The EMBO Journal vol.15 no.19 pp.5191–5201, 1996

Status of YopM and YopN in the *Yersinia* Yop virulon: YopM of *Y.enterocolitica* is internalized inside the cytosol of PU5-1.8 macrophages by the YopB, D, N delivery apparatus

Anne Boland, Marie-Paule Sory, Maite Iriarte, Corinne Kerbourch, Pierre Wattiau and Guy R.Cornelis[1]

Microbial Pathogenesis Unit, International Institute of Cellular and Molecular Pathology and Faculté de Médecine, Université Catholique de Louvain, UCL 74-49, B-1200 Brussels, Belgium

[1]Corresponding author

The *Yersinia* Yop virulon is an anti-host system made up of four elements: (i) a type III secretion system called Ysc; (ii) a system designed to deliver bacterial proteins into eukaryotic target cells (YopB, YopD); (iii) a control element (YopN); and (iv) a set of intracellularly delivered proteins designed to disarm these cells or disrupt their communications (YopE, YopH and possibly others). YopM, another Yop protein, binds thrombin and is thus presumed to act as an extracellular effector. Here, we analyzed YopM from *Y.enterocolitica* and we wondered whether it could also be delivered inside eukaryotic cells. To answer this question we applied the Yop–Cya reporter strategy. Hybrids made of 141 or 100 N-terminal residues of YopM fused to Cya were delivered inside PU5-1.8 macrophages by recombinant *Y.enterocolitica* strains. YopB and YopD were required as translocators. Leakage of the reporters into the macrophage culture supernatant during the bacterial infection increased strongly when YopN was missing, showing that YopN is involved in the control of delivery of YopM inside eukaryotic cells. YopN itself was not delivered into the macrophages. In conclusion, YopM is translocated inside the eukaryotic cells and its physiopathological role should be revised or completed.

Keywords: microbial pathogenesis/protein translocation/type III secretion/*Yersinia enterocolitica*/Yops

Introduction

Invasive pathogenic bacteria have in common the capacity to overcome the defence mechanisms of their animal host and to proliferate in its tissues. They all have their own life style and target organs, leading to a variety of symptoms and diseases, which suggested the existence of a great diversity among their virulence factors. However, recent data from several laboratories contradicted this view and revealed the existence of related major virulence systems in various pathogenic bacteria, including phytopathogens. The Yop virulon which enables *Yersiniae* (*Yersinia pestis*, *Y.pseudotuberculosis* and *Y.enterocolitica*) to survive and multiply in the lymphoid tissues of their host is such a conserved system. It consists basically of a dozen secreted proteins, called Yops, and their secretion apparatus, called Ysc (for Yop secretion). This apparatus, made up of some 22 proteins belongs to the type III family of secretory systems (Michiels *et al.*, 1991; Haddix and Straley, 1992; Rimpiläinen *et al.*, 1992; Plano and Straley, 1993; Allaoui *et al.*, 1994, 1995; Bergman *et al.*, 1994; Fields *et al.*, 1994; Woestyn *et al.*, 1994). It recognizes the Yops by a short N-terminal signal that is not cleaved off during secretion (Michiels *et al.*, 1990; Sory *et al.*, 1995) and it readily secretes hybrid proteins composed of the N-terminal part of a Yop fused to various prokaryotic or even eukaryotic proteins (Sory *et al.*, 1990, 1992, 1995; Michiels and Cornelis, 1991; Sory and Cornelis, 1994). Secretion of YopE, YopH, YopB and YopD also requires the presence, in the bacterial cytosol, of individual chaperones called SycE, SycH and SycD, respectively (Wattiau and Cornelis, 1993; Wattiau *et al.*, 1994; Frithz-Lindsten *et al.*, 1995; for review, see Wattiau *et al.*, 1996). The 12 *yop* genes as well as the 22 *ysc* and *lcr* genes encoding the Ysc secretion machinery are borne by a 70 kb plasmid called pYV. Expression of these genes is induced at 37°C, the host body temperature, and most of them constitute a regulon that is positively controlled by VirF, a transcriptional activator of the AraC family (Cornelis *et al.*, 1989). A negative regulatory system is superimposed on this positive temperature regulation: in bacterial cultures, *yop* genes expression is down-regulated in the presence of Ca^{2+} ions or when secretion is compromised by a mutation (Cornelis *et al.*, 1987; for reviews, see Cornelis, 1994; Forsberg *et al.*, 1994; Cornelis *et al.*, 1995; Straley and Perry, 1995). Inhibition of Yop secretion by Ca^{2+} involves at least one of the Yops themselves, namely YopN (LcrE), but the mechanism of its action is unknown (Forsberg *et al.*, 1991).

The function of all the Yops has not been elucidated yet, but a coherent picture is emerging. YopE is a cytotoxin that disrupts the actin microfilament structure of cultured HeLa cells (Rosqvist *et al.*, 1991). YopH is a protein tyrosine phosphatase (PTPase) related to eukaryotic PTPases (Guan and Dixon, 1990) that acts on tyrosine-phosphorylated proteins of macrophages (Bliska *et al.*, 1991, 1992); it is involved in the inhibition of bacterial uptake (Rosqvist *et al.*, 1988) and oxidative burst by cultured macrophages (Bliska and Black, 1995). YpkA (YopO) is a protein kinase showing extensive similarity to eukaryotic serine–threonine kinases (Galyov *et al.*, 1993). YopE and YopH produced by extracellular bacteria adhering to cultured HeLa cells or macrophages are translocated across both the prokaryotic and eukaryotic membranes to reach their cytosolic target (Rosqvist *et al.*, 1994; Sory and Cornelis, 1994; Persson *et al.*, 1995; Sory *et al.*, 1995). Delivery of YopE and YopH into eukaryotic cells requires not only an intact Ysc secretion apparatus but also the presence of YopB and/or YopD, probably acting as translocators (Rosqvist *et al.*, 1994; Sory and

Cornelis, 1994; Persson *et al.*, 1995; Sory *et al.*, 1995). This observation probably accounts for the role of YopD in the inhibition of the respiratory burst (Hartland *et al.*, 1994) and for the role of YopB and YopD in the inhibition of phagocytosis (Fällman *et al.*, 1995). Translocation of YopE and YopH across the eukaryotic cell membrane is ensured by the presence of a specific signal located in their 50 and 71 N-terminal residues, respectively (Sory *et al.*, 1995). The Syc binding site on YopE and YopH matches this translocation signal, and the chaperones become dispensable for Yop secretion when the translocation signal is removed. These two elements suggest that a role for the Syc chaperones could be to prevent the intrabacterial association of Yops with some components of the translocation apparatus, possibly YopB or YopD (Woestyn *et al.*, 1996).

YopE and YopH are delivered into cultured macrophages or HeLa cells in spite of the presence of Ca^{2+} in the usual eukaryotic cell culture media. This indicates that Ca^{2+} chelation is not an absolute requirement for Yop synthesis and release, and that the actual signal triggering Yop secretion *in vivo* would thus not be Ca^{2+} chelation but rather contact with a eukaryotic cell membrane. During translocation of YopE and YopH from the *Yersinia* cytosol to the cytosol of cultured HeLa cells, only small amounts of YopE or YopH are released into the culture medium, indicating that secretion occurs mainly at the contact zone between cell and bacteria (Rosqvist *et al.*, 1994; Persson *et al.*, 1995). Mutants that are unable to secrete YopN, and thus have lost Ca^{2+} repression, are also depolarized for YopE and YopH secretion; unlike the wild-type bacteria, they release large amounts of YopE or YopH in the eukaryotic cell culture supernatant (Rosqvist *et al.*, 1994; Persson *et al.*, 1995). YopN thus somehow controls Yop release. The fate of YopN during a cell culture infection by *Yersinia* has not been addressed yet.

