***Yersinia enterocolitica* type III secretion–translocation system: channel formation by secreted Yops**

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‘Type III secretion’ allows extracellular adherent bacteria to inject bacterial effector proteins into the cytosol of their animal or plant host cells. In the archetypal *Yersinia* system the secreted proteins are called Yops. Some of them are intracellular effectors, while YopB and YopD have been shown by genetic analyses to be dedicated to the translocation of these effectors. Here, the secretion of Yops by *Y. enterocolitica* was induced in the presence of liposomes, and some Yops, including YopB and YopD, were found to be inserted into liposomes. The proteoliposomes were fused to a planar lipid membrane to characterize the putative pore-forming properties of the lipid-bound Yops. Electrophysiological experiments revealed the presence of channels with a 105 pS conductance and no ionic selectivity. Channels with those properties were generated by mutants devoid of the effectors and by *lcrG* mutants, as well as by wild-type bacteria. In contrast, mutants devoid of YopB did not generate channels and mutants devoid of YopD led to current fluctuations that were different from those observed with wild-type bacteria. The observed channel could be responsible for the translocation of Yop effectors.

Keywords: ionic channel/translocation/*Yersinia*/YopB/YopD

Introduction

Several animal and plant pathogenic Gram-negative bacteria use a new type of system called ‘type III secretion’ to attack their host (for a review, see Hueck, 1998; Galan and Collmer, 1999). These systems, activated by contact with a host cell membrane, allow bacteria to inject proteins across the two bacterial membranes and the eukaryotic cell membrane to destroy or subvert the target cell. The Yop virulon, a weapon common to *Yersinia enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*, is an archetype of these systems (for reviews, see Cornelis and Wolf-Watz, 1997; Cornelis *et al.*, 1998). It consists of a secretion apparatus

(the Ysc apparatus) made of 25 proteins, and a set of 12 proteins called Yops that are exported by the Ysc apparatus. Some of these Yops are effectors (YopE, YopH, YpkA/YopO, YopP/YopJ, YopM and YopT) that are delivered by extracellular bacteria into the cytosol of macrophages where they destroy the cytoskeleton, subvert the signaling network and induce apoptosis. Others, such as YopB and YopD, have been shown by genetic analyses to be required for the translocation of the effectors across the eukaryotic cell membrane (Rosqvist *et al.*, 1994; Sory and Cornelis, 1994; Persson *et al.*, 1995; Boland *et al.*, 1996; Håkansson *et al.*, 1996; Francis and Wolf-Watz, 1998; Skrzypek *et al.*, 1998). Finally, another Yop, called YopN, is thought to participate in the regulation of the secretion apparatus (Forsberg *et al.*, 1991; Iriarte *et al.*, 1998).

YopB and YopD are encoded by the same large *lcrGVsycDyopBD* operon that also encodes SycD, the chaperone of YopB and YopD (Wattiau *et al.*, 1994; Neyt and Cornelis, 1999a), as well as LcrG and LcrV, two other proteins that have also been shown to be required for effector translocation (Skrzypek and Straley, 1993; Nilles *et al.*, 1997, 1998; Sarker *et al.*, 1998a,b; Pettersson *et al.*, 1999).

Yersinia pseudotuberculosis has a lytic activity on sheep erythrocytes, depending on contact and on the presence of YopB. This hemolysis could be inhibited by dextran 4, suggesting the presence of a pore in the lipid membrane (Håkansson *et al.*, 1996). In agreement with this hypothesis, infection of macrophages with *Y. enterocolitica* leads to the selective release or uptake of small-sized dyes such as BCECF [2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein] (623 Da) and LY-CH [lucifer yellow carbohydrazide] (443 Da) but not of Texas Red-X phalloidin (1490 Da). This permeation of infected cells to small dyes requires both YopB and YopD but not LcrG (Neyt and Cornelis, 1999b). In addition, YopB, eluted from SDS-PAGE, was shown to have a membrane-disrupting activity on artificial lipid bilayers (Håkansson *et al.*, 1996). Taken together, these observations suggest that some Yops are able to insert into and destabilize the lipid membrane. However, the membrane organization of the complex leading to lysis of erythrocytes and permeation of macrophages has never been described.

