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Pore-forming activity of type III system-secreted proteins leads to oncosis of *Pseudomonas aeruginosa*-infected macrophages

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Summary

The *Pseudomonas aeruginosa* cystic fibrosis isolate CHA induces type III secretion system-dependent but ExoU-independent oncosis of neutrophils and macrophages. Time-lapse microscopy of the infection process revealed the rapid accumulation of motile bacteria around infected cells undergoing the process of oncosis, a phenomenon we termed pack swarming. Characterization of the non-chemotactic CHA*cheZ* mutant showed that pack swarming is a bacterial chemotactic response to infected macrophages. A non-cytotoxic mutant, lacking the type III-secreted proteins PcrV, PopB and PopD, was able to pack swarm only in the presence of the parental strain CHA or when macrophages were pretreated with the pore-forming toxin streptolysin O. Interaction of *P. aeruginosa* with red blood cells (RBCs) showed that the contact-dependent haemolysis provoked by CHA requires secretion via the type III system and the PcrV, PopB/PopD proteins. The pore inserted into RBC membrane was estimated from osmoprotection experiments to be between 2.8 and 3.5 nm. CHA-infected macrophages could be protected from cell lysis with PEG3350, indicating that the pore introduced into RBC and macrophage membranes is of similar size. The time course uptake of the vital fluorescent dye, Yo-Pro-1, into infected macrophages confirmed that the formation of transmembrane pores by CHA precedes cellular oncosis. Therefore, CHA-induced macrophage death results from a pore-forming activity that is dependent on the intact *pcrGVHpopBD* operon.

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Introduction

Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen for humans and is one of the major agents responsible for nosocomial infections. This bacterium causes acute infections in immunocompromised individuals with pathologies such as cancer, AIDS, severe burns and wounds. Chronic respiratory infections resulting from *P. aeruginosa* and associated host inflammatory responses are the major causes of mortality in patients with cystic fibrosis (CF).

In addition to numerous extracellular-secreted virulence determinants, such as elastase, phospholipases and exopolysaccharide alginate (Salyers and Whitt, 1994), the majority of clinical *P. aeruginosa* isolates possess a specialized apparatus, the type III secretion system, dedicated to overcoming the host defences (Frank, 1997). The type III system is widespread in Gram-negative pathogens, including *Yersinia*, *Salmonella* and *Shigella*, and in plant pathogens such as *Pseudomonas syringae* and *Erwinia chrysanthemi* (Galan and Collmer, 1999). This unusual system requires close contact between infecting bacteria and the host cell to secrete and deliver toxic bacterial proteins directly to the cytosol of the host cell. The prototypic type III secretion system is that of *Yersinia* species (Cornelis *et al.*, 1998; Cornelis and Van Gijsegem, 2000). The *Yersinia* secretion system contains ≈20 proteins (*ysc*) that comprise the secretion apparatus spanning the two bacterial membranes. Six proteins (termed Yops) of *Yersinia* spp. are secreted through the *ysc* complex and injected into the eukaryotic cytosol. The translocation of Yop toxins across the eukaryotic membrane is thought to occur through a protein channel consisting of at least two proteins, YopB and YopD, inserted into a membrane by the bacteria (Cornelis and Van Gijsegem, 2000). Although type III secretons and putative translocation proteins are conserved among bacterial species, the diversity of effectors and their cellular targets results in the different phenotypes induced by pathogens (Galan and Collmer, 1999).

