of 10% 2-propanol, 450 m*M* $Ca(OAc)_2$ and 100 m*M* Na–MES pH 6.0. Crystals were obtained by mixing 4 µl of 6–8 mg ml^{-1} YopM with 4 µl of the precipitant. The first crystals usually appeared overnight. In two weeks, they grew to maximum dimensions of 1.5 × 1.0 × 1.0 mm (Fig. 1*b*). Cryoprotection could only be achieved by dipping the crystals into a 1:1:3 mixture of Paratone-N, heavy paraffin oil and silicone oil, followed by flash-freezing in liquid nitrogen.

The third crystal form (form 3) appeared under the same conditions as form 2, except that the precipitant also contained 20% ethylene glycol. These crystals looked like bi-truncated tetragonal bipyramids (Fig. 1*c*). Unlike form 2 crystals, these crystals grew very slowly over a period of four weeks and never exceeded 0.08 × 0.06 × 0.06 mm in size. These crystals could be readily frozen in their mother liquor.

The fourth crystal form of YopM (form 4) was found by adding 0.1–0.3 µl of 50 m*M* ethylmercury phosphate solution to the drops set up to produce form 2 crystals. Six- and eight-sided prismatic crystals (Fig. 1*d*) grew out of a copious brown precipitate in 1–3 d, reaching maximum dimensions of 1.5 × 1.5 × 1 mm in 5–7 d. Other mercury, lead, gold and platinum compounds were tried, but only the addition of ethylmercury phosphate produced crystals of a useful size. Cryoprotection of form 4 crystals was achieved by dipping the crystals into the same oil mixture as for form 2 crystals, taking great care to remove all the aqueous drops from the crystal faces. After such treatment, form 4 crystals could be flash-frozen in liquid nitrogen. Only modest-sized specimens were useful; crystals larger than 0.3 × 0.3 × 0.2 mm invariably developed cracks upon freezing.

A number of crystals (Figs. 1*e* and 1*f*) were obtained by the addition of 1–10 m*M* of transition-metal ions (Ni^{2+}, Co^{2+}, Mn^{2+} *etc.*) to the precipitant solutions used to grow form 2 crystals. Because it was difficult to reproduce these crystals and very difficult to cryoprotect them, they were not studied in further detail.