In the present work, we analyzed the insertion of Yops by *Yersinia* into artificial bilayers and we provide electrophysiological evidence for the formation of a channel involving YopB and YopD in this bilayer. This represents the first such demonstration with a type III secretion system.

Results

Insertion of YopB and YopD into lipid vesicles

In vitro, Yops can be released by incubating *Yersinia* cultures at 37°C in the absence of Ca²⁺. However, Yops

form large aggregates in the extracellular medium (Michiels *et al.*, 1990), which impairs their purification in a native state. To bypass protein purification steps that involve denaturation of the protein, we induced Yops synthesis and release in the presence of pure asolectin large unilamellar vesicles. Bacteria were grown in a Ca^{2+} -chelated medium and liposomes were added to the culture medium just before shifting the temperature to 37°C. After 4 h of incubation, bacteria were pelleted and the proteoliposomes were concentrated and purified by flotation on a sucrose gradient. The band of proteoliposomes was collected and washed to eliminate sucrose and electrostatically bound proteins.

Since previous experiments with red blood cells and macrophages have shown that Yop effectors prevent hemolysis (Håkansson *et al.*, 1996) and the passage of dyes (Neyt and Cornelis, 1999b), the first experiments were carried out with mutant bacteria (called HOPEMN) that do not produce these effectors (YopH, YopO, YopP, YopE, YopM and YopN) but tend to overproduce YopB, YopD and LcrV (Boland *et al.*, 1998; Neyt and Cornelis, 1999b). Figure 1A shows the electrophoretic pattern of proteins contained in the culture supernatant in the absence of liposomes and in the purified proteoliposomes. Two proteins with molecular weights corresponding to those of YopD and YopB were found to be associated with the liposomes (lane 2). Their identity was confirmed by N-terminal sequencing, and the molar ratio of YopD/YopB was evaluated to be ~ 5 (mol/mol). The lipid to protein ratio was 34 ± 4 (w/w, $n = 4$).

Yersinia inserts channels in lipid membranes

To evaluate the ability of the lipid-bound proteins to induce the formation of a channel, the proteoliposomes

Fig. 1. Analysis of Yops secreted and inserted into liposomes (12% SDS-PAGE, silver staining). Lanes 1: TCA precipitate of 0.1 ml of culture supernatant prepared as described in Materials and methods. Lanes 2: purified proteoliposomes (80 µg of phospholipid). The presence of lipids is responsible for the difference in migration observed between lanes 1 and 2. (A) HOPEMN mutant strain. (B) HOPEMND $\Delta 121-165$ mutant strain. The arrow indicates the position where the truncate is observed in lane 2 when the gel is overloaded (see text). In lane 1, the truncate appears clearly (★). (C) Wild-type strain. The letters on the right of the gels identify the Yop proteins.