P. aeruginosa strains secrete at least four effector proteins through their type III secretion system, namely

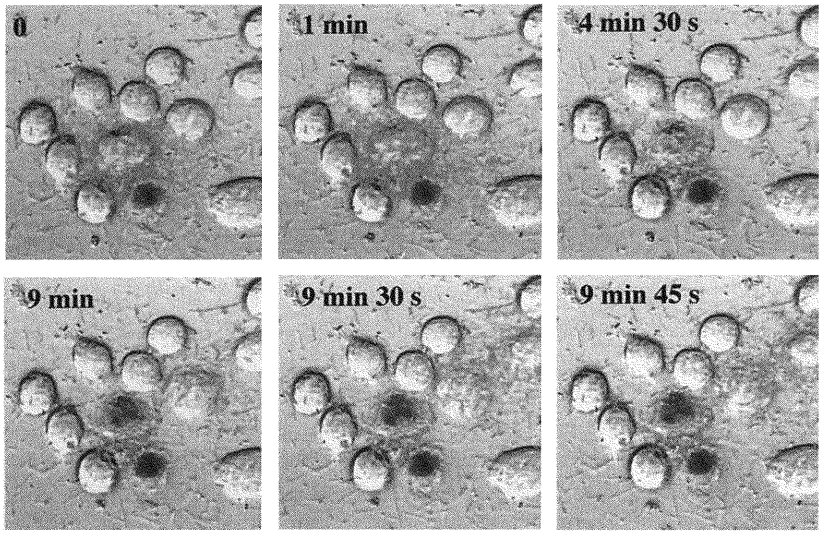

Fig. 1. Pack swarming of *P. aeruginosa* CHA during macrophage infection. J774 cells were infected with CHA (green) expressing GFP (MOI of 10) in medium containing EtBr. EtBr only enters dead cells (red). Infection was performed in Lab-Tek chambers at 37°C for 15–30 min and then taken for microscopy observations. Time-lapse was performed by xyt scan with a Zeiss confocal microscope for 10 min with images saved each 15 s. Image 0 represents the first scan saved.

ExoS, ExoT, ExoY and ExoU. ExoS and ExoT are related proteins with ADP-ribosylation activity (Frank, 1997; Liu *et al.*, 1997) and GTPase-activating activity *in vitro* towards low-molecular-weight GTP-binding proteins (Goehring *et al.*, 1999; Krall *et al.*, 2000). ExoY is an adenylate cyclase secreted by some strains of *P. aeruginosa* (Yahr *et al.*, 1998). Finally, ExoU (PepA) has been identified by two groups as a putative cytotoxin causing cell death of epithelial cells and macrophages (Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998). Results from several laboratories, including our own, strongly suggest that *P. aeruginosa* strains collected from diverse infection environments express different type III system-secreted effectors (or effector combinations), and this therefore results in different phenotypes on cultured cells. We recently reported the cytotoxic phenotype of a CF clinical isolate, CHA, on professional phagocytes: neutrophils and macrophages. When infected at a low multiplicity of infection (MOI of 10), such cells die by rapid oncosis characterized by cellular and nuclear swelling. The cytotoxic phenotype required a functional type III secretion system but was independent of the toxin ExoU (Dacheux *et al.*, 1999; 2000), suggesting a novel mechanism of cell intoxication used by some isolates of *P. aeruginosa*.

The work presented here provides new insights into the mechanism of CHA-induced macrophage death. Time-lapse microscopy of infection processes revealed unusual bacterial behaviour during macrophage infection, described as pack swarming. We show that pack swarming is a chemotactic response of bacteria towards molecules released from wounded macrophages, and we provide evidence for a novel, bacterial pore-forming activity that precedes macrophage cell death. This activity requires type III-secreted proteins encoded within the *pcrGVHpopBD* operon.

Results

CHA-induced oncosis is accompanied by bacterial pack swarming

In order to obtain more information concerning the rapid oncosis of J774 macrophages induced via the type III secretion system of the *P. aeruginosa* cytotoxic CF isolate CHA, the infection process was followed using time-lapse confocal microscopy (Fig. 1). The macrophages were grown in Lab-Tek chambers and infected at an MOI of 10 with fluorescent CHA expressing green fluorescent protein (GFP). Incubation was carried out for 15 min at 37°C in medium containing ethidium bromide (EtBr), a membrane-impermeant fluorescent dye that enters only dead cells and colours nuclei red by intercalation within DNA. Although the bacteria were uniformly distributed in the medium, even at 15–30 min after infection, highly motile bacteria have already started to accumulate around some macrophage cells, forming 'pack swarms'. [The term 'pack swarming', used in this work to describe the behaviour of *P. aeruginosa* during macrophage infection, should not be confused with the active surface motility, termed swarming, widespread among Gram-negative bacteria (Harshey, 1994), including *P. aeruginosa* (Kohler *et al.*, 2000).] Swarms were variable in bacterial density, but could reach several hundreds of bacteria (estimated by fluorescence intensity) in some cases. Obvious changes in the morphology of the 'attacked' macrophage were observed during pack swarming. These included membrane blebbing accompanied by cellular and nuclear swelling, cell flattening and detachment from the dish surface, in agreement with the previously reported characterization of CHA-induced cell oncosis (Dacheux *et al.*, 2000).

