The EMBO Journal Vol.17 No.7 pp.1907–1918, 1998

TyeA, a protein involved in control of Yop release and in translocation of *Yersinia* Yop effectors

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Extracellular *Yersinia* spp. disarm the immune system by injecting the effector *Yersinia* outer proteins (Yops) into the target cell. Yop secretion is triggered by contact with eukaryotic cells or by Ca^{2+} chelation. Two proteins, YopN and LcrG, are known to be involved in Yop-secretion control. Here we describe TyeA, a third protein involved in the control of Yop release. Like YopN, TyeA is localized at the bacterial surface. A *tyeA* knock-out mutant secreted Yops in the presence of Ca^{2+} and in the absence of eukaryotic cells. Unlike a *yopN* null mutant, the *tyeA* mutant was defective for translocation of YopE and YopH, but not YopM, YopO and YopP, into eukaryotic cells. This is the first observation suggesting that Yop effectors can be divided into two sets for delivery into eukaryotic cells. TyeA was found to interact with the translocator YopD and with residues 242–293 of YopN. In contrast with a *yopN* null mutant, a $yopN_{\Delta 248–272}$ mutant was also unable to translocate YopE and YopH. Our results suggest that TyeA forms part of the translocation-control apparatus together with YopD and YopN, and that the interaction of these proteins is required for selective translocation of Yops inside eukaryotic cells.
Keywords: bacteria–host-cell communication/microbial pathogenesis/signalling/type III secretion/*Yersinia*

Introduction

Yersinia enterocolitica resists the primary immune response of the host by injecting toxic effector *Yersinia* outer proteins (Yops) into the cells involved in this response. The apparatus allowing this pathogen–cell interaction is called the Yop virulon and is encoded by the pYV plasmid (for review see Cornelis and Wolf-Watz, 1997). The Yop virulon is composed of four elements: (i) a type III or contact secretion machinery, responsible for the secretion of Yop proteins and composed of at least 20 elements (Michiels *et al.*, 1991; Plano *et al.*, 1991; Allaoui *et al.*, 1994, 1995; Bergman *et al.*, 1994; Fields *et al.*, 1994; Woestyn *et al.*, 1994); (ii) a system involved in the recognition of eukaryotic cells and the control of the secretion, consisting of YopN and LcrG (Forsberg *et al.*, 1991; Skrzypek and Straley 1993; Rosqvist *et al.*, 1994; Boland *et al.*, 1996; Boyd *et al.*, 1998; Sarker *et al.*, 1998b); (iii) a complex of Yops, consisting of at least YopB, YopD and LcrV, required to translocate the effector Yops across the membrane of the target cell (Rosqvist *et al.*, 1994; Sory and Cornelis, 1994; Persson *et al.*, 1995; Sory *et al.*, 1995; Boland *et al.*, 1996; Håkansson *et al.*, 1996; Sarker *et al.*, 1998a; M.-P.Sory, C.Kerbourch and G.R.Cornelis, unpublished); and (iv) a set of toxic effector Yops, namely YopE, YopH, YopM, YopO (YpkA in *Yersinia pseudotuberculosis*) and YopP (YopJ in *Y.pseudotuberculosis*), that are internalized into the eukaryotic cells to disarm their functions (Rosqvist *et al.*, 1994; Sory and Cornelis, 1994; Persson *et al.*, 1995; Sory *et al.*, 1995; Boland *et al.*, 1996; Håkansson *et al.*, 1996; M.-P.Sory, C.Kerbourch and G.R.Cornelis, unpublished). YopH is a 51 kDa protein tyrosine phosphatase (Michiels and Cornelis, 1988; Guan and Dixon, 1990; Black and Bliska, 1997; Persson *et al.*, 1997) and YopO/YpkA is a 81 kDa serine/threonine kinase (Galyov *et al.*, 1993; Håkansson *et al.*, 1996). The enzymic properties of YopE, YopM and YopP/YopJ are not known. The action of YopE leads to the disruption of the actin microfilaments (Rosqvist *et al.*, 1991) while that of YopP/YopJ is required for apoptosis of macrophages (Mills *et al.*, 1997; Monack *et al.*, 1997). The secretion and subsequent injection of Yops is a phenomenon that occurs essentially in the area of contact between the bacterium and the eukaryotic cell. The protein YopN is involved in this polarized delivery since a *yopN* null mutant secretes more Yops into the eukaryotic cell medium than it injects into eukaryotic cells. This results in a very low ratio between the amount of Yops delivered into cells and Yops secreted into the culture medium (Forsberg *et al.*, 1994; Rosqvist *et al.*, 1994; Persson *et al.*, 1995; Boland *et al.*, 1996). From this observation it was inferred that YopN blocks the release of Yop effectors and translocators until contact between the bacterium and the eukaryotic cell is established and thus could function as a stop valve. In the absence of eukaryotic cells and at 37°C, Yop secretion can be artificially triggered by Ca^{2+} chelation. Mutants unable to synthesize YopN or LcrG are Ca^{2+}-blind in the sense that they secrete Yops in the presence as well as in the absence of Ca^{2+} (Forsberg *et al.*, 1991, 1994; Skrzypek and Straley, 1993; Boland *et al.*, 1996; Sarker *et al.*, 1998b). As a result of this deregulated secretion they are unable to grow at 37°C irrespective of the presence or the absence of Ca^{2+}. YopN is the product of the first gene of an operon which also encodes four other open reading frames (ORFs) that have not yet been characterized (Viitanen *et al.*, 1990; Forsberg *et al.*, 1991). In this paper we show that the product of the first ORF, called TyeA (Translocation of Yops into eukaryotic cells A), interacts with YopN and YopD and is required for translocation of YopE and YopH but not

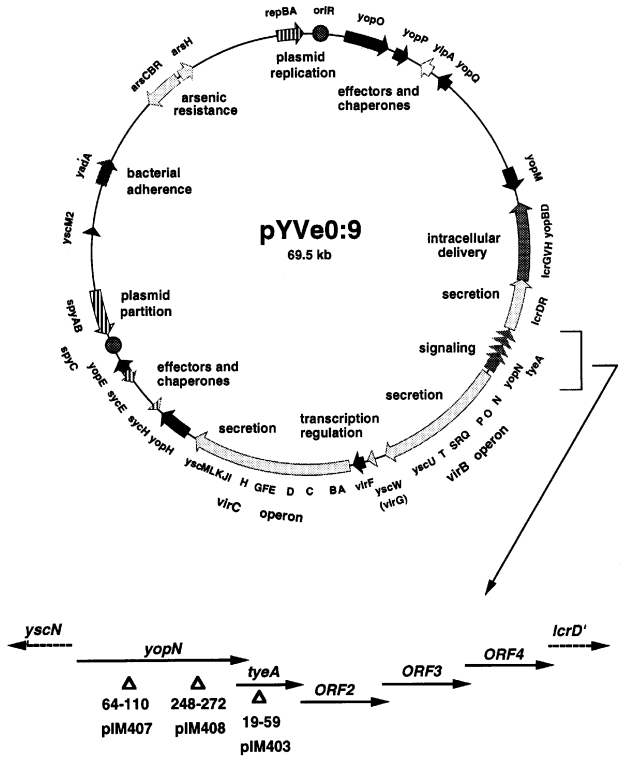


Fig. 1. Genetic map of the pYV plasmid of *Y.enterocolitica*. The region encoding YopN, TyeA and the surrounding genes is shown in more detail.

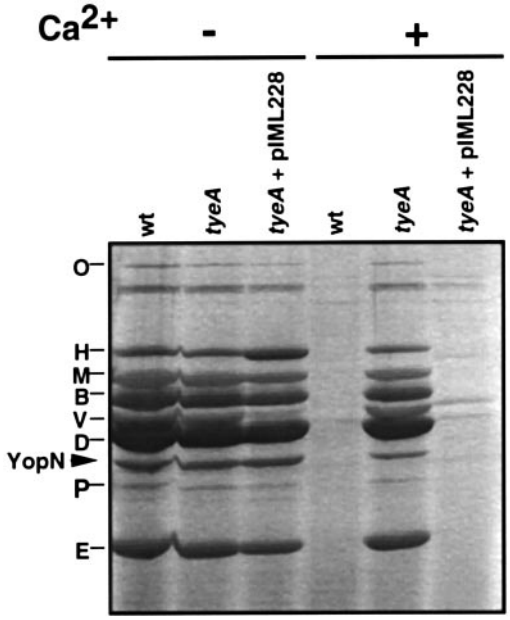


Fig. 2. Comparison of the Yop secretion pattern of the wild-type strain and the *tyeA* mutant. wt, wild-type MRS40 (pYV40); *tyeA*, *tyeA* mutant MIE40 (pIM403); *tyeA* pIML228, *tyeA* mutant MIE40 (pIM403) carrying plasmid pIML228 expressing P_{lac} *tyeA*. The strains were grown in BHI-OX ($-Ca^{2+}$) or BHI-Ca^{2+} ($+Ca^{2+}$). Culture supernatants were analysed by SDS–PAGE and stained with Coomassie Blue. Letters on the left indicate the different Yops.

YopM, YopO or YopP. This is the first observation suggesting that intracellular Yop effectors could be divided into two sets for delivery into eukaryotic cells.