were fused with a planar lipid bilayer. After formation of a stable asolectin membrane, proteoliposomes were added to the *cis* chamber under vigorous stirring, and an electrical potential difference between 50 and 70 mV was applied across the planar lipid bilayer. The amount of proteins added ranged from 3 to 8 µg and the amount of total phospholipids never exceeded 250 µg. In $\sim 90\%$ of our experiments, a fusion leading to a detectable jump of the bilayer conductance was observed within ~ 15 min. Stirring was then stopped immediately to prevent new fusion events. As shown in Figure 2A and B, upon application of a voltage, random fluctuations between discrete levels of current amplitude occurred, which suggested that a channel had been reconstituted in the planar lipid bilayer (for details and definitions of channel properties, see Homblé *et al.*, 1998). Gating was observed in symmetrical salt conditions [both sides of the membrane: phosphate buffer (PB) + NaCl from 25 mM to 1 M], as illustrated in Figure 2A for a single channel, and in asymmetrical salt conditions (*cis* side: PB + 100, 150 or 180 mM NaCl; *trans* side: PB + 5 mM NaCl) as shown in Figure 2B, where at least three channels were inserted in the planar lipid bilayer and were closing and re-opening. Since the amplitude of the current flowing through a channel at a given voltage was independent of salt concentration between 100 mM and 1 M NaCl (see below), the data obtained in salt conditions close to the physiological concentrations (100–180 mM salt) were pooled to draw current–voltage (I–V) curves (Figure 2C). The relationship between the magnitude of the current flowing through an open channel and the applied voltage was linear in both symmetrical and asymmetrical salt conditions. The unitary conductance of the channel (105 ± 4 pS, $n = 8$) was calculated from the slope of individual I–V curves. In asymmetrical conditions, the membrane potential under zero current conditions (the reversal potential) was -11.5 ± 0.3 mV ($n = 4$). Using the Goldman–Hodgkin–Katz equation, we calculated that it corresponds to a ratio of the permeability coefficient $P_{\text{Cl}^-}/P_{\text{Na}^+}$ of 1.4 ± 0.3 ($n = 4$). This value is very close to the ionic mobility ratio found in solution ($u_{\text{Cl}^-} = 1.52 u_{\text{Na}^+}$), indicating that the observed channel has no ionic selectivity. Figure 2D shows that the channel conductance was independent of salt concentration above 100 mM, suggesting ion flux saturation above this value. Saturation of conductance is a feature common to channels of different organisms (e.g. Coronado *et al.*, 1980; Homblé and Very, 1992).

Role of YopB, YopD and LcrG in channel formation

To assess the role of YopB, YopD and LcrG in the formation of the channel, we repeated the experiments using the HOPEMN strain in which additional mutations have been introduced. We first introduced the *lcrG* $\Delta 8-56$ allele (Sarker *et al.*, 1998b) giving strain HOPEMNG (Neyt and Cornelis, 1999b). Proteoliposomes incubated with these *lcrG* mutant bacteria contained YopB and YopD and gave channels that were similar to those generated by HOPEMN bacteria (not shown), indicating that LcrG is not directly involved in channel formation.

We then tested HOPEMND $\Delta 121-165$, a strain that contains an in-frame deletion from codon 121 to codon 165 of *yopD* and leads to the expression of a truncated 31 kDa YopD (Figure 1B, lane 1) deprived of its hydrophobic

domain (Mills *et al.*, 1997). When 80 μ g of proteoliposomes were loaded on a gel, only YopB was visualized upon silver staining (Figure 1B, lane 2), although the truncated YopD clearly had been released by the bacteria (lane 1). Overloading the gel with 400 μ g of these proteoliposomes allowed the detection of a very faint band at 31 kDa (data not shown). During eight fusion

experiments with these proteoliposomes, we never obtained any current traces similar to those presented in Figure 2A and B. Channel-like current fluctuations were observed but they were characterized by many short transitions to a broad range of various open state amplitudes (Figure 3). The difference between the current traces observed for HOPEMN and HOPEMND $_{\Delta 121-165}$ is best illustrated by comparing the amplitude histograms of the channel conductance from representative experiments (Figure 4). While the histogram obtained with the HOPEMN strain was characterized by two peaks corresponding to a single open state with a 105 pS conductance, histograms corresponding to the HOPEMND $_{\Delta 121-165}$ mutant showed a broad distribution of low conducting levels. Thus, the current fluctuation generated by the HOPEMND $_{\Delta 121-165}$ mutant clearly differed from the 105 pS channel described when both YopB and YopD are lipid bound, indicating that without YopD, no stable channel structure is inserted.

Finally, we tested the HOPEMNB strain that contains the *yopB* $_{\Delta 89-217}$ allele (Schulte *et al.*, 1996). With these YopB-deficient bacteria, no channel formation was detected ($n = 8$ symmetrical salt concentration 100 mM and asymmetrical salt concentration 5–100 mM) although YopD was associated with the lipid vesicles (data not shown).