Several cells surrounded by motile bacteria were followed in independent experiments using time-lapse

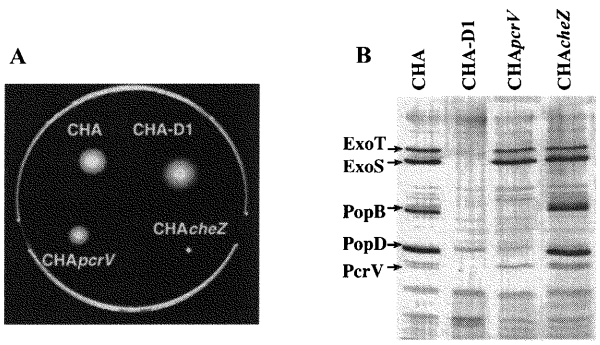

Fig. 2. Phenotypic characterization of *P. aeruginosa* strains.
A. Chemotactic phenotypes of CHA, CHA-D1, CHA*pcrV* and CHA*cheZ* were analysed on 0.3% LB agar plates. The difference in swimming motility between CHA and CHA*pcrV*, visible here, is not significant.
B. Secretion of type III system proteins ExoS, ExoT, PcrV, PopB and PopD. Supernatants (40 μl) from bacterial-induced cultures (calcium-depleted LB) were analysed by SDS–PAGE. The gel was developed by silver staining.

microscopy. Figure 1 represents selected micrographs of one example of the phenomenon. The whole sequence can be viewed as a time-lapse (QuickTime) movie at http://www.blackwell-science.com/products/journals/suppmat/mmi/2368/mmi2368sm.htm.

Approximately 2 min after maximal swarm formation, the selected cell takes up EtBr, suggesting that plasma membrane disintegration has occurred. At about the same time, the bacteria begin to disperse and, in some cases, a new swarm was formed around a neighbouring cell (see Fig. 1). Cell death, measured from the beginning of swarm formation until the incorporation of EtBr into the nuclei of a selected cell, occurs within 5–10 min on average. Pack swarming around cells undergoing the process of oncosis was observed with all motile CF isolates that were previously found to be cytotoxic towards neutrophils and macrophages (data not shown), showing that the phenomenon described is not unique to the CHA strain.

Interestingly, the same phenomenon has been reported for *Salmonella typhimurium* interacting with HeLa cells previously damaged by exposure to low pH (Uhlman and Jones, 1982). It has been postulated that HeLa cells, upon treatment with low pH, release a low molecular chemoattractant in the surrounding medium creating chemotactic gradients, which then attract the bacteria. It has been proposed that the *Salmonella* swarms disperse when the chemoreceptors are saturated, the phenomenon also observed in interactions of CHA with macrophages.

Pack swarming is a P. aeruginosa *chemotactic response*

Chemotaxis enables motile bacteria to sense changes in an environment and to respond to the concentration gradient of nutrients. The tactic response includes chemosensing by methyl-accepting receptor proteins (MCPs), phosphorelay by signal transduction proteins (Che) and locomotion of bacterial flagella (Stock and Surette, 1994). We hypothesized that the *P. aeruginosa* pack swarming around infected J774 cells, by analogy with the phenomenon described for *Salmonella*, could be related to the chemotactic response of bacteria towards molecule(s) released from infected macrophages. To test this hypothesis, we constructed an isogenic non-chemotactic mutant, CHA*cheZ*, in which the *cheZ* gene was inactivated (see *Experimental procedures*). The *cheZ* gene encodes a phosphatase that is necessary for correct signal transduction between membrane MCPs and flagella (Eisenbach, 1996; Boesch *et al.*, 2000). The test for chemotaxis, performed on 0.3% agar plates, confirmed that the insertion of the Gm cassette into *cheZ* on the CHA chromosome resulted in a non-chemotactic phenotype (Fig. 2A). The mutant was still motile in liquid medium. To investigate the pack swarming behaviour of CHA*cheZ*, infection of macrophages with CHA*cheZ* was followed by phase-contrast microscopy in several independent infection experiments. In contrast to the parental CHA strain, the CHA*cheZ* mutant was unable to swarm. Thus, the pack swarming by cytotoxic CHA during macrophage infection required an intact chemotactic signalling pathway.

The secretion of the type III system proteins ExoS, ExoT, PcrV, PopB and PopD *in vitro* under inducing (low calcium level) growth conditions by CHA*cheZ* was compared with secretion by the parental strain CHA. No obvious difference was observed between the two strains (Fig. 2B), showing that the *cheZ* mutation does not affect the type III system secretion. Microscopic observations suggested that the CHA*cheZ* mutant is slightly delayed in provoking cytotoxicity in initial periods of infection (15–30 min after infection), but rapidly reached the wild-type (CHA) level at prolonged incubation periods (not shown). However, the cytotoxicity test, using the release of lactate dehydrogenase (LDH) as a measure of cell death, did not allow measurement of this difference. We have therefore shown here that: (i) the phenomenon of pack swarming is a bacterial chemotactic response towards oncotic macrophages; and (ii) it plays a role in the very initial periods of *ex vivo* CHA–macrophage interactions.