Results

TyeA, a new protein involved in the control of Yop release

The first ORF situated downstream of the *yopN* gene encodes a protein of 92 amino acids which has not received much attention so far (Viitanen *et al.*, 1990; Forsberg *et al.*, 1991; Figure 1). Taking into account the compact character of the pYV plasmid, we considered that this small protein could be important in the Yop virulon. We engineered an in-frame deletion from codons 19–59 by directed mutagenesis, and the $orf1_{\Delta\ 19–59}$ allele was introduced into the pYV plasmid by allelic exchange. The mutant *Y.enterocolitica* strain was called E40 (pIM403). We also inactivated the chromosomal *blaA* gene encoding β-lactamase A. The *orf1 blaA* mutant strain was called MIE40 (pIM403).

Like the *yopN* mutant, the MIE40 (pIM403) mutant was Ca^{2+}-blind for growth and Yop secretion: at 37°C the mutant failed to grow and secreted all Yops irrespective of the presence of Ca^{2+} (Figure 2). To ensure that this phenotype was due to the *orf1* mutation and not to a polar effect on a downstream gene, we complemented the mutation using pIML228, a pBC18R derivative containing *orf1* under the control of the P_{lac} promoter. Introduction of pIML228 into MIE40 (pIM403) fully restored the wild-type phenotype for Yop secretion (Figure 2). The product of *orf1* is thus involved in the control of Yop release. For reasons that will become apparent later, we named it TyeA.

Production of TyeA is independent of Ca^{2+}

It has previously been shown that in contrast with the other Yops, YopN is produced at 37°C in the presence of Ca^{2+} but not secreted (Forsberg *et al.*, 1991). We wondered whether TyeA was also produced under the same conditions. To test this we grew the wild-type strain and the *tyeA* mutant in various conditions and analysed the total cell proteins by immunoblotting with a polyclonal anti-TyeA serum and a polyclonal anti-YopN serum. As a control the same cellular extracts were analysed for the presence of YopE. In the wild-type strain TyeA and YopN were present at 37°C even in the presence of Ca^{2+}, while YopE was not (Figure 3). None of the proteins was produced at 22°C. As already observed (Figure 2), YopN was produced in a *tyeA* mutant, indicating that TyeA is not required for YopN production.

TyeA is localized at the bacterial surface

The fact that YopN and TyeA are constitutively expressed at 37°C suggests that these two proteins could act as sensors for the *Yersinia* Yop virulon and thus be located at the bacterial surface. In a previous work, Forsberg *et al.* (1991) showed that at least some of the YopN produced is exposed at the bacterial surface. To analyse the localization of TyeA and YopN, we induced transcription of the *yop* genes at 37°C in the presence and absence of Ca^{2+}, fractionated cells and monitored the presence of the proteins by Western blotting. When wild-type bacteria were grown in low Ca^{2+} conditions (Figure 4, BHI-OX), YopN was mainly detected in the culture supernatant and in the Triton-insoluble membrane fraction, and was only weakly detected in the bacterial-soluble fraction. Localization of YopN was the same in the *tyeA* mutant, even though the level detected in the bacterial-soluble fraction

was very weak. In the same low Ca^{2+} conditions, TyeA was detected in the bacterial-soluble fraction and in the Triton-insoluble membrane fraction but not in the culture supernatant (Figure 4A).

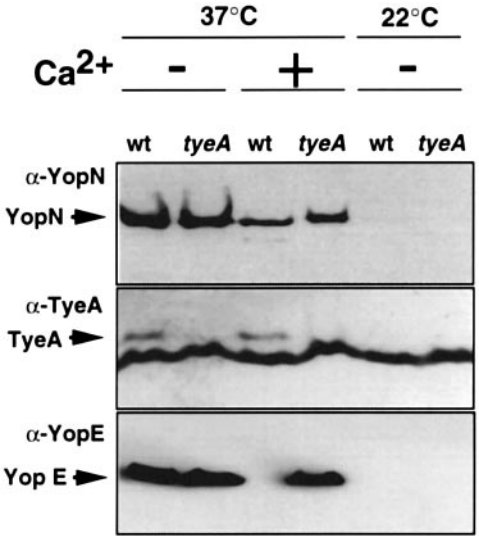
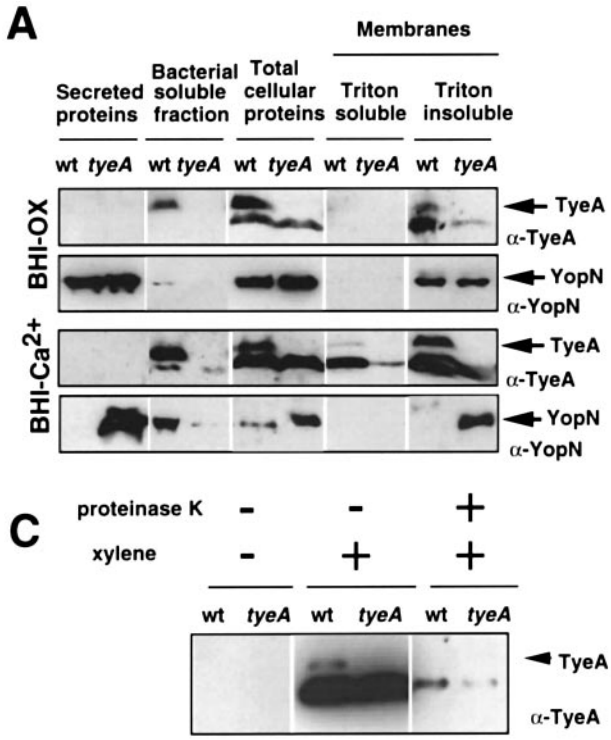


Fig. 3. Conditions of production of TyeA, YopN and YopE. Bacteria were grown in BHI-OX (–Ca^{2+}) or in BHI Ca^{2+} (+Ca^{2+}) at 37 or 22°C. Western blot analysis of total cell (O.D. = 1) using super signal ultra chemoluminescent as a substrate. wt, wild-type E40 (pYV40); *tyeA*, *tyeA* mutant E40 (pIM403).

When wild-type bacteria were grown in the presence of Ca^{2+}, YopN was detected in the bacterial soluble fraction, barely in the Triton-insoluble membrane fraction and not in the supernatant. In the Ca^{2+}-blind *tyeA* mutant, the amount of YopN in the Triton-insoluble membrane fraction was higher than in the soluble fraction and YopN appeared in the supernatant. TyeA was detected in the bacterial-soluble fraction and in the Triton-insoluble membrane fraction of wild-type bacteria (Figure 4A). We concluded from these observations (summarized in Figure 4B) that TyeA is present in the bacterial membrane, in the presence as well as the absence of Ca^{2+}. However, unlike YopN, TyeA was not massively released into the supernatant. We noticed that the anti-TyeA sera reacted unspecifically with a second lower molecular band that was weaker in the membrane fraction of the *tyeA* mutant than in the membrane fraction of the wild-type strain. We considered that the decrease in the amount of this second protein in the *tyeA* mutant was unspecific, because it was not observed in Figure 4C (see below).

To confirm that TyeA was partially located at the bacterial surface, we treated intact bacteria grown in low Ca^{2+} with xylene (Michiels *et al.*, 1990) and analysed the proteins extracted from the bacterial surface. Xylene extraction removed TyeA from the cell surface, indicating that TyeA was loosely associated with the membrane (Figure 4C). When bacteria were treated with proteinase K prior to xylene extraction, TyeA was not recovered (Figure 4C).



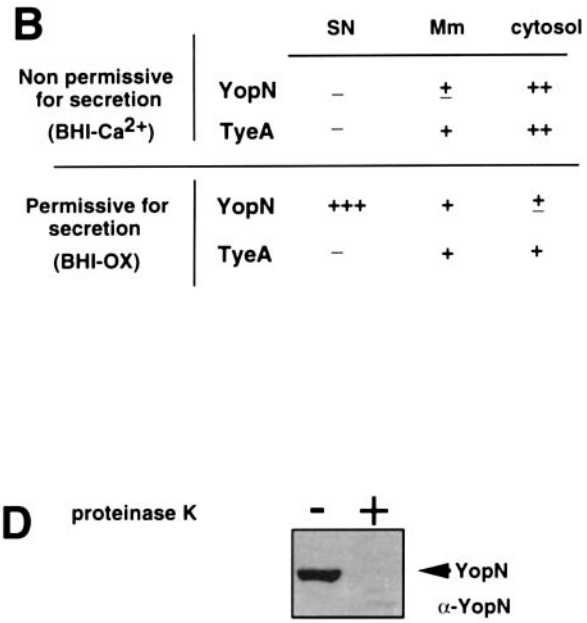


Fig. 4. Cellular localization of TyeA and YopN. (**A**) Bacteria were grown in BHI-OX or in BHI-Ca^{2+} at 37°C. Cells were fractionated as described by Michiels *et al.* (1990) and analysed by Western blot using super signal ultra chemoluminescent as a substrate. wt, wild-type E40 (pYV40); *tyeA*, *tyeA* mutant E40 (pIM403). The amounts loaded in the different lanes are: secreted proteins, the equivalent of 10 ml culture supernatant; soluble fraction, the equivalent of 2 ml of the culture; total cellular proteins, the equivalent of 1 O.D.; membranes, the equivalent of 3.5 ml of culture. (**B**) Interpretation of the results presented in Figure 3A: –, not detected; +, protein detected; SN, supernatant; Mm, membranes. (**C**) Total cells of the two strains grown in BHI-OX at 37°C were extracted with either PBS or xylene. Some samples were treated with proteinase K prior to xylene extraction. Western blot using super signal ultra chemoluminescent as a substrate. (**D**) An equivalent amount of total cells of MRS40 (pYV40) grown at 37°C in the presence of Ca^{2+} were treated with 500 μg/ml of proteinase K for 40 min at room temperature. The equivalent of 1 O.D. of cells was analysed by Western blot with a polyclonal anti-YopN antibody, using super signal ultra chemoluminescent as a substrate.