Effect of total Yops on channel formation

Our results show that effector polymutant bacteria could induce the formation of a channel with defined parameters (conductance and selectivity) in a planar lipid membrane. To investigate whether such a channel would be observed in the more complex environment represented by wild-type bacteria, we repeated the experiments with *Y. enterocolitica* E40. The protein content of the proteoliposomes and of the culture supernatant from the induction control were analyzed by SDS-PAGE (Figure 1C). Upon silver staining, seven bands were visualized in the control and were identified on the basis of their apparent molecular weights (Cornelis *et al.*, 1998): they correspond to YopH, YopM and YopB, LcrV, YopD and YopT, YopN, YopE and YopQ. When more culture supernatant was loaded, three more bands appeared, corresponding presumably to YopO, YopR and YopP. Lane 2 shows the protein pattern of the purified

Fig. 2. Typical current records and I-V curve obtained upon fusion of proteoliposomes (HOPEMN strain) with a planar lipid bilayer. (A) Current trace in symmetrical salt conditions: both *cis* and *trans* sides, PB + 180 mM NaCl. The arrow marks the time when the voltage is switched to -70 mV. (B) Current trace in asymmetrical salt conditions (*cis* side, PB + 100 mM NaCl; *trans* side, PB + 5 mM NaCl). The arrow marks the time when the voltage is switched to +90 mV. The letter 'c' refers to the closed state of the channel and the different levels of single channel current are identified by a number given on the right of the current traces. (C) Mean I-V curves. Values are the average of at least three independent (new batch of proteoliposomes and a new planar lipid bilayer) experiments realized in various symmetrical ($\blacktriangledown$, PB + 180 mM NaCl, or 150 or 100 mM NaCl) and asymmetrical salt conditions ($\triangle$, *trans* = PB + 5 mM NaCl; *cis* = PB + 180 mM NaCl, or 150 or 100 mM NaCl). Data were fitted by linear regression between -100 mV and +100 mV with $r^2 > 0.99$. (D) Effect of salt concentration on the single channel conductance. Experiments were performed in symmetrical NaCl concentrations at room temperature. The data were fitted with a Michaelis-Menten equation and gave a half-saturating NaCl concentration of 45 mM and a maximal conductance of 137 pS.

Fig. 3. Typical current record observed upon fusion of proteoliposomes (HOPEMND $\Delta_{121-165}$ strain) with a planar lipid membrane. This representative current trace has been selected in asymmetrical salt conditions: *cis* side, PB + 100 mM NaCl; *trans* side, PB + 5 mM NaCl. The arrow marks the time when the voltage is switched to +80 mV. The letter 'c' refers to the closed state (full line) of the channel, and the multiple open states (dashed lines) are identified by their corresponding conductance value calculated using Ohm's law.

Fig. 4. Amplitude histograms of the conductance levels for the HOPEMND $\Delta_{121-165}$ and HOPEMN mutant strains. The histograms were constructed from the frequency distributions of the current amplitude measured on representative current traces analyzed for 20 s and showing at least five gating events. The current values were converted into conductance (pS, x-axis) using Ohm's law. The y-axis indicates the percentage of total time spent at one given conductance level. One representative experiment was selected for each strain.

proteoliposomes. N-terminal sequencing allowed the identification of the proteins and a relative quantification of their proportion. The relative quantities were determined in two batches of proteoliposomes, and the results obtained did not differ by >1% (Table I). YopB and YopD were the two major proteins since they accounted for ~60% (mol/mol) of the total Yops associated with the liposomes, with an apparent stoichiometry of 6/3.5 (YopD/YopB, mol/mol). The effectors YopH, YopM and YopE and the hypothetical secretion plug YopN were bound to the vesicles in much lower amounts (between 6 and 15% of the total proteins, mol/mol). The low intensity band at 81 kDa was too weak to be sequenced and was identified as YopO on the basis of its molecular weight. Analyses