The interaction *Yersinia*–eukaryotic cell thus constitutes a new type of cell–bacteria interaction: *Yersiniae* possess an integrated anti-host system consisting of the Ysc secretion machinery, a delivery apparatus (YopB and/or YopD), a control element (YopN) and intracellular effectors (YopE, YopH) silencing or damaging host cells. Given its serine–threonine kinase activity, YpkA (YopO) (Galyov *et al.*, 1993) presumably belongs to the effectors group.

YopM, another major Yop protein that is required for full virulence (Leung *et al.*, 1990), is an acidic protein composed of a succession of six leucine-rich repeated structures (Leung and Straley, 1989). It presents some similarity with the α chain of the platelet membrane glycoprotein Ib, a protein which binds thrombin and the von Willebrand factor (Leung and Straley, 1989). This similarity suggested that YopM could also bind thrombin. In agreement with this hypothesis, *in vitro* studies showed that YopM-containing culture supernatants of *Y.pestis* inhibit platelet aggregation, whereas culture supernatants of a *yopM* mutant do not (Leung *et al.*, 1990). Purified YopM displays the same effect on platelet aggregation and binds thrombin *in vitro* (Reisner and Straley, 1992). By interacting with thrombin, YopM could interfere with the normal process of platelet aggregation and/or with platelet-mediated events of the inflammatory response, and thus play an important role in the initial stages of an infection (Reisner and Straley, 1992). In conclusion, these studies suggest that YopM is an extracellular effector.

Given that YopM is secreted by the Ysc secretion apparatus and released in the same *in vitro* conditions as the other Yops, we wondered whether YopM could also be involved in translocation of intracellular effectors or even be internalized itself into eukaryotic cells. In addition, we investigated the fate of YopN. Using the Yop–Cya approach that we developed recently (Sory and Cornelis, 1994; Sory *et al.*, 1995), we show that YopM, but not YopN, is delivered inside eukaryotic cells. This delivery requires the same factors as those required to deliver YopE and YopH. The interpretation of the role of YopM thus needs to be revised or completed.

Results

Analysis of the Y.enterocolitica yopM gene

Thus far, the *yopM* gene has been isolated and sequenced only in *Y.pestis* KIM5 (Leung and Straley, 1989). For the present study, we isolated and sequenced the homologous gene from the *Y.enterocolitica* strain W22703. The nucleic acid sequence was 95.6% identical to that from *Y.pestis* (Leung and Straley, 1989). In both sequences, there is an ambiguity on the start codon because of the presence of three ATGs in-frame in the 5′ end of the gene. To alleviate this ambiguity, we sequenced the N-terminal end of the protein recovered from the culture supernatant of *Y.enterocolitica* W22703. The observed sequence exactly matched the sequence translated from the second start codon (data not shown). In agreement with this data, the second start codon is preceded by a fairly good ribosome binding site while the first one is not. Translation of the sequence between start codons 1 and 2 would not give a peptide resembling a classical signal peptide. YopM from *Y.enterocolitica* is thus translated from the second ATG in the sequence, as is the case in *Y.pestis* (Reisner and Straley, 1992). Like all the Yops, it is secreted without removal of a signal peptide, as was also reported in *Y.pestis* (Reisner and Straley, 1992). YopM secreted by *Y.enterocolitica* is a 41 330 Da protein with a predicted isoelectric point of 4.09. It is made up of 367 residues as is its homolog from *Y.pestis*, and both proteins are 90.5% identical.

The only protein which is significantly similar to YopM is IpaH of *Shigella* (Hartman *et al.*, 1990); both proteins have 41.2% identity in a 177 amino acid overlap. Unfortunately, nothing is known at the moment about the function of IpaH. As in *Y.pestis*, YopM contains a succession of six repeated structures (Leung and Straley, 1989). These repeats, made up of 19 amino acids, are rich in leucine residues and they are related to the very common leucine-rich repeat (LRR) motifs (for a review, see Kobe and Deisenhofer, 1994). As a consequence of the presence of these motifs, YopM shows some similarity to a great number of proteins, including many proteins of the extracellular matrix (proteoglycan, lumican, fibromodulin), proteins located in the plasma membrane (GPIb), proteins involved in signal transduction (thyreotropin receptor precursor) and even proteins having an intracellular function (regulatory proteins involved in mitosis, adenylate cyclases).

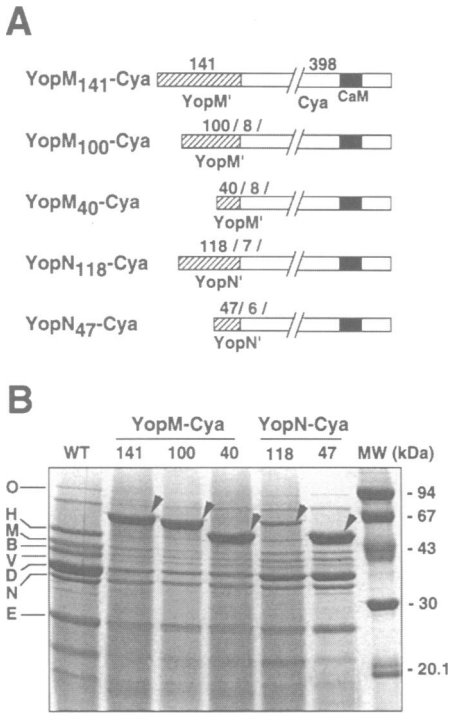

Fig. 1. (**A**) The YopM–Cya and YopN–Cya hybrid proteins. The numbers indicate the length, in amino acids, of Yop/hinge/Cya (no hinge in $YopM_{141}$–Cya; Cya is always 398 residues). The black box in the Cya domain represents the calmodulin binding site (CaM). (**B**) Secretion of the hybrid proteins represented in (A) by *Y.enterocolitica* E40 strain, after 4 h induction at 37°C, in a Ca^{2+}-deprived medium. The numbers on top of the lanes represent the number of residues of YopM or YopN present in the hybrid protein. WT: lane with proteins secreted by the wild-type host strain. The letters identify the secreted Yops. The arrows point to the secreted hybrids. SDS–PAGE stained with Coomassie blue.

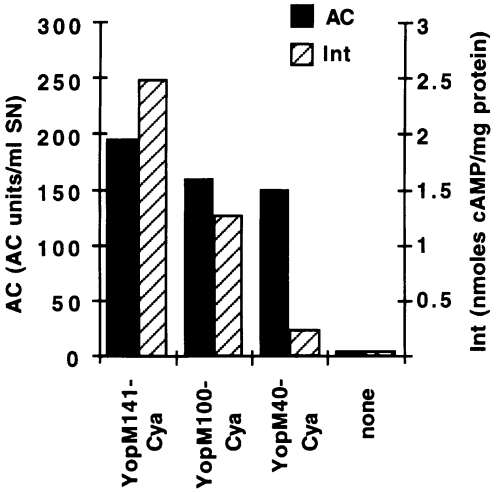

Fig. 2. Enzymatic activity of the secreted YopM–Cya reporters and their intracellular delivery. To measure the adenylate cyclase (AC) activity of the secreted hybrid proteins, bacteria were cultured in BHIox and induced for 1 h at 37°C. The AC activity (black columns) is expressed in AC units per ml of culture supernatant (left scale). The hatched columns represent the amount of cAMP produced in the cells (nmol/mg of protein, right scale).

YopM is not a component of the delivery apparatus

We first asked whether it was possible thatYopM was not an element of the delivery apparatus, like YopB and YopD. We thus monitored the delivery of YopE by a wild-type *Y.enterocolitica* and by mutants unable to synthesize YopM, using the $YopE_{130}$–Cya reporter (Sory and Cornelis, 1994; Sory *et al.*, 1995). Briefly, this hybrid between the N-terminal part of YopE and the calmodulin-dependent adenylate cyclase domain (Cya) of the *Bordetella pertussis* cyclolysin can only be enzymatically active in the presence of its co-factor, calmodulin, and substrate, ATP (for a review, see Mock and Ullmann, 1993). *Yersinia enterocolitica* engineered to secrete this reporter are added to monolayers of eukaryotic cells and cAMP is assayed subsequently on eukaryotic cell extracts. The accumulation of cAMP marks the internalization of the protein inside the eukaryotic cytosol, since this is the only compartment of the system where both co-factor and substrate are available (Sory and Cornelis, 1994).