Table I. Internalization of the $YopE_{130}$–Cya, $YopH_{99}$–Cya and $YopM_{100}$–Cya hybrid proteins in PU5-1.8 macrophages

Plasmids		Genotype	Yop–Cya	Cell cAMP[a]	AC in RPMI[b]
pYV	Other				
pYV40	pMS111	wt	$YopE_{130}$–Cya	6.9±1.9	4
pPW401	pMS111	*yopB*	$YopE_{130}$–Cya	0.2±0.2	ND
pIM41	pMS111	$yopN_{45}$	$YopE_{130}$–Cya	6.7±1.7	60
pIM407	pMS111	$yopN_{\Delta 64-110}$	$YopE_{130}$–Cya	6.7±1.8	52
pIM408	pMS111	$yopN_{\Delta 248-272}$	$YopE_{130}$–Cya	0.2±0.2	44
pIM403	pMS111	*tyeA*	$YopE_{130}$–Cya	0.2±0.2	29
pIM411	pMS111	$yopN_{45}$,*tyeA*	$YopE_{130}$–Cya	0.2	ND
pIM408	pMS111, pIML245	$yopN_{\Delta 248-272}$, $yopN^{+}$	$YopE_{130}$–Cya	6.1	ND
pIM403	pMS111, pIML228	*tyeA*,$tyeA^{+}$	$YopE_{130}$–Cya	8.8	ND
pYV40	pMSLH99	wt	$YopH_{99}$–Cya	5.1±1.2	ND
pPW401	pMSLH99	*yopB*	$YopH_{99}$–Cya	0.6±0.2	ND
pIM41	pMSLH99	$yopN_{45}$	$YopH_{99}$–Cya	1.9±0.9	ND
pIM407	pMSLH99	$yopN_{\Delta 64-110}$	$YopH_{99}$–Cya	3.5±0.9	ND
pIM408	pMSLH99	$yopN_{\Delta 248-272}$	$YopH_{99}$–Cya	0.6±0.2	ND
pIM403	pMSLH99	*tyeA*	$YopH_{99}$–Cya	0.7±0.3	ND
pYV40	pAB6	wt	$YopM_{100}$–Cya	2.6±1.3	ND
pPW401	pAB6	*yopB*	$YopM_{100}$–Cya	0.1±0.1	ND
pIM41	pAB6	$yopN_{45}$	$YopM_{100}$–Cya	3.2±1.5	ND
pIM407	pAB6	$yopN_{\Delta 64-110}$	$YopM_{100}$–Cya	4.5±1.9	ND
pIM408	pAB6	$yopN_{\Delta 248-272}$	$YopM_{100}$–Cya	6.3±1.1	ND
pIM403	pAB6	*tyeA*	$YopM_{100}$–Cya	6.7±1.9	ND

[a]Data (nmol cAMP/mg protein) are means ± SD of at least three experiments, each one done in duplicate. Experiments were carried out in the presence of cytochalasin D.
[b]Adenylate cyclase activity in the eukaryotic RPMI medium of macrophages (nmol cAMP/min/ml).
ND, not determined.

Similar experiments were performed to confirm surface localization of YopN. We treated intact bacteria grown in the presence of Ca^{2+} with proteinase K and analysed the total cell proteins by immunoblotting with a polyclonal anti-YopN serum. YopN was degraded by external proteases (Figure 4D).

These results indicate that TyeA, as well as YopN, is at least partially localized at the surface of bacteria and that TyeA is not required for the localization of YopN.

TyeA is required for delivery of the effectors YopE and YopH into eukaryotic cells

To determine whether TyeA is required for translocation, we compared the delivery of the $YopE_{130}$–Cya reporter (Sory and Cornelis, 1994) by the wild-type strain *Y.enterocolitica* E40 (pYV40), the *tyeA* mutant E40 (pIM403), the *yopN* mutant E40 (pIM41) (Boland *et al.*, 1996) and the *yopB* mutant E40 (pPW401) (Boland *et al.*, 1996). We infected PU5-1.8 macrophages and we measured not only the accumulation of cAMP inside the eukaryotic cell, but also the release of the $YopE_{130}$–Cya into the culture supernatant. Like the *yopN* mutant, the *tyeA* mutant released much more $YopE_{130}$–Cya into the RPMI supernatant than the wild-type strain (Table I). Nevertheless, like the *yopB* mutant, the *tyeA* mutant did not deliver $YopE_{130}$–Cya into macrophages (Table I). The wild-type phenotype was restored after introduction of plasmid pIML228 (P_{lac} *tyeA*) into the *tyeA* mutant (Table I).

To check this result using a morphological approach, we infected PU5-1.8 macrophages with $tyeA^{+}$ and $tyeA^{-}$ isogenic *Y.enterocolitica* overproducing YopE and analysed them by confocal microscopy. YopE appeared dispersed in the cytosol of macrophages infected with the $tyeA^{+}$ MRS40 (pYV40) (pMS3) bacteria (Figure 5B) but was not visible in the cytosol of cells infected with the $yopB^{-}$ MRS40 (pPW401) (pMS3) bacteria (Figure 5A) or the $tyeA^{-}$ MIE40 (pIM403) (pMS3) bacteria (Figure 5C), confirming that YopE was not translocated by the *tyeA* mutant. The $tyeA^{-}$ bacteria were overstained due to inefficient translocation (Figure 5C).

We then analysed the effect of *tyeA* on translocation of $YopH_{99}$–Cya (Sory *et al.*, 1995) and observed that the *tyeA* mutation, like the *yopB* mutation, also prevented translocation of $YopH_{99}$–Cya (Table I). Thus TyeA is required for translocation of effectors YopE and YopH, hence the designation of TyeA for Translocation of Yops into eukaryotic cells A.

TyeA is not required for delivery of the effectors YopM, YopO and YopP

We also analysed the delivery of $YopM_{100}$–Cya (Boland *et al.*, 1996) by a *tyeA* mutant. TyeA appeared to play no role in translocation of $YopM_{100}$–Cya (Table I). Translocation of YopO and YopP is more difficult to monitor because these proteins are translocated in lower amounts than YopE, YopH and YopM (Håkansson *et al.*, 1996; M.-P.Sory, C.Kerbourch and G.R.Cornelis, unpublished). Håkansson *et al.* (1996) observed that this difficulty can be circumvented by using *Y.enterocolitica* mutants unable to produce most of the Yop effectors. Thus, to study the effect of TyeA on translocation of YopO–Cya and YopP–Cya, we used MRS40 (pABL403), a polymutant of MRS40 deprived of *yopE*, *yopH*, *yopM*, *yopO* and *yopP*, and MRS40 (pAB409), the same strain mutated in *yopB* (Boland and Cornelis, 1998). We introduced a *tyeA* mutation into MRS40 (pABL403) and then tested the translocation of $YopO_{77}$–Cya, $YopO_{143}$–Cya, $YopP_{99}$–Cya, and as a control $YopE_{130}$–Cya, by these polymutant strains. Translocation of $YopO_{143}$–Cya was reduced 2-fold and translocation of $YopO_{77}$–Cya was increased 2-fold in the