Table I. Identification of the Yop proteins inserted in the liposomes

Mol. wt (kDa)	Identification	N-terminus	Relative quantity (mol/mol)
87	Yop O	ND	<1%
51	Yop H	MXLSLSDLHRQVSRLVQ	6%
44	Yop M	MFINPRNVSNFTLQE	12%
	Yop B	SALITHDRSTPVTGS	22%
36/34	Yop D	TINIKTDSPIITTSQ	38%
	Yop N	TTLHNLSYGNTPLRNEHPEI	6%
25	Yop E	MKISSFISTSLPLPASV	15%

ND = not determined.

of five independent proteoliposome preparations by densitometry showed that the patterns of the lipid-associated proteins were highly reproducible. By lipid and protein dosages, the mean phospholipid/protein ratio was estimated to be 28 ± 5 (w/w, $n = 4$). Association of Yops with the liposomes seems to be relatively specific since only seven out of the 12 secreted Yops were lipid bound.

The Yops-containing proteoliposomes were fused with a planar lipid bilayer. In asymmetrical salt conditions, upon voltage imposition, in most cases and during much of the recorded time, the channel remained locked in an open state. Occasionally, brief transitions to a closed state were observed at positive potentials (e.g. in Figure 5A) and at high negative potentials (over -70 mV). In symmetrical salt conditions, current fluctuations between open and closed states occurred frequently (Figure 5B). The relationship between the magnitude of the current flowing through an open channel and the applied voltage was linear in both symmetrical and asymmetrical salt conditions (Figure 5C). The unitary conductance of the channel (107 ± 5 pS, $n = 6$) was calculated from the slope of the I-V curves. The reversal potential measured in asymmetrical salt conditions was -14 ± 1 mV ($n = 3$), which corresponds to a ratio of the permeability coefficient P_{Cl^-}/P_{Na^+} of 1.9 ± 0.3 ($n = 3$). This indicates that the wild-type *Y. enterocolitica* strain inserts into liposomes a channel whose characteristics (conductance and ionic selectivity) are identical to those found with the HOPEMN mutant.

Discussion

A central question in *Yersinia* type III secretion is to understand how the effector Yops are transferred from the bacteria into the cytoplasm of the target cells. Håkansson *et al.* (1996) and then Neyt and Cornelis (1999b) have suggested that this transfer might be related to the presence of a pore in the lipid membrane. The diameter of this pore has been estimated to be between 1.6 and 2.3 nm. However, the membrane complex by which pore formation occurs has not yet been described. Using a strategy that bypasses any denaturing protein purification, we provide here electrophysiological evidence for the insertion of a channel by *Yersinia* into artificial lipid vesicles.

Bacteria unable to synthesize the effectors YopH, YopO, YopP, YopE, YopM and YopN induced the insertion of a channel with a conductance of 105 pS and no selectivity in a planar lipid bilayer. In experiments in which more than one channel was reconstituted (for instance, see Figure 2B), the magnitude of the maximal current was an

Fig. 5. Typical current records and I-V curve obtained upon fusion of proteoliposomes (wild-type strain) with a planar lipid bilayer. (A) Asymmetrical salt conditions: *cis* side, PB + 180 mM NaCl; *trans* side, PB + 5 mM NaCl. (B) Symmetrical salt conditions. Both the *cis* and *trans* sides contained PB + 180 mM NaCl. The closed (c) and open (1) states of the channel are indicated. The arrows mark the time when the voltage is switched to +70 mV. (C) Mean I-V curves. Closed circles: symmetrical salt conditions (both the *cis* and *trans* sides: PB + 180 mM NaCl). Values are the average of three independent (new batch of proteoliposomes and a new planar lipid bilayer) experiments and the standard errors are shown. Open symbols: asymmetrical salt conditions (*cis* side, PB + 180 mM NaCl; *trans* side, PB + 5 mM NaCl). Four independent experiments were performed. Since closures were not observed at all voltages, different symbols are used when closures were observed in four experiments out of four (Δ), three out of four ($\diamond$), two out of four ($\square$) and one out of four voltages ($\circ$). Data were fitted by a linear regression between -100 and +100 mV with $r^2 > 0.99$.