We introduced plasmid pMS111 encoding $YopE_{130}$–Cya into wild-type *Y.enterocolitica* W22703(pYV227) and in its *yopM* insertion mutants W22703(pBM15) and W22703(pBM21) (Mulder *et al.*, 1989). Upon infection of HeLa cells with the three strains, we detected 1.7, 1.4 and 2.7 nmol cAMP/mg of protein, respectively. By comparison, infection with a *yopBD* mutant only generates 0.01 nmol of cAMP/mg of protein (Sory and Cornelis, 1994). This indicated that YopM is not required for delivery of YopE inside eukaryotic cells and hence could be an effector Yop.

Construction and characterization of YopM reporters

To determine whether YopM is delivered inside eukaryotic cells or in the extracellular compartment, we engineered plasmids pAB12, pAB6 and pAB13 encoding $YopM_{141}$–Cya, $YopM_{100}$–Cya and $YopM_{40}$–Cya, respectively. The three YopM–Cya reporters are represented in Figure 1A.

The plasmids encoding these hybrids were introduced in *Y.enterocolitica* E40(pYV40), and the *yop* regulon, which includes the engineered genes, was induced by a temperature shift in a Ca^{2+}-deprived medium. The secreted proteins were analyzed by SDS–PAGE. As shown in Figure 1B, all the hybrid proteins were secreted efficiently. To check whether they were enzymatically functional after secretion, we assayed the adenylate cyclase (AC) activity in the culture supernatants after 1 h of heat induction. All of the culture supernatants containing a YopM–Cya protein had an activity ranging from 150 to 200 AC U/ml of culture supernatant (Figure 2, black columns), while the supernatant of the wild-type bacteria only had an activity of 0.08 U/ml. All the hybrid proteins constructed were thus secreted efficiently and were enzymatically active.

YopM is internalized in PU5-1.8 macrophages

Recombinant *Y.enterocolitica* E40(pYV40) producing either $YopM_{141}$–Cya, $YopM_{100}$–Cya or $YopM_{40}$–Cya were used to infect PU5-1.8 macrophages. All the experiments were carried out in the presence of cytochalasin D which prevents phagocytosis of the bacteria themselves but not translocation of hybrid Yop–Cya proteins (Sory *et al.*, 1995). Infection with strain E40 producing $YopM_{141}$–Cya or $YopM_{100}$–Cya led to a significant increase in intracellular cAMP after 2 h (see Table I), showing that $YopM_{141}$–Cya and $YopM_{100}$–Cya were internalized

Table I. Internalization of the Yop–Cya hybrid proteins in PU5-1.8 macrophages

Plasmids		Genotype	Yop–Cya	Cell cAMP
pYV	Other			
–	–	–	–	0.08 ± 0.06
pYV40	–	wt	–	0.07 ± 0.01
pYV40	pAB12	wt	$YopM_{141}$–Cya	2.51 ± 0.97
pYV40	pAB6	wt	$YopM_{100}$–Cya	1.28 ± 0.76
pYV40	pAB13	wt	$YopM_{40}$–Cya	0.26 ± 0.16
pMSL41	pAB6	*yscN*[a]	$YopM_{100}$–Cya	0.05 ± 0.04
pYV40	pMS111	wt	$YopE_{130}$–Cya	9.29 ± 0.36[b]
pYV40	pMSLH99	wt	$YopH_{99}$–Cya	3.72 ± 0.69[b]
pAB41	pAB6	*lcrH*[c]	$YopM_{100}$–Cya	0.12 ± 0.11
pPW401	pMS111	*yopB*	$YopE_{130}$–Cya	0.08 ± 0.03[b]
pPW401	pMSLH99	*yopB*	$YopH_{99}$–Cya	0.11
pPW401	pAB6	*yopB*	$YopM_{100}$–Cya	0.08 ± 0.04
pMSL42	pMS111	*yopD*	$YopE_{130}$–Cya	0.11 ± 0.03[b]
pMSL42	pMSLH99	*yopD*	$YopH_{99}$–Cya	0.013
pMSL42	pAB6	*yopD*	$YopM_{100}$–Cya	0.06 ± 0.03
pYV40	pAB11	wt	$YopN_{118}$–Cya	0.06 ± 0.03
pYV40	pMSL12	wt	$YopN_{47}$–Cya	0.06 ± 0.03

Data (nmol cAMP/mg protein) are means ± SD of at least three experiments, each done in duplicate.
[a]Secretion mutant.
[b]Data are means ± SD of triplicate samples.
[c]Mutant unable to produce the SycD chaperone and hence to secrete YopB and YopD.
Experiments were carried out in the presence of cytochalasin D.

efficiently in the cytosol of macrophages. Infection with strain E40 producing $YopM_{40}$–Cya only resulted in a small increase in the level of intracellular cAMP, indicating that $YopM_{40}$–Cya was only very poorly internalized (see Table I). Infection with a *yscN* mutant encoding $YopM_{100}$–Cya but deficient in Yop secretion did not give rise to any increase in cAMP, showing that the process required Yop secretion and was not due to release of proteins by lysis of bacteria. The fact that $YopM_{141}$–Cya and $YopM_{100}$–Cya caused an increase in intracellular cAMP while $YopM_{40}$–Cya did not (see Figure 2, hatched columns), in spite of the fact that the three proteins had approximatively similar enzymatic activities (see Figure 2, black columns), strongly suggests that YopM is internalized in eukaryotic cells and that the domain of YopM required for this process is localized within the first 100 amino acids of the protein.

YopB and YopD individually are required for the internalization of YopM, YopE and YopH

The internalization of YopE and YopH in eukaryotic cells necessitates the presence of YopD and/or YopB (Rosqvist *et al.*, 1994; Sory and Cornelis, 1994; Persson *et al.*, 1995; Sory *et al.*, 1995), two Yops encoded by the *lcrGVHyopBD* operon. We investigated whether internalization of YopM also required these factors. We first introduced our recombinant plasmids in *Y.enterocolitica* E40 (pAB41), a *lcrH* non-polar mutant unable to secrete YopD and YopB because of a lack of the SycD chaperone (Wattiau *et al.*, 1994). This strain secreted the $YopM_{141}$– and $YopM_{100}$–Cya proteins to the same extent as the wild-type parental strain, but in macrophage infection experiments it failed to induce any accumulation of cAMP (see Table I). YopD and/or YopB were thus required for the internalization of YopM, as is the case for YopE and YopH.

To investigate further the individual role of YopD and YopB, we constructed a *yopD* mutant, E40 (pMSL42), and a non-polar *yopB* mutant, E40(pPW401). In Ca^{2+} depletion conditions, both mutants secreted normal amounts of all the Yops, except YopD or YopB respectively (not shown). We used these mutants to determine the individual role of YopB and YopD in delivery of $YopM_{100}$–Cya. As shown in Table I, the hybrid was not delivered in macrophages by the mutants. Similar results were obtained with $YopM_{141}$–Cya (not shown). YopD and YopB were thus both necessary for translocation of YopM.

Thus far, the role of YopB and YopD in translocation of YopE and YopH could only be deduced from the analysis of a *yopD* mutant (Rosqvist *et al.*, 1994) and polar *yopBD* mutants (Sory and Cornelis, 1994; Persson *et al.*, 1995; Sory *et al.*, 1995). To determine their individual role in translocation of YopE and YopH, we infected macrophages with the E40 strain and its isogenic *yopB* and *yopD* mutants producing $YopE_{130}$–Cya or $YopH_{99}$–Cya (Sory and Cornelis, 1994; Sory *et al.*, 1995). As with $YopM_{100}$–Cya, we did not detect any cAMP increase in cells infected with the mutants, while cAMP was raised significantly upon infection with the wild-type strain (see Table I).

In conclusion, YopB and YopD were both required for translocation of YopE, YopH and YopM.