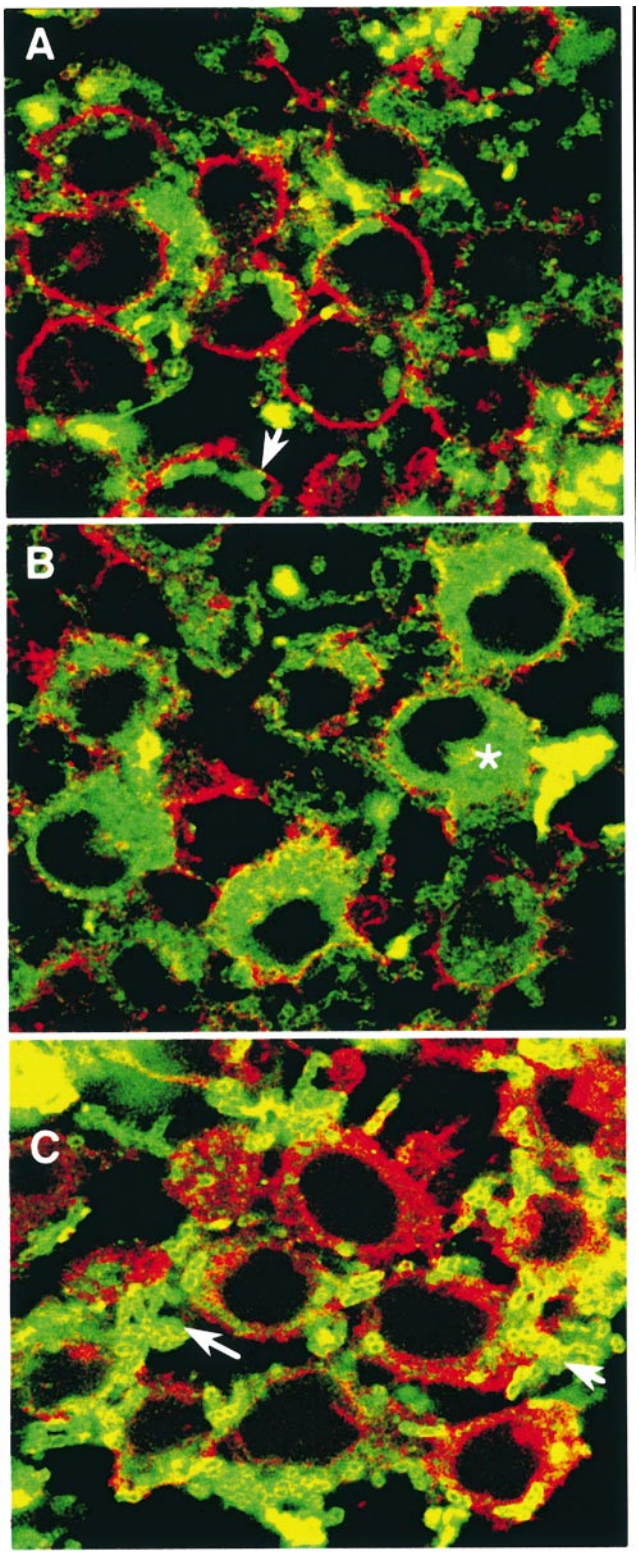


Fig. 5. Delivery of YopE inside PU5-1.8 macrophages. Confocal microscopy showing PU5-1.8 macrophages after 3 h of infection with (**A**) the *yopB* mutant strain MRS40 (pPW401) (pMS3), (**B**) the wild-type strain MRS40 (pYV40) (pMS3), or (**C**) the *tyeA* mutant strain MIE40 (pIM403) (pMS3) overexpressing YopE. YopE is immunostained with FITC. The cell membranes are stained with Texas-red-labelled wheat germ agglutinin. Arrows indicates bacteria. * indicates YopE inside cytosol of the target cell.

tyeA mutant compared with the parental strain (Table II). Translocation of $YopP_{99}$–Cya was increased 4-fold compared with the parental strain. To confirm that TyeA has no influence on the effect of YopP in eukaryotic cells, we measured the apoptotic effect of the wild-type strain and the *tyeA* mutant strain as described by Mills *et al.* (1997). We also tested the deregulated mutant $yopN_{45}$ since YopP is translocated by this mutant (M.-P.Sory, C.Kerbourch and G.R.Cornelis, unpublished). As a control we infected macrophages with the secretion mutant *yscN* (Woestyn *et al.*, 1994; Sory *et al.*, 1995). The infected cells were examined by phase-contrast microscopy to record general cell morphology and the total number of cells, and by fluorescence (480 nm) microscopy to record the number of TUNEL-positive nuclei and the morphology of these nuclei. The *tyeA* mutant, as well as the $yopN_{45}$ mutant, induced apoptosis to approximately the same level as the wild-type strain (Figure 6A and B). These results show that YopP-mediated induction of apoptosis, like translocation of YopP, is independent of TyeA. We conclude from these experiments that TyeA is not essential for translocation of YopM, YopO and YopP.

TyeA binds to the second coiled-coil domain of YopN

To determine whether TyeA controls Yop secretion and translocation through interaction with YopN, we analysed the capacity of TyeA to bind YopN. Plasmid pIML217, encoding both GST–YopN and TyeA, was introduced into *Escherichia coli* LK111 in order to overproduce the two proteins simultaneously. The GST–YopN protein was purified from the cell-free extract with glutathione–Sepharose beads and analysed by SDS–PAGE for the recovery of TyeA. GST–YopN was recovered together with an ~10.7 kDa protein that reacted with an anti-TyeA polyclonal antibody (data not shown) indicating that TyeA binds to YopN (Figure 7A). The sequence analysis of YopN predicts that the regions spanning amino acids 62–108 and 248–272 could form coiled-coil structures. Since such structures are known to be involved in protein–protein interactions, we wondered whether these domains could be responsible for the interaction with TyeA. To study this we constructed a series of plasmids encoding TyeA and GST–YopN hybrids deprived of one or other of these regions. We also engineered a plasmid encoding TyeA and GST fused to amino acids 242–293 of YopN. The GST–YopN proteins were purified with glutathione–Sepharose beads and analysed by SDS–PAGE. The hybrid proteins containing truncated forms of YopN were very unstable, but apparently TyeA was not purified with GST–$YopN_{\Delta 64–110}$ or GST–$YopN_{\Delta 248–272}$ (Figure 7A), even though the *E.coli* extracts were prepared under the same conditions. However, TyeA was copurified with GST–YopN and with GST fused to amino acids 242–293 of YopN, but not with GST alone (Figure 7A). We also constructed and tested plasmid pIM216 encoding GST–YopN. Interestingly, GST–YopN was almost undetectable in *E.coli* extracts in the absence of TyeA (data not shown).

To study whether TyeA and YopN also interact in *Yersinia*, we introduced plasmid pIMLF225 encoding GST–TyeA or as a control pGEX-KG encoding GST alone, in the wild-type strain MRS40 (pYV40), the $yopN_{45}$ mutant MIE40 (pIM41), the $yopN_{\Delta 64–110}$ mutant, MIE40

Table II. Internalization of the $YopE_{130}$–Cya, $YopO_{143}$–Cya, $YopO_{77}$–Cya, and $YopP_{99}$–Cya hybrid proteins in PU5-1.8 macrophages

Plasmids		Genotype	Yop–Cya	Cell cAMP[a]
pYV	Other			
pABL403	pMS111	*yopO,yopE,yopH,yopM,yopP*	$YopE_{130}$–Cya	3.0
pAB409	pMS111	*yopO,yopE,yopH,yopM,yopP,yopB*	$YopE_{130}$–Cya	0.1
pIM415	pMS111	*yopO,yopE,yopH,yopM,yopP,tyeA*	$YopE_{130}$–Cya	0.2
pIM415	pMS111, pIML228	*yopO,yopE,yopH,yopM,yopP,tyeA,tyeA*$^+$	$YopE_{130}$–Cya	3.4
pABL403	pCD10	*yopO,yopE,yopH,yopM,yopP*	$YopO_{143}$–Cya	5.5±1.1
pAB409	pCD10	*yopO,yopE,yopH,yopM,yopP,yopB*	$YopO_{143}$–Cya	0.1±0.1
pIM415	pCD10	*yopO,yopE,yopH,yopM,yopP,tyeA*	$YopO_{143}$–Cya	2.5±0.8
pIM416	pCD10	*yopO,yopE,yopH,yopM,yopP,yopN*$_{\Delta 248–272}$	$YopO_{143}$–Cya	2.2
pABL403	pCD11	*yopO,yopE,yopH,yopM,yopP*	$YopO_{77}$–Cya	1.4±0.3
pAB409	pCD11	*yopO,yopE,yopH,yopM,yopP,yopB*	$YopO_{77}$–Cya	0.2±0.1
pIM415	pCD11	*yopO,yopE,yopH,yopM,yopP,tyeA*	$YopO_{77}$–Cya	3.3±1.3
pABL403	pMSK3	*yopO,yopE,yopH,yopM,yopP*	$YopP_{99}$–Cya	0.8±0.3
pAB409	pMSK3	*yopO,yopE,yopH,yopM,yopP,yopB*	$YopP_{99}$–Cya	0.4±0.3
pIM415	pMSK3	*yopO,yopE,yopH,yopM,yopP,tyeA*	$YopP_{99}$–Cya	3.4±1.2

[a]Data (nmol cAMP/mg protein) are means ± SD of at least three experiments, each one done in duplicate. Experiments were carried out in the presence of cytochalasin D.

(pIM407) and in the *yopN*$_{\Delta 248–272}$ mutant MIE40 (pIM408). Bacteria were grown for 1 h at 37°C, expression of GST or GST–TyeA was induced by adding IPTG to a final concentration of 0.1 mM and the cells were harvested 1 h later. After sonication, GST and GST–TyeA were purified from the cell-free extract with glutathione beads and the purified proteins were analysed by immunoblot for the presence of YopN. YopN was recovered in large amounts with GST–TyeA (Figure 7B, lower panel, lane 1). The truncated $YopN_{\Delta 64–110}$ was also recovered, but in smaller amounts than complete YopN (Figure 7B, lower panel, lane 3). Interestingly, only a very low amount of $YopN_{\Delta 248–272}$ copurified with GST–TyeA (Figure 7B, lower panel, lane 4). The amount of $YopN_{\Delta 64–110}$ and $YopN_{\Delta 248–272}$ in the total cells was equivalent (Figure 7B, upper panel, lanes 3 and 4). As expected, no YopN copurified with GST–TyeA in the *yopN*$_{45}$ mutant (Figure 7B, lane 2) and YopN does not copurify with GST in the wild-type strain (Figure 7B, lane 5). We conclude from these results that TyeA and YopN interact in *Yersinia* and that TyeA binds the second coiled-coil domain of YopN.

The YopN–TyeA interaction is required for translocation of YopE and YopH

To further understand the role of YopN, and in particular that of the coiled-coil domains, we deleted domains 64–110 and 248–272 of the protein. The alleles *yopN*$_{\Delta 64–110}$ and *yopN*$_{\Delta 248–272}$ were introduced along with *blaA* mutation into pYV40 by allelic exchange giving strains MIE40 (pIM407) and MIE40 (pIM408). The *yopN*$_{\Delta 64–110}$ and *yopN*$_{\Delta 248–272}$ mutants were Ca^{2+}-blind and secreted all the Yops, including the truncated YopN protein, in the presence and absence of Ca^{2+} (Figure 8A and B). As observed before, the truncated YopN proteins were less abundant than the entire YopN protein. They were thus Ca^{2+}-blind like the *yopN*$_{45}$ mutant described previously (Boland *et al.*, 1996). The wild-type phenotype was mostly restored after introduction of plasmid pIML245 encoding YopN only, but not after introduction of plasmid pIML228 encoding TyeA, indicating that the mutations were non-polar on downstream genes.