integer multiple of the unitary conductance (105 pS), indicating that the channel has a single state of conductance. A channel of the same conductance and selectivity was observed in the absence of LcrG, suggesting that this protein does not play a significant role in channel formation. On the contrary, proteoliposomes incubated with

yopD mutant bacteria gave current fluctuations that were very different from the 105 pS channel described above. Finally, proteoliposomes incubated with bacteria deficient in YopB did not give rise to any channel activity. This strongly suggests that both YopB and YopD, but not LcrG, are required to form a channel. So far, we cannot exclude that LcrV might play a role in channel formation, although this was not detected by gel electrophoresis. However, since the *lcrV* mutant does not secrete YopB and YopD (Sarker *et al.*, 1998a), testing this mutant would not give any additional information.

Gram-negative bacteria are known to release porins into the growth medium, and the OmpC porin of *Y. enterocolitica* has been shown to induce the formation of a cation-selective channel with a 1.3 nS conductance in 1 M KCl (Brzostek *et al.*, 1989). We can exclude the possibility that OmpC would be responsible for the channel observed in this study since it has electrical properties completely different from those of the channel described herein.

Our results are in perfect agreement with those showing that *Yersinia* has a hemolytic and cell permeabilizing activity that is dependent on YopB and YopD, but not on LcrG (Håkansson *et al.*, 1996; Neyt and Cornelis, 1999b). They give direct evidence for pore formation, supporting the idea that permeation to small molecules and lysis of erythrocytes is the consequence of the insertion of a channel in the cell plasma membrane.

YopB and YopD contain hydrophobic regions (amino acids 168–208 and 224–258 for YopB and 122–152 for YopD) as well as hypothetical coiled-coil sequences (Lupas *et al.*, 1991) that are often involved in protein–protein interaction. YopB and YopD have been shown to be already associated in the bacterial cytoplasm prior to secretion, bound together to a common chaperone (Neyt and Cornelis, 1999a). These data as well as those presented here suggest that YopB and YopD could insert together in the eukaryotic plasma membrane and would form a channel in this membrane. Their interaction with the lipid membrane most probably is mediated by their hydrophobic domains. In agreement with this hypothesis, we observed that a YopD mutant deleted of this domain (amino acids 121–165) has a much lower ability to bind to the pure lipid vesicles.

In addition to its role in channel formation, YopD was shown to act as a negative regulator of Yop production and to be translocated itself across the plasma membrane (Francis and Wolf-Watz, 1998; Lee *et al.*, 1998; Williams and Straley, 1998). The latter observation suggests either that YopD interacts with YopB to form a stable channel, exposing domains to the cytoplasmic phase, or that sequential events are taking place, whereby YopD participates in the translocation of effectors and is then released into the cell cytoplasm.

The question of the stoichiometry of YopB and YopD in the lipid membrane is quite puzzling. In our experiments, the YopD/YopB molar ratio in the membrane varies from ~2 for the wild-type strain to ~5 for the mutant deleted of the effector proteins. However, although the stoichiometry of the complex seems to differ, the conductance and ionic selectivity of the inserted channel are identical. This could reflect the different proportions of YopB and YopD secreted by the two strains and a differential insertion of YopB and/or YopD individually or as a

complex. The stoichiometry would then represent the total amount of YopB and YopD in the membrane and not the stoichiometry of the channel. From this point of view, insertion of a higher amount of YopD is not expected to modify the channel properties since YopB is required for channel formation. The stoichiometry of the active channel will be investigated further in the future.