Construction and characterization of a yopN mutant.

To investigate further the internalization process of Yops in macrophages by *Y.enterocolitica*, we studied the importance of YopN in this process. We cloned *yopN* from *Y.enterocolitica* W22703 and created an out-of-frame deletion of 445 bp, leaving only 45 codons of *yopN*. We replaced the wild-type *yopN* by the mutated allele ($yopN_{45}$) in the pYV plasmid of strain E40, giving strain E40(pIM41). The mutant was first tested for *in vitro* Yop secretion, in the absence of eukaryotic cells. At 37°C, this strain secreted all the Yops, with the exception of YopN,

in the presence as well as in the absence of Ca^{2+} (Figure 3). The mutant was thus 'Ca^{2+}-blind', as already described in *Y.pseudotuberculosis* (Forsberg *et al.*, 1991).

To characterize the interaction between our *yopN* mutant and eukaryotic cells, we monitored the internalization of $YopH_{99}$–Cya. We performed kinetic experiments with the wild-type strain, the *yopN* mutant, the *lcrH* mutant unable to secrete YopD and YopB and the *yscN* secretion mutant taken as a negative control. At each time point, we recorded intracellular delivery of $YopH_{99}$–Cya by assaying cAMP produced inside the macrophages during the infection. To gain an idea of the amount of $YopH_{99}$–Cya which is not delivered inside the macrophages but released in the supernatant, we assayed the AC activity in the cell culture medium. The *yopN* mutant released much more $YopH_{99}$–Cya in the cell culture medium than the wild-type strain and the *lcrH* mutant (Figure 4A), while the *yscN* mutant did not release any AC activity (data not shown). The opposite situation was observed inside the macrophages: the level of intracellular cAMP increased during the course of the infection with the wild-type strain but not with the *yopN* mutant, indicating that this mutant delivered virtually no hybrid protein inside the macrophages (Figure 4B). This experiment showed that secretion of $YopH_{99}$–Cya by a wild-type *Y.enterocolitica* is a polarized phenomenon when it is induced by contact with eukaryotic cells; it also showed that a *yopN* mutant secretes Yops in an unpolarized manner, which results in a very low rate of internalization. *Y.enterocolitica* thus behaved exactly as *Y.pseudotuberculosis* in that respect (Rosqvist *et al.*, 1994; Persson *et al.*, 1995).

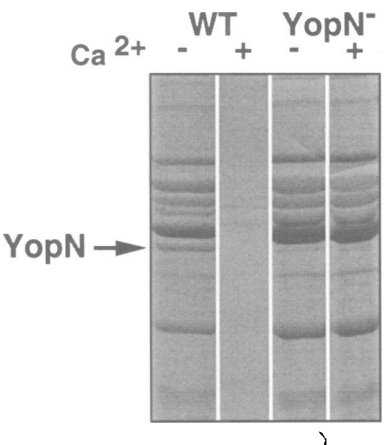

Fig. 3. Yop secretion by the *Y.enterocolitica* wild-type E40(pYV40) and the *yopN* mutant E40(pIM41), in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of Ca^{2+}. Bacteria were grown in BHIox or BHI-Ca^{2+}, and Yop secretion was induced for 4 h at 37°C. SDS–PAGE stained with Coomassie blue.

YopN is involved in intracellular targeting of YopM

We then turned to the analysis of $YopM_{100}$–Cya. Again, we monitored cAMP inside the macrophages and the AC activity in the cell culture supernatant. $YopM_{100}$–Cya was released in the supernatant by the *yopN* mutant, and to an even higher extent than $YopH_{99}$–Cya (Figure 4C). $YopM_{100}$–Cya was, nevertheless, delivered inside the macrophages by the *yopN* mutant, as indicated by the accumulation of cAMP (Figure 4D). Moreover, the amount of cAMP that accumulated inside the macrophages upon infection with the *yopN* mutant was higher than upon infection with the wild-type strain (Figure 4D). The situation was thus somewhat different from that observed with YopH: the *yopN* mutant secreted YopM in an unpolarized manner but it did, nevertheless, translocate YopM very efficiently inside eukaryotic cells (see Figure 4B and D).

To clarify this point, we wanted to compare the amounts of hybrid protein released in the supernatant and delivered inside the macrophages and to calculate the percentage of

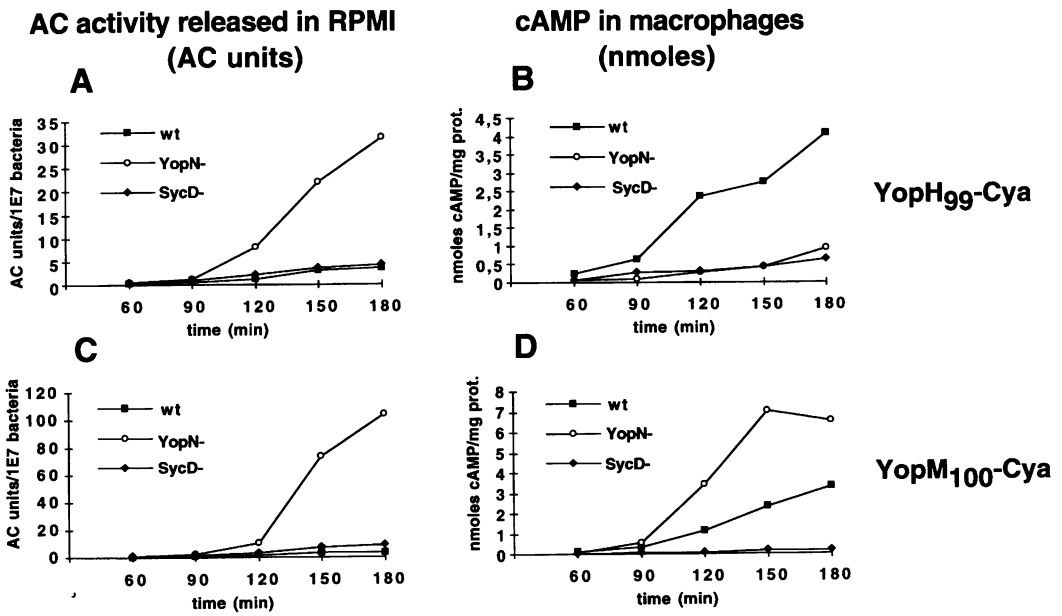

Fig. 4. Kinetics of internalization and release of the $YopH_{99}$–Cya and $YopM_{100}$–Cya hybrid reporters by a wild-type strain (■), a *yopN* mutant (○) and a *sycD* mutant (◆). The amounts of reporter released in RPMI are expressed in AC units per 10^7 bacteria. The internalization results are expressed in nmoles of cAMP produced in the macrophages per mg of total proteins. Note that the scales are different for each graph.
(**A**) $YopH_{99}$–Cya released in RPMI containing infected macrophages. (**B**) $YopH_{99}$–Cya internalized in the macrophages. (**C**) $YopM_{100}$–Cya released in RPMI containing infected macrophages. (**D**) $YopM_{100}$–Cya internalized in the macrophages.