We then tested the effect of the deletion of each of these two coiled-coils on translocation of Yop effectors into eukaryotic cells. We compared the delivery of $YopE_{130}$–Cya, $YopH_{99}$–Cya and $YopM_{100}$–Cya reporters inside PU5-1.8 macrophages (Sory *et al.*, 1995; Boland *et al.*, 1996) by the wild-type strain and by the different *yopN* mutants. The *yopN*$_{\Delta 64–110}$ mutant could deliver $YopE_{130}$–Cya, $YopH_{99}$–Cya and $YopM_{100}$–Cya but was completely depolarized like the *yopN*$_{45}$ mutant (Table I). This suggests that $YopN_{\Delta 64–110}$ is not functional and confirms that YopN is dispensible for translocation of Yop proteins. Surprisingly, the *yopN*$_{\Delta 248–272}$ mutant was unable to deliver $YopE_{130}$–Cya and $YopH_{99}$–Cya but could deliver $YopM_{100}$–Cya and $YopO_{143}$–Cya, just like the *tyeA* mutant (Tables I and II). The *yopN*$_{\Delta 248–272}$ mutant complemented with pIML245 (P_{lac} *yopN*) was able to deliver $YopE_{130}$–Cya, indicating that the phenotype of this mutant was not due to a polar effect on *tyeA* (Table I). Thus the phenotype of the *yopN*$_{\Delta 248–272}$ mutant was different from that of *yopN* null mutant but identical to that of the *tyeA* mutant. This suggests that once a functional YopN is inserted in the control complex, its second coiled-coil domain interacts with TyeA and that this interaction is required for translocation of YopE and YopH but not YopM.

TyeA is not involved in removing the YopN plug

We considered whether TyeA would be required for translocation of YopE and YopH even in the absence of YopN. To clarify this point we engineered a *yopN*$_{45}$ *tyeA* double mutant [E40 (pIM411)] and analysed its capacity to deliver a $YopE_{130}$–Cya reporter into macrophages. If the role of TyeA is solely to remove YopN, which is supposed to act as a plug on the secretion apparatus (Forsberg *et al.*, 1991; Rosqvist *et al.*, 1994), then this double mutant should be able to translocate the Yop effectors. The double mutant *yopN*$_{45}$ *tyeA* did not induce cAMP accumulation inside the cytosol of infected PU5-1.8 macrophages (Table I). These results indicate that TyeA itself is required for translocation of YopE, independently of YopN.

TyeA binds to YopD

Since TyeA is required for translocation, it could interact with the translocation apparatus. To investigate the binding

of TyeA to YopD, a component of the translocation machinery, we constructed plasmid pIML241 which encodes GST–TyeA and YopD, and plasmid pCN29 encoding GST and YopD. The GST-hybrid proteins were purified with glutathione–Sepharose beads and analysed by SDS–PAGE. The presence of YopD was monitored by immunoblotting using monoclonal anti-YopD because GST–TyeA and YopD have the same mass (35 kDa). YopD was copurified with GST–TyeA but not with GST, demonstrating that TyeA interacts specifically with YopD (Figure 9A).

To study whether TyeA and YopD also interact in *Yersinia*, we introduced plasmid pIMLF225 encoding GST–TyeA, or as a control pGEX-KG encoding GST alone, in the wild-type strain MRS40 (pYV40). Bacteria were grown for 1 h at 37°C, expression of GST or GST–TyeA was induced as before and the cells were harvested 1 h later. After sonication, GST and GST–TyeA were purified from the cell-free extract with glutathione beads and the purified proteins were analysed by immunoblot for the presence of YopD using monoclonal anti-YopD. Even though the amount of YopD was the same in the total cells of bacteria producing GST or GST–TyeA (Figure 9B, upper panel), the amount of YopD copurified with GST–TyeA was significantly higher than the amount of YopD copurified with GST (Figure 9B, lower panel). Our results indicate that TyeA interacts specifically with YopD in *Yersinia*.

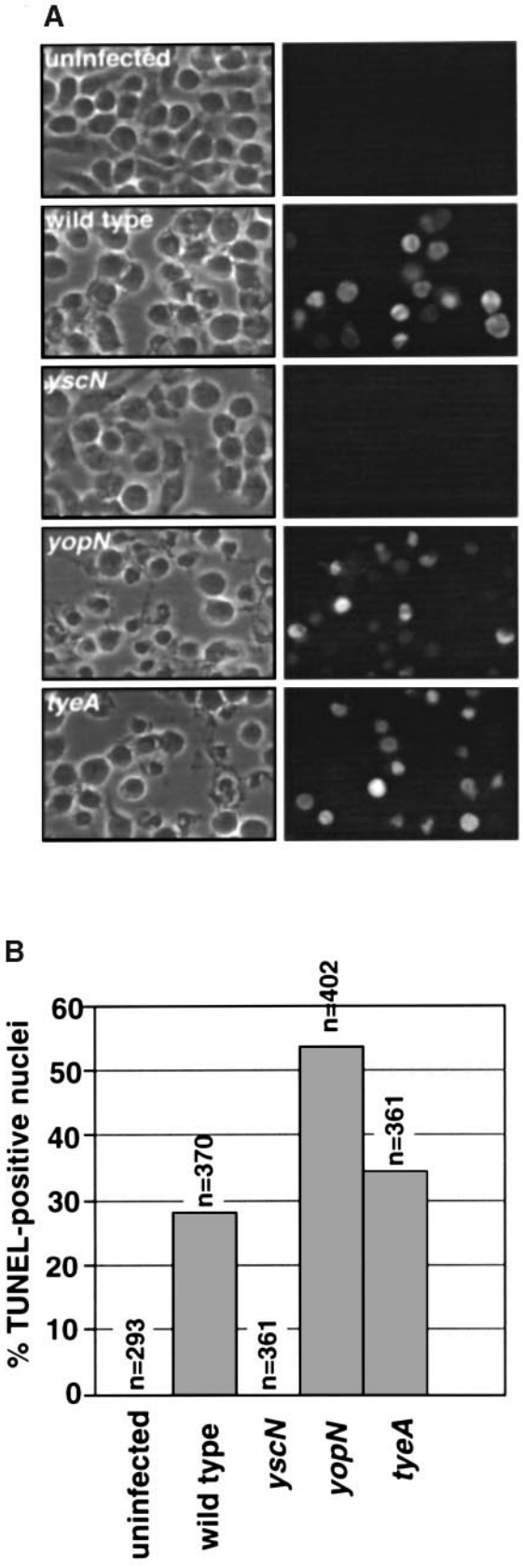


Fig. 6. Role of TyeA in *Y.enterocolitica*-induced apoptosis. (**A**) Phase contrast (left) and fluorescence (right) images depicting J774A.1 cells 4 h post-infection. The results of the cell infection (cell morphology) with the various *Y.enterocolitica* strains is shown by phase contrast and the TUNEL reaction (nuclear DNA fragmentation) by epifluorescence. (**B**) Percent TUNEL-positive nuclei in J774A.1 cells 4 h post-infection. n values refer to the number of total cells evaluated per treatment. All strains were tested a minimum of three times. The results shown are from one representative experiment. wild-type, E40 (pYV40); *yscN*, E40 (pMSL41); *yopN*, E40 (pIM41); *tyeA*, E40 (pIM403).

Discussion

In this paper we analysed the role of the TyeA protein, which is encoded immediately downstream from YopN. A mutant deprived of TyeA was deregulated for Yop secretion in the sense that it secreted Yops even in the presence of Ca^{2+} ions or the absence of eukaryotic cells. This phenotype, similar to *yopN* and *lcrG* mutants (Forsberg *et al.*, 1991; Skrzypek and Straley 1993; Rosqvist *et al.*, 1994; Boland *et al.*, 1996; Sarker *et al.*, 1998b), suggested that TyeA is involved, together with YopN and LcrG, in the control of Yop release. In accordance with this hypothesis, we observed that TyeA binds to YopN and, like YopN, fractionates with the Triton-insoluble membrane fraction which contains the outer membrane proteins. Thus Yop release is controlled by a complex of at least three proteins including YopN, TyeA and LcrG.