Previous observations indicated that YopB, YopD and LcrG are required for translocation of Yop effectors across the eukaryotic cell plasma membrane (Rosqvist *et al.*, 1994; Sory and Cornelis, 1994; Sory *et al.*, 1995; Boland *et al.*, 1996; Francis and Wolf-Watz, 1998; Sarker *et al.*, 1998b) but the role of YopB in translocation has been challenged recently by Lee and Schneewind (1999). If YopB is not involved in translocation, the channel we describe here would be an independent way to attack cells rather than a translocation apparatus.

In conclusion, we demonstrated that *Y. enterocolitica* incubated with an artificial lipid membrane induces the formation of a channel that involves both YopB and YopD. Since YopB and YopD have been shown in some reports to be essential for the translocation of *Yersinia* effectors into the cell cytoplasm, it is very likely that this channel is related in some way to effector translocation, but our results do not allow us to draw conclusions about this question. However, they suggest that liposomes represent a potentially interesting system to address it. The putative translocation mechanism of the channel will be characterized by addition of a purified Yop effector to vesicles containing this reconstituted channel.

Materials and methods

Bacterial strains and growth conditions

Yersinia enterocolitica MRS40, referred to here as the wild-type strain, is the *blaA* mutant of strain E40 (Sarker *et al.*, 1998b). The *Y. enterocolitica* HOPEMN strain is MRS40(pIM417) and carries the mutated alleles *yopO*_{Δ65–558}, *yopE*₂₁, *yopH*_{Δ1–352}, *yopM*₂₃, *yopP*₂₃ and *yopN*₄₅ (Neyt and Cornelis, 1999b). The *Y. enterocolitica* HOPEMND_{Δ121–165} strain is MRS40(pCN4007), a derivative of MRS40(pIM417) with an in-frame deletion of codons 121–165 from the *yopD* gene (Mills *et al.*, 1997). This strain produces and secretes a 31 kDa truncated YopD product. Strains HOPEMNB [MRS40(pCN4008)] and HOPEMNG [MRS40(pCN4006)] contain the *yopB*_{Δ89–217} and *lcrG*_{Δ8–57} alleles, respectively (Neyt and Cornelis, 1999b).

The *Y. enterocolitica* strains were plated routinely on tryptic soy agar and cultured in brain–heart infusion (BHI, Difco Laboratories, Detroit, MI) supplemented with 0.4% glucose (w/w) and 20 mM MgCl₂. Selective agents were nalidixic acid (35 µg/ml) and arsenate (0.4 mM).

Liposome preparation

A stock solution of asolectin in chloroform was prepared after purification (according to Kagawa and Racker, 1971) of the mixed soybean phospholipids from Sigma.

After evaporation of the organic solvent under N₂ flux and overnight drying under vacuum, the lipid film was rehydrated with phosphate-buffered saline (PBS; 137 mM NaCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 10 mM KCl buffer, pH 7.4). Large unilamellar vesicles were then prepared in the same buffer at room temperature according to the procedure of Hope *et al.* (1985) by using an extruder (Lipex Biomembranes Inc; Vancouver, Canada) (pores 0.1 µm diameter). The liposome concentration was estimated by measuring the phospholipid content, derived from the inorganic phosphorus content, as described by Mrsny *et al.* (1986).

Induction of the Yop regulon, analysis of the Yops

The protocol we developed for induction of Yops secretion is largely adapted from Michiels *et al.* (1990). *Yersinia* grown overnight at room temperature (22–26°C) were inoculated to an OD at 600 nm of 0.2 in