Table II. Percentage of Yop–Cya hybrid protein internalized in the macrophages by the wild-type strain and the *yopN* mutant

Plasmids		Yops produced	Cyclase activity		% Intracellular
pYV	Other		in RPMI + MØ[a]	in MØ lysate[b]	
Study of $YopE_{130}$–Cya					
pYV40	pMS111	all	21.42	26.76	55.5
pIM41	pMS111	all except YopN	199.55	15.69	7.3
pMSL41	pMS111	none	0.15	2.75	–
Study of $YopH_{99}$–Cya					
exp.1					
pYV40	pMSLH99	all	1.50	1.33	46.99
pIM41	pMSLH99	all except YopN	23.59	0.75	3.08
pMSL41	pMSLH99	none	0.38	0.55	–
exp.2					
pYV40	pMSLH99	all	1.26	1.59	55.79
pIM41	pMSLH99	all except YopN	7.77	0.54	6.49
pMSL41	pMSLH99	none	1.15	0.50	–
Study of $YopM_{141}$–Cya					
pYV40	pAB12	all	5.27	1.49	22.04
pIM41	pAB12	all except YopN	128.91	1.61	1.15
pMSL41	pAB12	none	0.65	0.37	–
Study of $YopM_{100}$–Cya					
exp.1					
pYV40	pAB6	all	4.90	1.05	17.64
pIM41	pAB6	all except YopN	112.64	2.73	2.37
pMSL41	pAB6	none	0.55	0.33	–
exp.2					
pYV40	pAB6	all	3.02	0.76	20.11
pIM41	pAB6	all except YopN	77.53	1.13	1.44
pMSL41	pAB6	none	0.48	0.33	–

All the results are expressed in AC units per 10^7 bacteria.
[a]Adenylate cyclase activity detected in the RPMI medium in the presence of macrophages.
[b]Adenylate cyclase activity detected inside the macrophages.

internalization: instead of measuring the amount of cAMP synthesized inside the macrophages during the course of infection, we assayed the AC activity in the macrophage lysate as well as in the culture supernatant. This was done for $YopM_{141}$–Cya and $YopM_{100}$–Cya produced by the wild-type strain, the *yopN* mutant and the *yscN* mutant. The results are shown in Table II. Upon infection with the wild-type strain producing the two YopM–Cya hybrids, ~20% of the cyclase activity was recovered in the macrophages. In the absence of YopN, there was an increase of extracellular delivery but also of intracellular delivery, which is in agreement with our previous observation. Nevertheless, the loss of YopN resulted in a 10-fold decrease of the relative amount of reporter delivered inside macrophages (~2% instead of 20%). Thus, YopN controlled YopM polarized release but it was not required for YopM translocation.

In view of these results, we also wanted to quantify the influence of YopN on the delivery of YopE and YopH. Polarization appeared to be slightly more efficient for $YopE_{130}$–Cya and $YopH_{99}$–Cya than for YopM–Cya: ~50% of the hybrids were directed to the macrophage cytosol by the wild-type strain, while infection with the *yopN* mutant only permitted <8% of internalization in both cases. Thus, YopN clearly played a role in the polarized delivery of YopE and YopH inside the macrophages, as shown previously (Rosqvist *et al.*, 1994; Persson *et al.*, 1995).

We conclude from this that YopN controls polarized delivery of YopM inside the macrophage cytosol as it does for YopE and YopH, but the control seems to be less stringent for YopM.

YopN is not internalized in PU5-1.8 macrophages

The fact that YopN is involved in the control of polarization does not exclude the possibility that it could itself be internalized inside macrophages. To address this question, we engineered plasmids encoding $YopN_{118}$–Cya and $YopN_{47}$–Cya (Figure 1A). As for the other Yop–Cya hybrids, they were secreted efficiently (Figure 1B) and were enzymatically active (Figure 5, black columns). We infected cultured PU5-1.8 macrophages with *Y.enterocolitica* E40 producing either $YopN_{118}$–Cya or $YopN_{47}$–Cya. The amounts of intracellular cAMP detected after 2 h of infection were as low as those detected in macrophages infected with a wild-type *Y.enterocolitica* E40 strain (see Table I). This result indicated that the two YopN–Cya hybrid proteins constructed were not internalized during the contact between bacteria and macrophages, and suggested that YopN itself was probably not internalized in macrophages. The internalization results are summarized in Figure 5 (hatched columns).

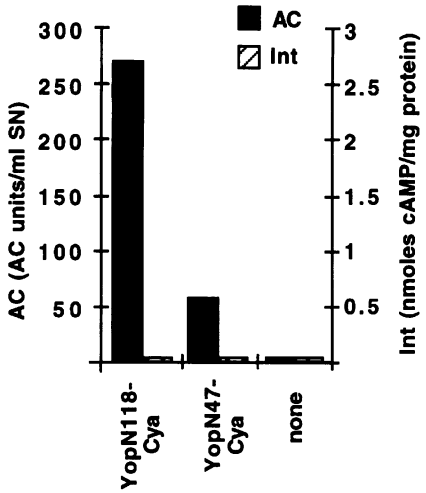

Fig. 5. Enzymatic activity and intracellular delivery of the secreted YopN–Cya reporters. To measure the adenylate cyclase (AC) activity of the secreted hybrid proteins, bacteria were cultured in BHIox and induced for 1 h at 37°C. The AC activity (black columns) is expressed in AC units per ml of culture supernatant (left scale). The hatched columns represent the amount of cAMP (nmoles) produced in the cells per mg of protein (right scale).

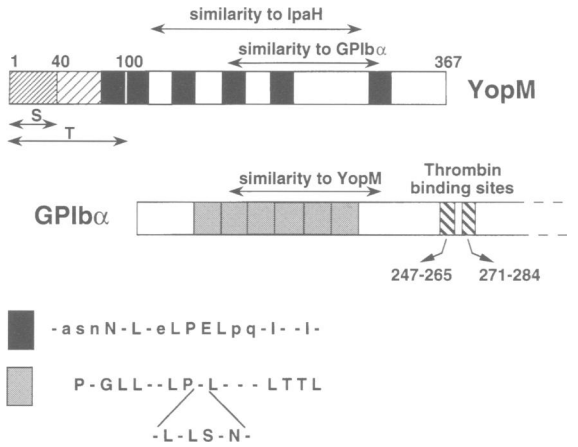

Fig. 6. Schematic representation of YopM and GPIbα. The repeats present in the proteins are represented by black (YopM) or gray (GPIbα) boxes. The corresponding consensus sequences are shown below (Leung and Straley, 1989). The numbers represent the residues of the proteins. The indicated similarity between both proteins was reported by Leung and Straley (1989). The secretion (S) and translocation (T) domains of YopM were determined in this work.

Discussion

YopE and YopH are delivered inside the eukaryotic cytosol by extracellular bacteria adhering to the cell surface. Previous work showed that the polarized delivery process requires YopD, YopN and possibly YopB (Rosqvist *et al.*, 1994; Sory and Cornelis, 1994; Persson *et al.*, 1995; Sory *et al.*, 1995). The experiments reported here show that YopB is also individually required. All these discoveries indicate that the Yop virulon forms an integrated system allowing extracellular bacteria to communicate with the host cell's cytosol by the injection of effector proteins. The virulon is composed of the type III Ysc secretion apparatus and three categories of Yops: intracellular effectors (YopE, YopH), a delivery apparatus (YopB, YopD) and a control element (YopN). The delivery apparatus comprises at least YopB and YopD; other Yops could be involved, in particular LcrV that is encoded by the same operon. Demonstration of the involvement of LcrV is difficult, however, since non-polar *lcrV* mutants present an unexplained complete lack of Yop secretion (Bergman *et al.*, 1991; Skrzypek and Straley, 1995).

The early hypothesis about the role of YopM as an extracellular effector that interacts with thrombin and interferes with the onset of the inflammatory response leads to the conclusion that some Yops are intracellular effectors while others are extracellular agents. Since YopE, YopH and YopM are secreted by the same apparatus (Michiels *et al.*, 1991; Allaoui *et al.*, 1994, 1995; Bergman *et al.*, 1994; Fields *et al.*, 1994; Woestyn *et al.*, 1994), we wondered whether YopM could also be translocated inside eukaryotic cells as are YopE and YopH. Hybrid proteins consisting of 100 or 141 residues of YopM fused to Cya reached the cytosol of infected macrophages in conditions which did not allow entry of the bacteria. The values obtained were in the same range as those recorded earlier for YopE–Cya and YopH–Cya hybrids, indicating that YopM follows the same route as YopE and YopH. The conclusion that YopM is delivered inside eukaryotic cells is strengthened by the fact that internalization of YopM–Cya required the presence of YopD and YopB, as is the case for YopE and YopH. Moreover, the transfer was controlled by YopN for all three Yops. All these experiments demonstrate that YopM is translocated across the eukaryotic cell membrane and thus may act as an intracellular effector, like YopE or YopH.