In addition to its role in control of Yop release, TyeA is also required for translocation of YopE and YopH. TyeA operates through interaction with YopN and with YopD, a component of the translocation apparatus. This suggests that the complex which recognizes eukaryotic cells and controls Yop secretion is also actively involved in translocation. Previous work has shown that YopN is dispensible for translocation (Rosqvist *et al.*, 1994; Boland *et al.*, 1996). However, TyeA has a phenotype dominant over YopN because the double mutant $yopN_{45}$ *tyeA* does not translocate YopE. Thus TyeA itself is required for trans-

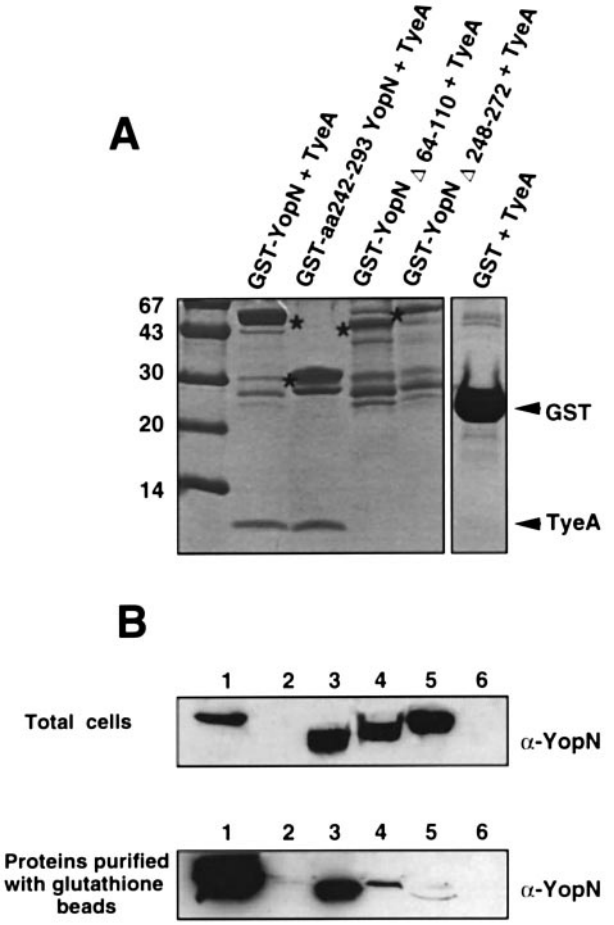


Fig. 7. (A) Binding of YopN to TyeA in *E.coli*. The various GST–YopN hybrids and TyeA were produced simultaneously in *E.coli* LK111. The GST-hybrid proteins were purified from the cell-free extracts with glutathione–Sepharose beads and analysed by SDS–PAGE and Coomassie Blue staining for the recovery of TyeA. Note the instability of GST–YopN$_{\Delta 64–110}$ and GST–YopN$_{\Delta 248–272}$. The numbers on the left indicate molecular mass (kDa). * indicates the different GST–YopN hybrids. (**B**) Binding of YopN with TyeA in *Y.enterocolitica*. GST–TyeA (lanes 1, 2, 3 and 4) or GST (lanes 5 and 6) were produced in *Y.enterocolitica* MRS40 (pYV40) (lanes 1 and 5), in the *yopN*$_{45}$ mutant MIE40 (pIM41) (lanes 2 and 6), in the *yopN*$_{\Delta 64–110}$ mutant MIE40 (pIM407) (lane 3) and in the *yopN*$_{\Delta 248–272}$ mutant MIE40 (pIM408) (lane 4). GST hybrids were purified from the cell extract with glutathione–Sepharose beads and analysed by Western blot with a polyclonal antibody against YopN. The upper panel shows the amount of YopN (lanes 1 and 5), YopN$_{\Delta 64–110}$ (lane 3), or YopN$_{\Delta 248–272}$ (lane 4) present in the total cells. The lower panel shows proteins copurified with GST–TyeA, YopN (lane 1), YopN$_{\Delta 64–110}$ (lane 3) and YopN$_{\Delta 248–272}$ (lane 4).

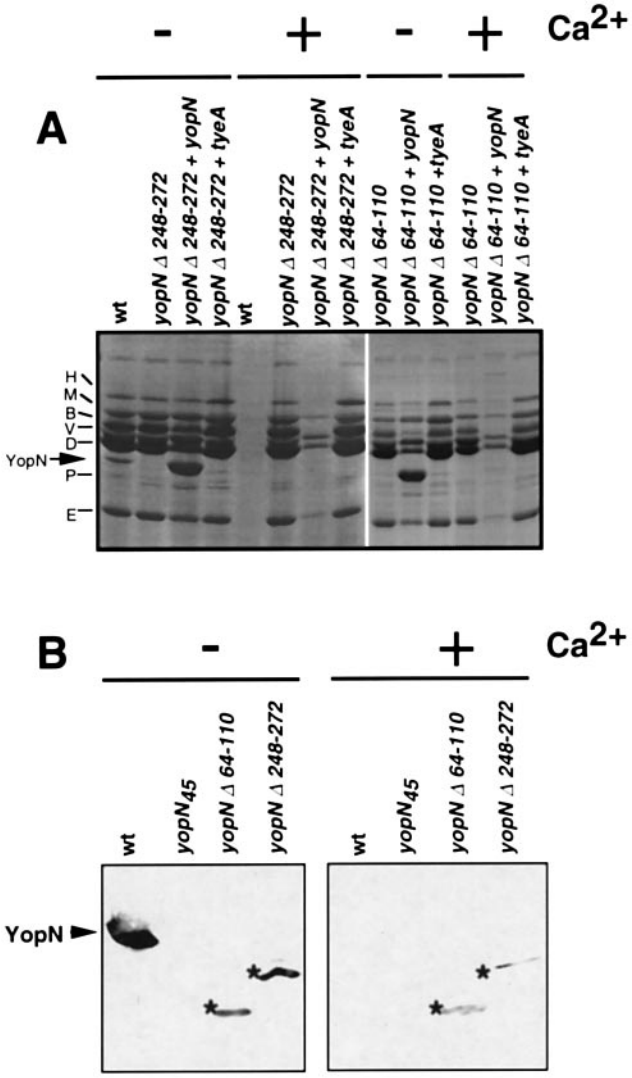


Fig. 8. Phenotypes of the various *yopN* mutants. Yops secreted by the wild-type strain MRS40 (pYV40), the *yopN*$_{\Delta 64–110}$ mutant MIE40 (pIM407) and the *yopN*$_{\Delta 248–272}$ mutant MIE40 (pIM408). Bacteria were grown in BHI-OX ($-Ca^{2+}$) or in BHI-Ca^{2+} ($+Ca^{2+}$) at 37°C. P$_{lac}$ *yopN* was in plasmid pIML245, P$_{lac}$ *tyeA* was in plasmid pIML228. (**A**) SDS–PAGE and Coomassie Blue staining. (**B**) Western blot analysis (using 4-chloro-1-naphthol as a substrate) with a polyclonal anti-YopN serum showing that the truncated YopN proteins are present although not visible on Coomassie Blue. Letters on the left indicate the different Yops. ***** indicates the YopN truncated proteins.

location, independently of the presence of YopN. Nevertheless, the fact that a *yopN*$_{\Delta 248–272}$ mutant, producing a truncated YopN protein deleted of the TyeA-binding domain has the same phenotype as a *tyeA* mutant, suggests that when YopN is inserted in the control complex, its interaction with TyeA is required for translocation of YopE and YopH. In contrast, another mutant *yopN*$_{\Delta 64–110}$ which encodes a YopN truncated protein where the TyeA-binding domain is still present, behaved as a *yopN* null mutant for release and translocation of Yops. This could be due to the fact that deletion of amino acids 64–110 of YopN induces significant changes in the conformation of the protein and could inhibit its insertion in the control complex.

Our observation that TyeA is required for translocation of YopE and YopH, but not YopM, YopO or YopP, was also unexpected. Yops would thus form two subsets: on one hand YopE and YopH and on the other hand YopM, YopO and YopP. There is already one known difference between these two groups: the two Yop effectors that require TyeA for their translocation into eukaryotic cells, YopE and YopH, are each known to have an intrabacterial chaperone. These chaperones, SycE and SycH respectively, are required for normal secretion of the relevant Yop (Wattiau and Cornelis, 1993; Wattiau *et al.*, 1994, 1996; Frithz-Lindsten *et al.*, 1995; Cheng *et al.*, 1997) and specifically bind to the Yop domain that is necessary for translocation into eukaryotic cells (Woestyn *et al.*, 1996). Since these chaperones remain inside the bacterium, they must be released from the Yop prior to secretion and

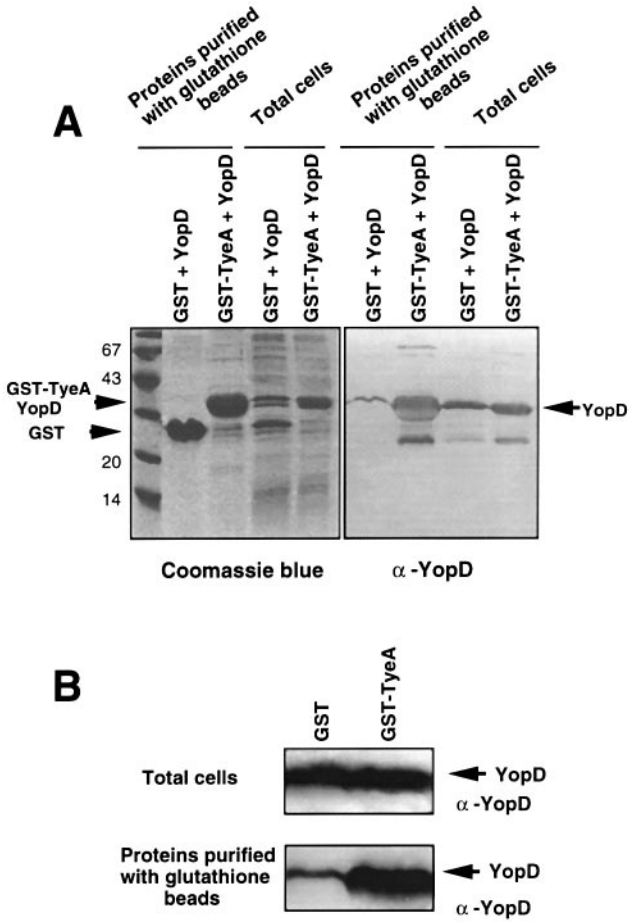


Fig. 9. (**A**) Interaction between TyeA and YopD in *E.coli*. Plasmid pCN29 (GST + YopD) and pIML241 (GST–TyeA + YopD) were introduced in *E.coli* LK111. The GST-hybrid proteins were purified from the cell free extracts with glutathione–Sepharose beads and analysed by SDS–PAGE for the recovery of the second protein. Left: SDS–PAGE and Coomassie Blue staining. Right: Western blot analysis with a monoclonal anti-YopD serum (Bodeus *et al.*, 1990) and using 4-chloro-1-naphthol as a substrate. Molecular mass standards (kDa) are shown on the left. (**B**) Interaction between TyeA and YopD in *Y.enterocolitica.* GST and GST–TyeA were produced in *Y.enterocolitica* MRS40 (pYV40). GST hybrids were purified from the cell extract with glutathione–Sepharose beads and analysed by Western blot with a monoclonal antibody against YopD. The upper panel shows the amount of YopD present in the total cells. The lower panel shows proteins copurified with glutathione beads.

translocation. No chaperone has yet been identified for YopO, YopP or YopM. Since TyeA is required for translocation of YopE and YopH, but not YopO, YopP and YopM, one could hypothesize that TyeA plays a role in removing the SycE and SycH chaperones prior to translocation.