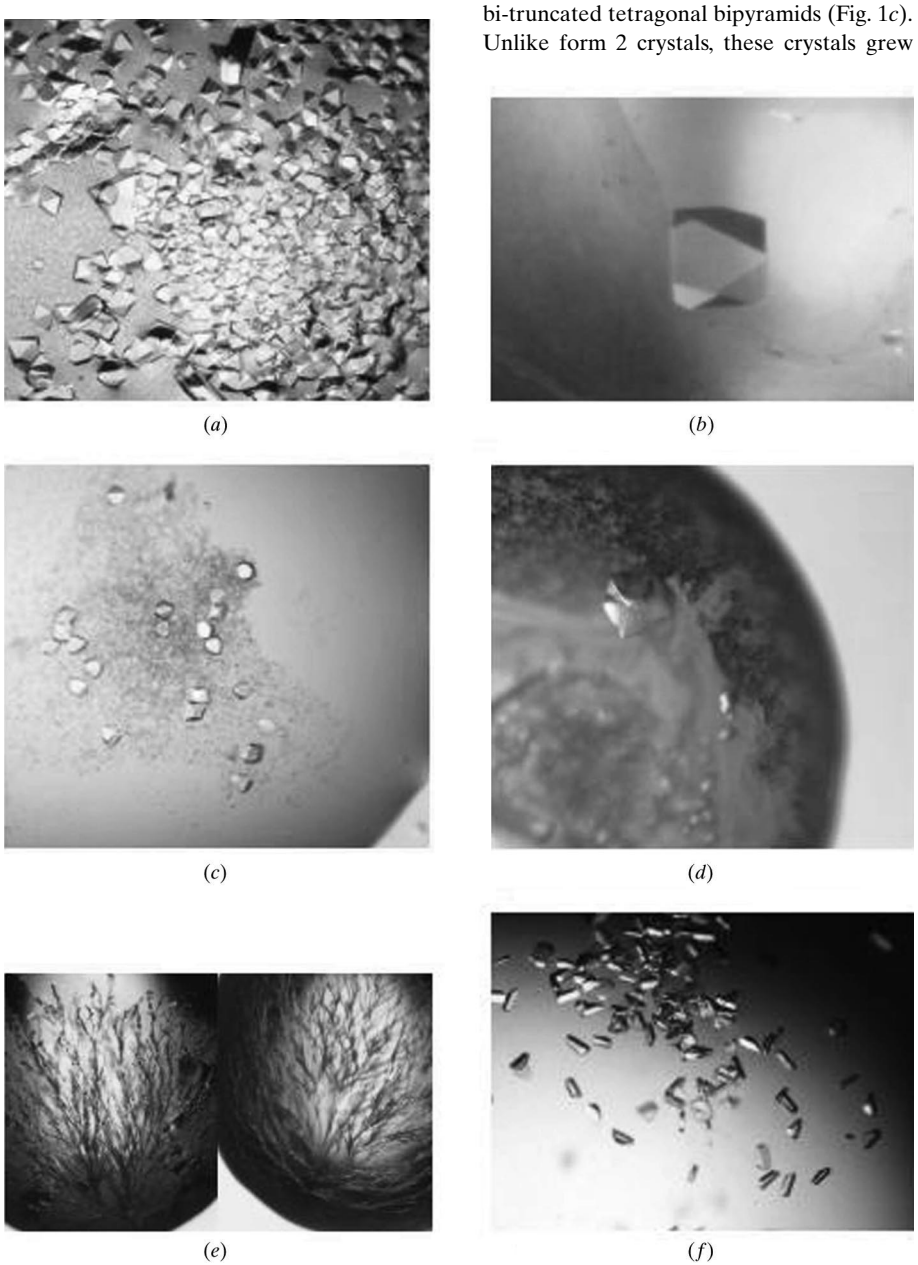

Figure 1
Crystal forms of YopM. (*a*, *b*) Form 2 crystals, (*c*) form 3 crystals, (*d*) form 4 crystals, (*e*, *f*) crystals obtained by addition of transition-metal ions.

4. X-ray diffraction analysis

The diffraction properties of the various YopM crystal forms are summarized in Table 1. Several considerations influenced our choice of crystals for data collection: form 1 was found to be irreproducible from one batch of protein to another. Large unit-cell dimensions and a modest range of diffraction made form 2 crystals an unlikely candidate. Form 3 crystals belonged to a high-symmetry tetragonal space group and had the smallest unit-cell volume of all the crystal forms of YopM, which made data collection considerably easier and faster. Despite their small size, form 3 crystals diffracted to 2.4 Å resolution. Form 4 was also an attractive candidate because these crystals were likely to contain mercury-derivatized protein (YopM has three free cysteines), which would render them suitable for multiwavelength anomalous diffraction (MAD) studies.

A 2.4 Å native data set for a cryoprotected form 3 crystal was collected at beamline X9-B at the National Synchrotron Light Source. Owing to the small size of the crystal, the exposure time had to be set to

Table 1
Essential crystallographic data.

Values for the highest resolution shell are given in parentheses.

Crystal form	1	2	3	4
Space group	—	*P*4(?)	$P4_222$	$C222_1$
Unit-cell parameters *a*, *b*, *c* (Å) ($\alpha = \beta = \gamma = 90^\circ$)	—	315.32, 315.32, 121.71	109.36, 109.36, 101.50	71.73, 121.85, 189.79
Diffraction limit (Å)	3.5	3.8	2.4	1.9
Asymmetric units per unit cell	—	4 (*P*4)/8 (*P*422)	8	8
Molecules per asymmetric unit	—	32–16–8/16–8–4	**1**–2	1–**2**–3
V_M (Å^3 Da^{-1})		1.9–3.8–7.7	3.3–1.5	4.2–2.1–1.4
Solvent content (%)		36–68–84	**62**–20	71–**41**–15
Completeness (%)	—	—	95.2 (93.6)	90.1 (89.5)†
$I/\sigma(I)$	—	—	12.4 (3.3)	14.1 (5.2)†
Redundancy	—	—	3.1	3.3
R_{sym}	—	—	0.10 (0.34)	0.07 (0.25)†

† Statistics calculated for the 2.4 Å data set.

5 min per 1.0° oscillation. Cryogenic data sets for crystal form 4 were collected to 2.8 and 2.4 Å, using laboratory and synchroton sources, respectively. These crystals diffracted to ∼1.9 Å at the synchrotron, but the data collection was limited to 2.4 Å by the crystal-to-detector distance, which had to be large enough to avoid spot overlaps arising from high mosaicity and a long unit-cell axis. It is most likely that the form 3 crystals contain one molecule of protein per asymmetric unit, while the form 4 crystals contain two monomers per asymmetric unit (Table 1).

We have recently established conditions for soaking heavy atoms into form 3 crystals and we expect to solve the structure either by MAD or by multiple isomorphous replacement in the near future.

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