10 ml of BHI supplemented with 5 mM EGTA. The culture was incubated with rotary shaking (150 r.p.m.) for 1.5 h at room temperature and then shifted to 37°C and grown for an additional 4 h. Just before the temperature shift, liposomes were added to the culture to a final concentration of 8 mg/ml. The incubation time at 37°C was determined by comparing the amount of proteins inserted in the liposomes after 1, 2, 3 and 4 h by densitometric analyses of Coomassie Blue-stained gels (data not shown). This amount reached a steady-state maximal level after 4 h. The bacteria were then pelleted by centrifugation for 6 min at 14 000 g (Sigma 201 centrifuge, B. Braun Biotech International, Rotor No.12001). The 10 ml liposome-containing supernatant was concentrated by a 30 min centrifugation at 126 000 g at 4°C (Beckman L7 ultracentrifuge, SW 60 rotor) on a 0.5 ml 80% sucrose layer. Concentrated liposomes formed a large turbid band at the top of that layer. This band was collected and mixed with an equal volume of 80% sucrose solution. A 25–5% sucrose gradient was then poured on top of the liposome suspension. An overnight centrifugation at 126 000 g at 4°C allowed separation of proteoliposomes from proteins that were not associated with liposomes, these free proteins remaining at the bottom of the gradient. The band containing the proteoliposomes was collected and the proteoliposomes were washed in PBS NaCl 0.8 M and centrifuged for 1 h at 126 000 g to remove the sucrose from the samples and eliminate the proteins that may interact electrostatically with liposomes. The pellet was resuspended in PBS and the concentration of phospholipid was estimated as described above. Total protein contents of proteoliposomes were estimated using the Folin Ciocalteu test (Lowry *et al.*, 1951).

An induction control, in which liposomes were replaced by an equal volume of PBS added to the culture medium, was run as described above. The proteins from the culture supernatant were precipitated overnight in 10% trichloroacetic acid (TCA) and pelleted by a 15 min centrifugation at 14 000 g. The experimental procedure was optimized with the wild-type strain and then applied to the mutant strains.

Yop protein patterns in the culture supernatant and in the proteoliposomes were analyzed by 12% SDS–PAGE. After electrophoresis, the proteins were visualized either by reverse staining according to Fernandez-Patron *et al.* (1995), by silver staining or by Coomassie Blue staining. The first procedure allows the proteins to be electroblotted directly on a PVDF membrane and sequence analysis to be performed. The peptide N-terminal amino acid sequence was determined by automated Edman degradation using a Beckman LF 3400 D protein-peptide microsequencer equipped with an on-line Gold 126 microgradient high-pressure liquid chromatography (HPLC) system and a model 168 Diode Array detector (Beckman Instruments, Inc., Fullerton, CA). All samples were sequenced using standard Beckman sequencer, procedure 4. The phenylthiohydantoin amino acid derivatives were identified quantitatively by reverse phase HPLC on an ODS Spherogel micro column (3 µm diameter particles, 2 × 150 mm, Beckman Instruments). All sequencing reagents were from Beckman.

Electrophysiological measurements

Bilayers were formed from 1%, w/v asolectin dissolved in *n*-decane on a 200 µm hole drilled in a styrene co-polymer partition separating two chambers by using the Mueller–Rudin technique (1962). The two chambers were connected to the voltage generator through Ag/AgCl electrodes and a bridge of 3 M KCl, 2% agar. The *cis* chamber was connected to the headstage input of a Biologic RK 300 patch clamp amplifier (Claix, France). The *trans* chamber was held at ground. Details of the apparatus and recording techniques are given elsewhere (Fuks and Homblé, 1995). The currents were digitized at 1 kHz and low-pass filtered at 300 Hz (five pole Tchebicheff filter). Data acquisition and analysis were performed using the PAT V7.0 program (courtesy of John Dempster, University of Strathclyde, Strathclyde, UK). Electrical potential differences were defined as *cis* with respect to *trans*.

The two chambers were filled with a phosphate buffer (PB; 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄ pH 7.4) supplemented with various NaCl concentrations given in the text. In the *cis* chamber, PB also contained 1 mM CaCl₂ to promote the fusion of liposomes with the lipid bilayer. Planar lipid bilayers were formed in either symmetrical or asymmetrical NaCl conditions.

Pure lipid vesicles or liposomes prepared as described above with the HOPEMN strain but in the presence of Ca²⁺ (no induction of Yops secretion) had no effect on the planar lipid bilayer conductance.

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