The observation that delivery of various Yops can be uncoupled raises the appealing possibility that some Yops could be delivered specifically to particular cell types. The analysis of cell types other than macrophages and epithelial cells for translocation might be interesting in this respect. In this hypothesis, we speculate that the recognition complex at the bacterial surface recognizes different receptors at the surface of eukaryotic cells. Information on these receptors is still scarce, but a first element appeared recently. LcrG has been shown to bind to heparan sulfate proteoglycans, and exogenous heparin can interfere with translocation into HeLa cells but this has not been shown to occur in macrophages (Boyd *et al.*, 1998; A.P.Boyd and G.F.Cornelis, unpublished data). In other examples such as pseudorabies virus, pathogen ligand–proteoglycan interactions precede a second interaction between another ligand and a specific cell receptor (Zsak *et al.*, 1991; Karger and Mettenleiter, 1993). Enteropathogenic *E.coli*'s interaction with its target cell requires an initial adherence through the bundle-forming pilus followed by an intimate adherence mediated by the intimin (Rosenshine *et al.*, 1996). In the *Yersinia* system, TyeA could be a second bacterial ligand recognizing another specific receptor.

Finally, a homologue of TyeA has been identified in the type III secretion system of *Pseudomonas aeruginosa* (Yahr *et al.*, 1997). TyeA also shows similarity (25% identity over 97 amino acid residues) with the C-terminal portion of SsaL, a component of the type III secretion apparatus of *Salmonella typhimurium* pathogenicity island 2 (Hensel *et al.*, 1997). This reinforces our view that TyeA is an important part of the type III secretion–translocation system.

Materials and methods

Bacterial strains, plasmids and growth conditions

Yersinia enterocolitica W22703 (pYV227) (Cornelis and Colson, 1975) and E40 (pYV40) (Sory *et al.*, 1995) belong to serotype O:9. *Yersinia enterocolitica* KNG22703 (Kaniga *et al.*, 1991) and MRS40 (Sarker *et al.*, 1998b) are *blaA* mutants of strains W22703 and E40 respectively. *Escherichia coli* LK111 received from M.Zabeau (Gent, Belgium) was used for standard genetic manipulations. *Escherichia coli* CJ236 (*dut, ung*) (Kunkel *et al.*, 1987) was used for site-directed mutagenesis. *Escherichia coli* SM10 λ *pir*$^{+}$ constructed by Miller and Mekalanos (1988) was used to deliver the mobilizable plasmids in *Y.enterocolitica*. The plasmids used in this study are listed in Table III.

The strains were routinely grown in tryptic soy broth. The Ca^{2+} requirement for growth at 37°C and induction of the Yop virulon was performed as described by Cornelis *et al.* (1987) and Michiels *et al.* (1990). Yops were prepared from culture supernatants and analysed by SDS–PAGE and Western blotting as described by Cornelis *et al.* (1987). Cell fractionation and xylene extraction were performed as described by Michiels *et al.* (1990). In some samples, proteinase K was added to give a final concentration of 500 μg prior to xylene extraction. Immunoblots were developed using horseradish peroxidase, and as a substrate either 4-chloro-1-naphthol or super signal ultra chemoluminescent substrate (Pierce).

Expression and purification of hybrid GST-fusion proteins

The expression and purification of the fusion proteins was performed basically as described by Smith and Johnson (1988) and according to Pharmacia Biotech.

Preparation and affinity purification of antibodies against TyeA, YopN and YopE

The GST–TyeA fusion protein was purified on glutathione beads and then cleaved with thrombin protease to release the TyeA protein with an additional 13 amino acids at the N-terminus (GSPGISGGGGGIH). This protein was >95% pure as judged by SDS–PAGE. Approximately 1 mg of protein was used to inoculate a rabbit. The TyeA serum was absorbed on sonicated extracts of the *tyeA* mutant E40 (pIM403). Yops released from a 37°C culture of *Y.enterocolitica* KNG22703 (pPW2269) (pIM152) overproducing YopN, or *Y.enterocolitica* E40 were subjected to SDS–PAGE. The protein of interest, YopN or YopE, was eluted from the gel and used to inoculate a rabbit. For purification of the antibodies, the relevant protein antigen was transferred to a nitrocellulose membrane. The membrane strip containing the protein of interest was cut out and affinity-purification of the serum was performed as described by Lebrun *et al.* (1996).

Table III. Plasmids

Plasmid	Genotype or description	Reference
pYV derivatives		
pYV40	WT pYV plasmid from strain E40	Sory *et al.* (1995)
pAB409	pYV40 *yopO*$_{\Delta 65-558}$,*yopE*$_{21}$,*yopH*$_{\Delta 1-352}$,*yopM*$_{23}$,*yopP*$_{23}$,*yopB*$_{\Delta 89-217}$	Boland and Cornelis (1988)
pABL403	pYV40 *yopO*$_{\Delta 65-558}$,*yopE*$_{21}$,*yopH*$_{\Delta 1-352}$,*yopM*$_{23}$,*yopP*$_{23}$	Boland and Cornelis (1988)
pIM41	pYV40 *yopN*$_{45}$ (encodes a truncated YopN of 45 aa)	Boland *et al.* (1996)
pPW401	pYV40 *yopB*$_{\Delta 89-217}$ (translocation mutant)	Boland *et al.* (1996)
pMSL41	pYV40 *yscN*$_{\Delta 169-177}$ (secretion mutant)	Woestyn *et al.* (1994); Sory *et al.* (1995)
pYV227	WT pYV plasmid from strain W22703	Cornelis *et al.* (1987)
pIM403	pYV40 *tyeA*$_{\Delta 19-59}$ (pYV40 mutated with pIM210)	this work
pIM407	pYV40 *yopN*$_{\Delta 64-110}$ (pYV40 mutated with pIML222)	this work
pIM408	pYV40 *yopN*$_{\Delta 248-272}$ (pYV40 mutated with pIML224)	this work
pIM411	pYY40 *yopN*$_{45}$-*tyeA*$_{\Delta 19-59}$ (pIM41 mutated with pIM210)	this work
pIM415	pYV40 *yopO*$_{\Delta 65-558}$,*yopE*$_{21}$,*yopH*$_{\Delta 1-352}$,*yopM*$_{23}$,*yopP*$_{23}$,*tyeA*$_{\Delta 19-59}$	this work
pIM416	pYV40 *yopO*$_{\Delta 65-558}$,*yopE*$_{21}$,*yopH*$_{\Delta 1-352}$,*yopM*$_{23}$,*yopP*$_{23}$,*yopN*$_{\Delta 248-272}$	this work
Clones and vectors		
pAB6	pTM100 P$_{yopM}$ *yopM*$_{100}$–*cyaA′*$^{+}$ (encodes YopM$_{100}$–Cya)	Boland *et al.* (1996)
pCD10	pTM100 P$_{sycE}$ *yopO*$_{143}$-*cyaA′*$^{+}$ (encodes YopO$_{143}$–Cya)	this work
pCD11	pTM100 P$_{sycE}$ *yopO*$_{77}$-*cyaA′*$^{+}$ (encodes YopO$_{77}$–Cya)	this work
pBC18R	pTZ18R *oriT*$_{RK2}$	China *et al.* (1990)
pCN29	pGEX-KG + PCR amplified *yopD* (encodes GST + YopD)	Sarker *et al.* (1998a)
pGEX-KG	ori$_{pBR322}$, P$_{tac}$, *gst*$^{+}$	Guan and Dixon (1991)
pIM146	pBluescriptII KS^{-} P$_{yopN}$ *yopN*$^{+}$	Boland *et al.* (1996)
pIM152	pBC18R *yopN*$^{+}$ *yscN*$^{+}$	this work
pIM176	pBluescriptII KS^{-} + 2.7 kb *Eco*RV fragment of pYV227: encoding YopN,TyeA,ORF2,ORF3 and ORF4	this work
pIM180	pIM176$_{\Delta 1167bp}$ *Bam*HI–*Bgl*II (contains 52 codons of *yopN*, *tyeA*,*ORF2*,*ORF3* and *ORF4*)	this work
pIM187	pIM180$_{\Delta 790bp}$ *Hin*dIII (contains 52 last codons of *yopN* and *tyeA*)	this work
pIM205	pIM187$_{\Delta 123bp}$ (encodes TyeA$_{\Delta 19-59}$)	this work
pIML217	pGEX-KG + PCR amplified *yopN* +*tyeA* (encodes GST–YopN + TyeA)	this work
pIML219	pBluescriptII KS^{-} *yopN*	this work
pIML220	pBluescriptII KS^{-} *yopN*$_{\Delta 64-110}$	this work
pIML221	pBluescriptII KS^{-} *yopN*$_{\Delta 248-272}$	this work
pIMLF225	pGEX-KG + PCR amplified *tyeA* (encodes GST–TyeA)	this work
pIML228	pBC18R + P$_{lac}$ *tyeA (Bgl*II–*Hin*dIII fragment of pIML217)	this work
pIML231	pIML217Δ 769 bp (encodes GST fused to aa242–293 of YopN + TyeA)	this work
pIMLF233	pGEX-KG + PCR amplified *yopN*$_{\Delta 64-110}$ and *tyeA* (encodes GST–YopN$_{\Delta 64-110}$ + TyeA)	this work
pIMLF234	pGEX-KG + PCR amplified *yopN*$_{\Delta 248-272}$ and *tyeA* (encodes GST–YopN$_{\Delta 248-272}$ + TyeA)	this work
pIML241	pIMLF225 + *yopD* (encodes GST–TyeA + YopD)	this work
pIML245	pBC18R p$_{lac}$ *yopN*	this work
pIML251	pGEX-KG + *Bam*HI–*Sal*I fragment of pIML217 containing *tyeA* (encodes GST + TyeA)	this work
pMSLH99	pTM100 P$_{yopH}$ *yopH*$_{99}$–*cyaA′*$^{+}$ (encodes YopH$_{99}$–Cya)	Sory *et al.* (1995)
pMS111	pTM100 *sycE*$^{+}$P$_{yopE}$ *yopE*$_{130}$–*cyaA′*$^{+}$ (encodes SycE and YopE$_{130}$–Cya)	Sory and Cornelis (1994)
pMSK3	pTM100 P$_{yopE}$ *yopP*$_{99}$–*cyaA′*$^{+}$ (encodes YopP$_{99}$–Cya)	M.-P.Sory, C.Kerbourch and G.R.Cornelis (unpublished)
pMS3	pACYC184 + oriT$_{RK2}$ + *yopE* + *sycE*	Sory *et al.* (1992)
Suicides and mutators		
pIM210	pKNG101 + *Sal*I–*Xba*I fragment of pIM205 (encodes TyeA$_{\Delta 19-59}$)	this work
pIML222	pKNG101 + *Sal*I–*Xba*I fragment of pIML220 (encodes YopN$_{\Delta 64-110}$)	this work
pIML224	pKNG101 + *Sal*I–*Xba*I fragment of pIML221 (encodes YopN$_{\Delta 248-272}$)	this work
pKNG101	*ori*$_{R6K}$ *sacBR oriT*$_{RK2}$ *strAB*	Kaniga *et al.* (1991)
pKNG105	*ori*$_{R6K}$ *sacBR oriT*$_{RK2}$ *strAB,blaA′ luxAB blaA″*	Kaniga *et al.* (1991)