The fact that YopM is internalized in the cytosol of eukaryotic cells and thus probably has an intracellular target does not rule out the possibility that YopM could be multifunctional and play a role in the extracellular compartment. Our data show indeed that there is more leakage of YopM than of YopE and YopH during delivery inside the macrophages. However, a few other elements suggest that it will be important to analyze the interaction with thrombin in more detail in order to confirm the role of YopM as a thrombin binding agent. First, no thrombin binding site has been identified on YopM so far. Second, the domains of the GPIbα that are involved in the interaction with thrombin have now been identified and they lie between amino acids 271 and 284, and 247 and 265 (De Marco *et al.*, 1994; Gralnick *et al.*, 1994), which is outside the region sharing homology with YopM (amino acids 83–207; Leung and Straley, 1989) (Figure 6).

With regard to the intracellular action of YopM, we cannot present any hypothesis so far. Comparison of the YopM protein with various databases did not reveal any striking similarity which could suggest a working hypothesis. YopM has some similarity to a great number of proteins, but these similarities are probably only due to the presence of the very common LRR motifs, which are present in proteins with various functions and different cell localizations. These similarities are thus probably not significant with regard to the function of YopM. The elucidation of the intracellular localization of YopM, now in progress, might be more indicative.

We have shown previously that YopE and YopH are modular proteins consisting of a short N-terminal secretion domain followed by a translocation domain of some 35–50 residues and finally by an effector domain (Sory *et al.*,

1995). We have shown here that a YopM–Cya hybrid containing only 40 N-terminal residues of YopM is secreted but is almost not delivered inside macrophages, while hybrids containing >100 YopM residues are translocated. This suggests that the overall organization of YopM is the same as that of YopE and YopH. The secretion signal is contained within the first 40 residues and the translocation domain is located within the first 100 residues. Since $YopM_{40}$–Cya was only slightly injected into macrophages and since the translocation domain probably ends before the first repeat, we hypothesize that it ends between residues 40 and 75, as is the case for YopE and YopH. Figure 6 gives the proposed organization of YopM.

A point which needs to be clarified is the involvement of a chaperone. Indeed, the two other Yops that are translocated across eukaryotic membranes, namely YopE and YopH, possess a specific chaperone and these chaperones are required for secretion of these Yops outside the bacterial cell (Wattiau and Cornelis, 1993; Wattiau *et al.*, 1994). Woestyn *et al.* (1996) suggested that proteins which are going to be internalized in eukaryotic cells need a chaperone to avoid intrabacterial interaction with some components of the translocation machinery. Based on this model, we would also expect the existence of a chaperone for YopM. Such a chaperone has not been identified thus far, but all the pYV genes have not yet been analyzed and candidate genes presently are being investigated.

Here, we have also investigated the fate of YopN. We confirmed the observation of H.Wolf-Watz and colleagues that YopN controls the polarized delivery of YopE and YopH (Rosqvist *et al.*, 1994; Persson *et al.*, 1995) and we extended these observations to YopM. We also constructed two YopN–Cya hybrids and we did not observe their internalization in macrophages, which obviously fits with the idea of YopN being a control element of the delivery apparatus (Rosqvist *et al.*, 1994). In *Shigella*, the control of Ipa release depends on IpaB and IpaD. Menard *et al.* (1994) showed that *ipaB* and *ipaD* mutants release large amounts of Ipas while the wild-type *Shigella* does not. They propose that IpaB and IpaD act as anti-secretion factors by directly blocking the secretion apparatus when *Shigella* does not interact with eukaryotic cells. IpaB resembles YopB of *Y.enterocolitica* but our *yopB* mutant did not exhibit the 'leaky' phenotype of *ipaB* mutants. Moreover, there is no *Yersinia* counterpart of IpaD. This suggest that both systems could be somewhat different and that the homologs do not necessarily play the same role in both organisms. *In vitro*, the *yopN* mutant presents a 'leaky' phenotype which evokes that of the *ipaB* and *ipaD* mutants. It is thus conceivable that YopN plays a role resembling that of IpaB and IpaD in *Shigella* and acts as a cork to block secretion of the translocator and effector Yops. Contact between YopN and a eukaryotic receptor at the cell surface would relieve this blockage and allow secretion, assembly of the delivery apparatus and translocation of effectors. Persson *et al.* (1995) reached the same conclusion after their analysis of YopH delivery by *Y.pseudotuberculosis*. Figure 7 illustrates the delivery of Yops in a eukaryotic cell by *Y.enterocolitica*.

In conclusion, we have shown that YopM is delivered by *Yersinia* inside macrophages, just like YopE and YopH. We have also shown that YopN exerts a control function for YopM and is probably not introduced itself in macrophages. The reassessment of the fate of YopM reinforces the idea that the Yop virulon forms an integrated anti-host system consisting of: (i) the Ysc secretion machinery; (ii) a delivery apparatus; (iii) a control system; and (iv) intracellular Yop effectors.

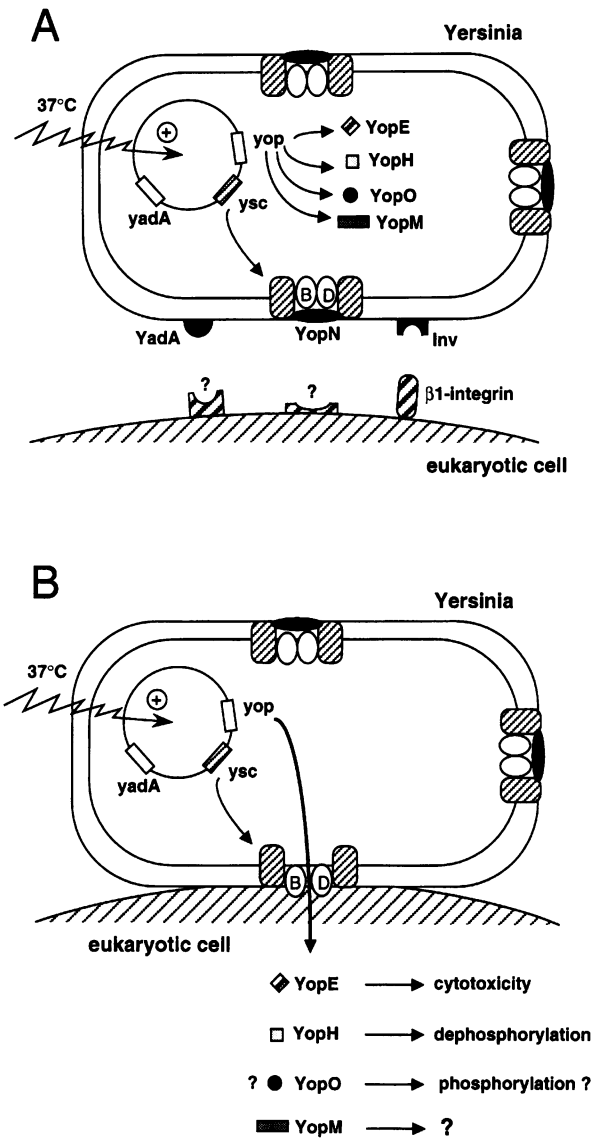

Fig. 7. Model showing *Y.enterocolitica* facing a eukaryotic cell. Before contact with the eukaryotic cell, the YopN protein acts as a cork limiting the release of Yop effectors in the surrounding environment (**A**). Upon YadA- and Inv-mediated intimate contact with the eukaryotic cell, YopN is removed and transfer of the YopE, YopH, YopM (and possibly YopO) effectors across the eukaryotic cell's membrane occurs (**B**); the complete process of translocation requires the secretion apparatus (Ysc) and both the YopB and YopD proteins.