Evidence for pore formation by CHA via type III system proteins

As the rapid oncosis of CHA-infected macrophages requires a functional type III secretion system, the pack swarming behaviour of two non-cytotoxic mutants was investigated during infection. The CHA-D1 (Dacheux *et al.*,

Table 1. Pack swarming of *P. aeruginosa* mutant strains during macrophage infections.

	Co-incubation with			
Strain observed[a]	DMEM only	CHA	Streptolysin O	α-toxin
CHA	+	ND	ND	+
CHA-D1	–	+	+	–
CHA*pcrV*	–	+	+	–
CHA*cheZ*	–	–	ND	ND

a. CHA-D1, CHA*pcrV* and CHA*cheZ* were labelled with GFP for pack swarming observations in co-infection experiments. Pack swarming, observed in several independent experiments, is symbolized by (+) for presence and (–) for absence. ND, not done.

1999) strain is deficient in type III secretion, because of the disruption of the *exsA* gene encoding the transcriptional activator of the system (Frank, 1997). CHA*pcrV* contains a Tn*5*Tc insertion in the *pcrV* gene within the *pcrGVHpopBD* operon. This mutant has been isolated as non-cytotoxic on neutrophils by a screen of the CHATn5Tc library (D. Dacheux, unpublished). CHA*pcrV* is deficient in the secretion of three type III system proteins, PcrV, PopB and PopD, but has an intact type III secretion apparatus, as it secretes at least two type III secreted proteins *in vitro*, ExoT and ExoS (Fig. 2B). CHA-D1 and CHA*pcrV* are not cytotoxic towards macrophages over a 2 h period of infection. Although these mutants have intact flagella and are motile on 0.3% agar swimming plates (Fig. 2A), microscopic observation showed that they never pack swarmed during infection of macrophages (Table 1). This suggests that the functionality of the type III secretion system and, more precisely, the presence of three secreted proteins, PcrV, PopB and PopD, is a prerequisite for pack swarming. To test whether the non-cytotoxic mutants CHA-D1 and CHA*pcrV* were able to swarm if an active type III secretion system was provided, macrophages were co-infected with the mutants (MOI of 10) and the parental strain CHA (MOI of 2). To distinguish the strains in co-infection experiments, CHA-D1 and CHA*pcrV* were transformed with a plasmid expressing GFP. In co-infection experiments, both mutants were motile and formed swarms around infected macrophages, confirming that the activity of the intact type III secretion system (in strain CHA) provides a chemotactic signal, necessary for pack swarming. We postulated that the chemoattractant molecule is slowly released from infected cells, creating the chemotactic gradient that attracts motile bacteria. Thus, to investigate further the origin of a signal molecule(s), macrophages were independently pretreated with two prototypes of pore-forming bacterial toxins, streptolysin O and staphylococcal α-toxin (Bhakdi *et al.*, 1996), and then infected with non-cytotoxic mutants, CHA-D1^{GFP} and CHA*pcrV*^{GFP}. The permeability of macrophage membranes resulting from the toxin treatment was verified by the uptake of a membrane-impermeant fluorescent dye Yo-Pro-1 (Molecular Probes). Treatment of cells by both toxins resulted in the rapid uptake of Yo-Pro-1, indicating efficient pore formation. Non-cytotoxic mutants accumulated rapidly around streptolysin O-treated macrophages. In contrast, the treatment of macrophages with α-toxin resulted in Yo-Pro-1 uptake, but was not sufficient to allow the formation of swarms by non-cytotoxic mutants (Table 1). The difference in action of the two toxins is related to the size of the pores they form in cell membranes; α-toxin forms small, transmembrane pores of 0.6–1 nm diameter, whereas the pores formed by streptolysin O are up to 30 nm in diameter. As the