Cloning and sequencing of yopN and the downstream region

A 2690 bp *Eco*RV fragment from pYV22703 containing yopN and downstream region was cloned into the *Sma*I site of pBluescript KS^{-} (Stratagene) yielding plasmid pIM176. Both strands of the 2690 bp fragment were sequenced using Taq cycle sequencing kit (Amersham) and an automated sequencer (Licor). The sequences of *yopN* and *tyeA* have been submitted to the DDBJ/EMBL/GenBank databases under accession number AF033863. DNA and proteins were analysed with the Blast (Altschul *et al.*, 1990, Gish and States, 1993) and FastA (Pearson and Lipman, 1988) programmes. The prediction of coiled-coils domains was performed with the algorithm described by Lupas *et al.* (1991).

Construction of the tyeA non polar mutant

tyeA is the second gene of the operon *yopNtyeAORF2ORF3ORF4* (Figure 1). The Shine–Dalgarno site of *tyeA* is localized 33 bp upstream of the stop codon of *yopN*. Hence, the 20 bp of the 5′ end of *tyeA* overlap with the 20 bp of the 3′ end of *yopN*. Likewise, the Shine–Dalgarno of ORF2 is situated 28 bp upstream from the stop codon of *tyeA*. The 14 bp of the 3′ end of *tyeA* thus overlap with the first 14 bp of the ORF2. The *tyeA* mutant was constructed by directed mutagenesis. To make a *tyeA* non-polar mutation on downstream genes we designed primer MIPA319 (5′-GCTCCTCATCGCTAAACTTGTTAACCAGT-GCG-3′) which hybridized to nucleotides 41–56 (with a mismatch at position 51, to introduce a *Mun*I site) and nucleotides 80–95 of *tyeA*. Directed mutagenesis (Kunkel *et al.* 1987) on plasmid pIM187 using this primer results in an in-frame deletion of amino acids 19–59 of *tyeA* and modification of amino acid 17 (Asn replaces Asp). The deletion was checked by sequencing. The mutated allele (*tyeA*$_{\Delta 19-59}$) was subcloned in the suicide vector pKNG101 and introduced in the pYV plasmid of *Y.enterocolitica* to produce E40 (pIM403). In a second step we inactivated the chromosomal gene encoding β-lactamase A (*blaA*; Cornelis and

Abraham, 1975) by inserting the *luxAB* genes using the mutator plasmid pKNG105, as described by Kaniga *et al.* (1991). The *tyeA blaA* mutant strain was designated MIE40 (pIM403). To construct the E40 (pIM411) $yopN_{45}$ *tyeA* double mutant, pIM210, the mutator plasmid for *tyeA* was crossed with the *Y.enterocolitica* $yopN_{45}$ mutant E40 (pIM41) (Boland *et al.*, 1996).

Construction of yopN mutants

Alleles $yopN_{\Delta 64–110}$ and $yopN_{\Delta 248–272}$ were constructed by site-directed mutagenesis as described by Kunkel *et al.* (1987), using pIM146 (Boland *et al.*, 1996) DNA as a template. Plasmid pIML220 encoding $YopN_{\Delta 64–110}$ was created with MIPA 379 (5′-ATATTGGGGCTGTTACT-ACGCTCGGAGAAGAC-3′). This primer hybridized to nucleotides 175–189 and 331–347 of *yopN*. Plasmid pIML221 encoding $YopN_{\Delta 248–272}$ was created with MIPA 378 (5′-AAATCCTTTAACCTGGTCTTGTTG-ACTTTGTCGCT-3′) which hybridized to nucleotides 725–741 and 816–834. The mutated alleles were subcloned in the suicide vector pKNG101 giving plasmids pIML222 and pIML224, and were introduced in *Y.enterocolitica*. The E40 $yopN_{\Delta 64–110}$ was called E40 (pIM407) and the E40 $yopN_{\Delta 248–272}$ was designated E40 (pIM408). In a second step we inactivated the chromosomal gene encoding β-lactamase A (*blaA*; Cornelis and Abraham, 1975). The corresponding strains were called MIE40 (pIM407) and MIE40 (pIM408).

Yersinia enterocolitica-induced apoptosis

Cell culture, J774A.1 macrophage infection and assessment of apoptosis were performed as described by Mills *et al.* (1997).

Cell infection and fluorescence labelling

Macrophages grown on a coverslip were infected with bacteria pregrown for 1 h at 37°C and prepared as described previously (Sory and Cornelis, 1994). They were then fixed in 4% paraformaldehyde, 0.1% glutaraldehyde for 20 min and permeabilized in PBS, pH 7.4, 0.2% Triton X-100 for 15 min. YopE was labelled by indirect immunofluorescence with an affinity-purified anti-YopE antiserum (10 μg/ml in 0.1% PBS-bovine serum albumin) plus goat anti-rabbit fluoresceine isothiocyanate-conjugated immunoglobulins (Dakopatts). Samples were mounted in 50% Mowiol (Polysciences, Inc.) containing 100 mg/ml diazabicyclo-octane (DABCO, Sigma). Cells were examined on a confocal laser scanning microscope equipped with dual detectors and an argon-krypton laser for simultaneous scanning of two different fluorochromes (MRC1024, Bio-Rad Laboratories, Richmond, CA).

Yop translocation assay

Maintenance of PU5-1.8 macrophages and translocation assays were carried out essentially as described by Sory and Cornelis (1994). Translocation of $YopE_{130}$–Cya was determined 2 h post-infection, $YopH_{99}$–Cya and $YopM_{100}$–Cya at 3 h post-infection, and $YopO_{143}$–Cya, $YopO_{77}$–Cya and $YopP_{99}$–Cya at 2.5 h post-infection. Release of $YopE_{130}$–Cya into the culture medium was monitored as described by Sory and Cornelis (1994).

Acknowledgements

We thank Corinne Kerbourch for excellent technical assistance. We are grateful to Cécile Neyt for providing the unpublished construct pCN29 and to Cecile Geuÿen for a critical reading of the manuscript. A.B. is supported as a research assistant from the Belgian Fonds National de la Recherche Scientifique. A.P.B. is recipient of a Brenninkmeijer fellowship attributed by I.C.P. This work was supported by the Belgian Fonds National de la Recherche Scientifique Médicale (Convention 3.4595.97), the Direction générale de la Recherche Scientifique-Communauté Française de Belgique (Action de Recherche Concertée 94/99–172) and by the 'Interuniversity Poles of Attraction Program: Belgian State, Prime Minister's Office, Federal Office for Scientific, Technical and Cultural affairs' (PAI 4/03). Confocal microscopy was funded by grant 9.4531.94F from FRSM (Loterie Nationale).

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Received November 20, 1997; revised January 28, 1998; accepted February 10, 1998