Materials and methods

Bacterial strains, plasmids and growth conditions

This work was carried out with *Y.enterocolitica* W22703(pYV227) (Cornelis and Colson, 1975) and E40(pYV40) (Sory *et al.*, 1995), two strains that belong to serotype O:9. The plasmids are listed in Table III. Plasmids encoding the Yop–Cya hybrid proteins were introduced in *Y.enterocolitica* by conjugation. Bacterial growth conditions, induction of the *yop* regulon and Yop protein analyses were described by Cornelis *et al.* (1989) and Sory *et al.* (1992). The culture media used for *Yersinia* were Brain Heart Infusion (BHI), BHI supplemented with 20 mM sodium

Table III. Plasmids

Plasmids	Relevant characteristics	References
pYV		
pAB41	pYV40 *lcrH16* (does not direct YopB and YopD secretion because of a lack of SycD)	Sory *et al.* (1995)
pBM15	pYV227 *yopM*::Tn*2507* (does not encode YopM)	Mulder *et al.* (1989)
pBM21	pYV227 *yopM*::Tn*2507* (does not encode YopM)	Mulder *et al.* (1989)
pIM41	pYV40 $yopN_{45}$ (pYV40 mutated with pIM150; does not encodeYopN)	this work
pMSL41	pYV40 $yscN_{\Delta 169-177}$ (secretion mutant)	Sory *et al.* (1995)
pMSL42	pYV40 *yopD::pMSL31* (pYV40 mutated by integration of pMSL31; does not encode YopD)	this work
pPW401	pYV40 $yopB_{\Delta 89-217}$ (pYV40 mutated with pPW75; does not encode YopB)	this work
pYV227	wild-type pYV plasmid of *Y.enterocolitica* W22703	Cornelis and Colson (1975)
pYV40	wild-type pYV plasmid of *Y.enterocolitica* E40	Sory *et al.* (1995)
Clones and vectors		
pAA17	pBC18R $yscN^+$, $yopN_{118}{}^-$ (contains the first 118 codons of *yopN*)	Woestyn *et al.* (1994)
pAA31	pBC18R $yscN^+$, $yopN_{47}{}^-$ (contains the first 47 codons of *yopN*)	Woestyn *et al.* (1994)
pAB5	pBluescript SK– P_{yopM} $yopM_{100}{}^+$ (PCR fragment encoding the first 100 residues of YopM cloned in the *Pst*I–*Hin*dIII sites of the vector)	this work
pAB6	pTM100 P_{yopM} $yopM_{100}$-$cyaA'^+$ (*Bam*HI–*Hin*dIII fragment from pAB5 subcloned in pMSL18; encodes $YopM_{100}$–Cya)	this work
pAB9	pBluescript SK– P_{yopM} $yopM_{141}{}^+$ (PCR fragment encoding the first 141 residues of YopM cloned in *Pst*I–*Hin*dIII sites of the vector)	this work
pAB10	pTM100 P_{yopM} $yopM_{141}{}^+$, $cyaA'^-$ (*Bam*HI–*Hin*dIII fragment from pAB9 subcloned in pMSL18; contains the first 141 codons of *yopM* and the *cyaA'* gene)	this work
pAB11	pTM100 P_{yopN} $yopN_{118}$-$cyaA'^+$ (*Bam*HI–*Sma*I fragment of pSWL1 cloned in pTM100; encodes $YopN_{118}$–Cya)	this work
pAB12	pTM100 P_{yopM} $yopM_{141}$-$cyaA'^+$ (*Bgl*II–*Hin*dIII digestion of pAB10, limited action of mung bean nuclease and religation; tencodes $YopM_{141}$–Cya)	this work
pAB13	pTM100 P_{yopM} $yopM_{40}$-$cyaA'^+$(*Nsi*I–*Hin*dIII deletion of pAB10, T4 polymerase treatment and religation; encodes $YopM_{40}$–Cya)	this work
pIM144	pBluescript KS– P_{yopN} $yopN^+$ (cloning of a 1160 bp PCR fragment in the *Cla*I site of the vector; contains the complete *yopN* gene)	this work
pIM146	pBluescript KS– P_{yopN} $yopN^+$ (subcloning of a *Sal*I–*Eco*RV fragment of pIM144 in *Sal*I–*Sma*I sites of pBluescript KS–)	this work
pIM149	pBluescript KS– P_{yopN} $yopN_{45}{}^+$ (*Pst*I restriction, T4 polymerase trimming and religation of pIM146; out-of-frame deletion of *yopN* after codon 45)	this work
pMS107	pIC20H $cyaA'^+$ (contains nucleotides 4–1197 of *cyaA*)	Sory and Cornelis (1994)
pMS111	pTM100 $sycE^+$, P_{yopE} $yopE_{130}$-$cyaA'^+$ (encodes SycE and $YopE_{130}$–Cya)	Sory and Cornelis (1994)
pMSL12	pTM100 P_{yopN} $yopN_{47}$-$cyaA'^+$ (cloning of a *Bam*HI–*Pst*I fragment from pAA31 in pMS111; encodes $YopN_{47}$–Cya)	this work
pMSL18	pTM100 P_{yopH} $yopH_{1200}{}^+$, $cyaA'^-$ (contains the first 1200 nucleotides of *yopH* and the *cyaA'* gene)	Sory *et al.* (1995)
pMSLH99	pTM100 P_{yopH} $yopH_{99}$-$cyaA'^-$ (encodes $YopH_{99}$–Cya)	Sory *et al.* (1995)
pSWL1	pBC18R $yopN_{118}$-$cyaA'^+$ (*Xho*I–*Sma*I fragment of pMS107 cloned in pAA17; encodes $YopN_{118}$–Cya)	this work
pTM100	pACYC184-oriT_{RK2}	Michiels and Cornelis (1991)
Suicide vectors and mutators		
pIM150	pKNG101 $yopN_{45}{}^-$ (*Sal*I–*Xba*I fragment of pIM149 cloned in pKNG101)	this work
pKNG101	ori_{R6K} *sac*BR^+ $oriT_{RK2}$ *str*AB^+ (suicide vector)	Kaniga *et al.* (1991)
pKNG160	ori_{R6K} mob_{RK2} *tet* (suicide vector)	K.Kaniga (unpublished)
pMSL31	pKNG160 $yopD'^+$(*Bgl*II–*Eco*RV 349 bp internal fragment of *yopD* (nucleotides 231–580) cloned in pKNG160)	this work
pPW75	pKNG101 $yopB_{\Delta 89-217}{}^-$	Schulte *et al.* (1996)

oxalate, 20 mM $MgCl_2$ and 0.4% glucose (BHIox) or BHI supplemented with 5 mM $CaCl_2$ and 0.4% glucose (BHI-Ca^{2+}).

DNA sequencing of yopM

To sequence *yopM*, a 2549 bp *Hin*cII fragment from the *Eco*RI no. 6 fragment of pYV22703 (Mulder *et al.*, 1989) was cloned in pBC19R and pBC18R. The resulting plasmids (pBM290 and pIM153) were submitted to gradual and unidirectional deletion by exonuclease III as recommended by the manufacturer (Promega). The sequence was determined on both strands using a LICOR automatic sequencer. The sequence was submitted to EMBL (accession number Z69926). DNA and protein sequences were analyzed using the Blast (Altschul *et al.*, 1990; Gish and States, 1993) and FastA programs (Pearson and Lipman, 1988).

Construction of the pYV mutants

To construct the *yopN* mutant, a 1527 bp fragment containing the entire *yopN* gene was amplified by PCR, using amplimers MIPA 238 5′-CCCGGAATTCCGGGCGCACACCAGGCACTA-3′ and MIPA 239 5′-CCGCGGATCCGCGGGCGAAAGGGGGTAG-3′ (Viitanen *et al.*, 1990). This fragment was cleaved with *Cla*I and the central 1160 bp fragment containing the entire *yopN* gene was cloned in the *Cla*I site of pBluescript KS–, yielding plasmid pIM144. The *Pst*I site of the multiple cloning site was eliminated, giving plasmid pIM146. An out-of-frame 445 bp deletion was introduced in *yopN* by a *Pst*I digestion followed by T4 polymerase treatment and religation, resulting in plasmid pIM149. The *Sal*I–*Xba*I fragment of pIM149 containing the mutated *yopN* allele, called $yopN_{45}$, was then cloned in the same sites of the suicide vector pKNG101 to generate the *yopN* mutator, pIM150. The $yopN_{45}$ allele was then introduced by double recombination into the pYV plasmid of *Y.enterocolitica* E40, giving E40(pIM41).

The *yopD* mutant, *Y.enterocolitica* E40(pMSL42), was obtained by disruptive integration of pMSL31 into *yopD*. pMSL31 is a derivative of pKNG160 containing the 349 bp *Bgl*II–*Eco*RV internal fragment of *yopD* (Håkansson *et al.*, 1993).

The *yopB* mutant, *Y.enterocolitica* E40(pIM41), was obtained by replacement of the wild-type *yopB* gene by the allele $yopB_{\Delta 89-217}$, using the mutator plasmid pPW75 (Schulte *et al.*, 1996). This non-polar mutant encodes a YopB protein deprived of a hydrophobic domain (residues 89–217).

Construction of the YopM–Cya hybrid proteins

A fragment containing the first 300 nucleotides of *yopM* (*yopM'*) and its promoter was amplified by PCR using amplimers MIPA 230 5'-TAGCCTGCAGCTTTCAATGGTAGAAGA-3' (introducing a *Pst*I site in 5') and MIPA 231 5'-CGACAAGCTTTACATGACGCCACTA-3' (introducing a *Hin*dIII site in 3'). This *Pst*I–*Hin*dIII fragment was first cloned in the corresponding sites of pBluescript SK–, giving plasmid pAB5; a *Bam*HI–*Hin*dIII fragment from pAB5 was then used to replace the *Bam*HI–*Hin*dIII fragment of pMSL18, which contains the first 1200 nucleotides of *yopH* (Sory *et al.*, 1995). In the resulting plasmid pAB6, the *yopM* promoter and the first 300 nucleotides of *yopM* are fused to nucleotides 4–1197 of *cyaA'*, encoding a hybrid protein of 506 residues, composed of the first 100 amino acids of YopM fused to Cya ($YopM_{100}$–Cya).

Amplimers MIPA 230 and MIPA 227 5'-CGGCAAGCTTACTG-ATTATTAGAGACACC-3' were used to amplify a fragment containing the *yopM* promoter and the first 141 amino acids of YopM; again, the *Pst*I–*Hin*dIII fragment was cloned in pBluescript SK– (plasmid pAB9) and a *Bam*HI–*Hin*dIII fragment was substituted in pMSL18, giving pAB10. To put both fragments in-frame, a *Bgl*II–*Hin*dIII digestion followed by mung bean nuclease treatment and religation was performed on pAB10; the resulting plasmid, pAB12, encodes a 539 codon hybrid protein composed of the first 141 amino acids of YopM fused to Cya ($YopM_{141}$–Cya).

Plasmid pAB13 encoding the hybrid protein $YopM_{40}$–Cya (446 amino acids) was obtained by deleting a *Nsi*I–*Hin*dIII fragment of pAB10 and treating with T4 polymerase before religation.

Construction of the YopN–Cya hybrid proteins

Plasmid pMSL12, encoding $YopN_{47}$–Cya, was obtained by cloning a 790 bp *Bam*HI–*Pst*I fragment of pAA31 (Woestyn *et al.*, 1994) containing the first 141 bp of *yopN* (Viitanen *et al.*, 1990) in the same restriction sites of pMS111 (Sory and Cornelis,1994).

Plasmid pAB11, encoding the hybrid protein $YopN_{118}$–Cya, was constructed by cloning a *Bam*HI–*Sma*I fragment of pSWL1 in pTM100. pSWL1 is a multicopy plasmid encoding $YopN_{118}$–Cya; it was obtained by cloning the *Xho*I–*Sma*I fragment of pMS107 containing *cyaA'* (Sory and Cornelis, 1994) in pAA17, which contains the first 118 codons of YopN (Woestyn *et al.*, 1994).

Adenylate cyclase activity assay of the hybrid proteins

The enzymatic activity of all the hybrid proteins was assayed on the supernatant of heat-induced cultures of *Y.enterocolitica*. Briefly, a freshly isolated transconjugant colony was cultured overnight in 5 ml of BHI and then diluted to an optical density at 600 nm of 0.1 in 10 ml of BHIox, supplemented with the relevant antibiotics. After 2 h growth at room temperature with shaking, bacteria were induced at 37°C for 1 h. One ml of the culture was then centrifuged for 5 min (7500 *g*) at 4°C; the supernatant was removed and centrifuged again for 20 min in the same conditions. The AC activity was then assayed by incubating 50 µl of supernatant with 2 mM ATP and 0.1 µM calmodulin at 30°C and monitoring cAMP synthesis, as described by Sory and Cornelis (1994). The AC activity was expressed in AC units per ml of culture supernatant. One AC unit corresponds to synthesis of 1 nmol of cAMP/min.

Eukaryotic cell growth conditions and assays

HeLa cells and PU5-1.8 mouse monocyte–macrophage cells (ATCC TIB 61) were routinely grown and used for the internalization experiments in 24-well tissue culture dishes, as decribed by Sory and Cornelis (1994).

All the experiments were performed in the presence of cytochalasin D, added 30 min prior to infection at a final concentration of 5 µg/ml (stock solution was 2 mg/ml in dimethylsulfoxide). After addition of 10^7 bacteria per well (m.o.i. = 10), cell cultures were incubated for 2 h at 37°C, in an 8% CO_2 atmosphere; after infection, cells were washed three times with RPMI and lysed in denaturing conditions (HCl 50 mM, Triton 0.1%, 100°C for 5 min). cAMP was then extracted and assayed by an enzyme immunoassay (Biotrak®, Amersham) as described by Sory and Cornelis (1994) and results expressed in nmoles of cAMP per mg of total proteins. The amount of total proteins was determined by the Bradford method (1976) (Bio-Rad Protein Assay).

To monitor the release of Yop–Cya hybrids in the culture medium of infected macrophages, we proceeded as follows: the culture medium was collected, centrifuged for 5 min at 7500 *g*, 4°C to eliminate bacteria; the supernatant was then centrifuged further for 20 min in the same conditions, and AC activity was assayed on 50 µl of supernatant; samples were incubated with 2 mM ATP and 0.1 µM calmodulin at 30°C for 15 min for the kinetic experiments and for 10 min in the other cases, as described by Sory and Cornelis (1994). The results were expressed in AC units per 10^7 bacteria.

To assay AC activity inside the macrophages, cells were washed as described above and lysed with 1 ml of NaCl 0.9%–Triton X-100 0.1% solution; the extract was cleared by centrifugation at 7500 *g* at 4°C, supplemented with 2 mM ATP and 0.1 µM calmodulin (Sigma) and incubated for 10 min at 30°C. Results were again expressed in AC units per 10^7 bacteria. This assay could be disturbed by the amount of cAMP produced inside the cells during the infection but we observed that this was not the case: the amount of intracellular cAMP was negligible in comparison with the amount of cAMP produced during the *in vitro* AC assay (~50-fold difference) and hence did not disturb the assay.

Acknowledgements

We thank Isabelle Lambermont for excellent technical assistance. We also thank M.Loiseau and K.Conrath (Smith Kline Biologicals) for sequencing the N-terminus of YopM, and K.Kaniga for the gift of plasmid pKNG160. We acknowledge T.Michiels, S.Woestyn and J.-C.Vanooteghem for critical reading of the manuscript. A.B. is a research assistant from the Belgian 'Fonds National de la Recherche Scientifique'. This work was supported by grants from the Belgian FRSM (Fonds National de la Recherche Scientifique Médicale, convention 3.4627.93) and the 'Actions de Recherche Concertées' 94/99-172 from the 'Direction générale de la Recherche Scientifique—Communauté Française de Belgique'. This work was also supported by the 'Human Capital and Mobility program' from the European Union (contract ERBCHRXCT930171).

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Received on March 8, 1996; revised on June 19, 1996