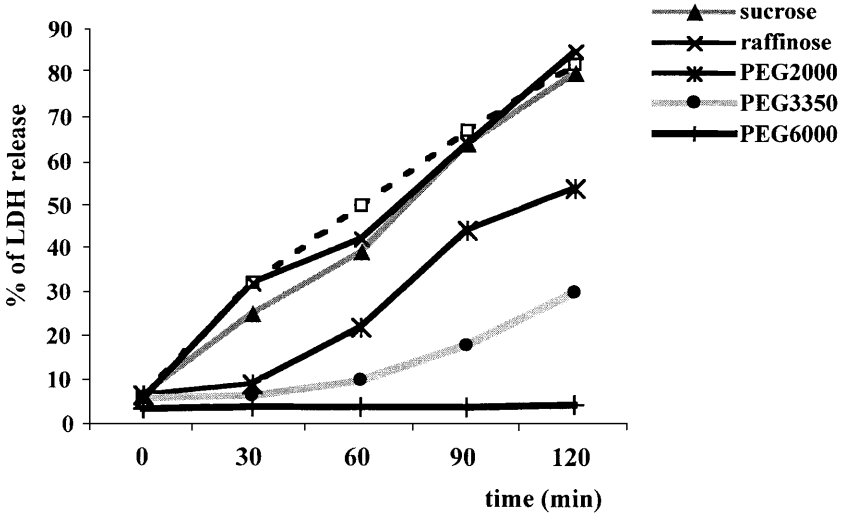

Fig. 3. Osmoprotection of CHA-infected macrophages. J774 macrophages grown in 24-well plates were infected with *P. aeruginosa* CHA (MOI of 5) grown to an OD_{600} of 1.0–1.5. DMEM was supplemented with 30 mM osmoprotectants, as indicated. Cytotoxicity was followed over a 2 h period by measuring LDH release using a cytotoxicity detection kit (Roche).

cytotoxicity of CHA towards macrophages and the action of streptolysin O are able to provide the same signal required for pack swarming behaviour, it is likely that the type III-secreted proteins have a pore-forming activity resulting in the formation of pores > 1 nm in diameter.

The ability of different sized carbohydrates to protect cells from lysis has been used previously to demonstrate pore formation by the RTX family of cytolysins and to estimate the size of the pore (Lobo and Welch, 1994). Polyethylene glycols (PEGs) of various molecular weights have also been used to size pores whose formation is induced by pathogens such as *Gardnella vaginalis* (Moran *et al.*, 1992) and *Legionella pneumophila* (Kirby *et al.*, 1998) or, more recently, to determine the size of pores inserted by *Shigella flexneri* type III system-secreted proteins into erythrocyte membranes (Blocker *et al.*, 1999). To determine whether the cytototoxicity of CHA towards macrophages was caused by the formation of a pore inserted into the cell membrane, we performed osmoprotection experiments. Osmoprotectants of different sizes were added to DMEM at a concentration of 30 mM, and LDH release was followed over a 2 h period of infection of macrophages. Although sucrose (0.9 nm diameter) and raffinose (1.3 nm diameter) did not prevent macrophage lysis, PEGs with molecular weights of 2000 (2.8 nm diameter) and 3350 (3.5 nm diameter) showed reproducible delay in LDH release. PEG6000 (5 nm diameter) added to the medium during infection completely inhibited the release of LDH from CHA-infected macrophages (Fig. 3).

Characterization of P. aeruginosa*-induced, contact-dependent haemolysis*

To investigate further the pore-forming activity of the type III system-secreted proteins of *P. aeruginosa*, interactions with sheep red blood cells (RBCs) were studied (Fig. 4A). Similar results were obtained with human RBCs (data not shown). Strains were mixed with RBCs in RPMI-1640 at a MOI of 1, incubated for 60 min at 37°C, and haemolytic activity was determined by measuring the release of haemoglobin, as described in *Experimental procedures*. No haemolysis could be detected without prior centrifugation of the bacteria and RBC mixture (Fig. 4A). When bacteria were mixed with RBCs and immediately centrifuged at 1500 *g*, the CHA strain lysed between 80% and 90% of cells within 60 min. The mutant strains, CHA-D1 and CHA*pcrV*, showed only low (< 20%) haemolytic activity, indicating that a large part of the haemolytic activity induced by CHA is dependent on type III system-secreted proteins. To show that the haemolysis induced by CHA was dependent on cell-to-cell contact, bacterial culture supernatants were also tested for their ability to lyse RBCs. Similar to CHA-D1 and CHA*pcrV*, the bacterial culture supernatants retained only residual (< 20%) haemolytic activity. Macroscopic analysis of CHA and CHA-D1 on sheep blood agar plates showed that the two strains are able to secrete other types of haemolytic factor(s) in the same manner (J. Croize, personal communication). Thus, the residual activity detected in our experiments is probably the result of factor(s) secreted independently of the type III secretion system.

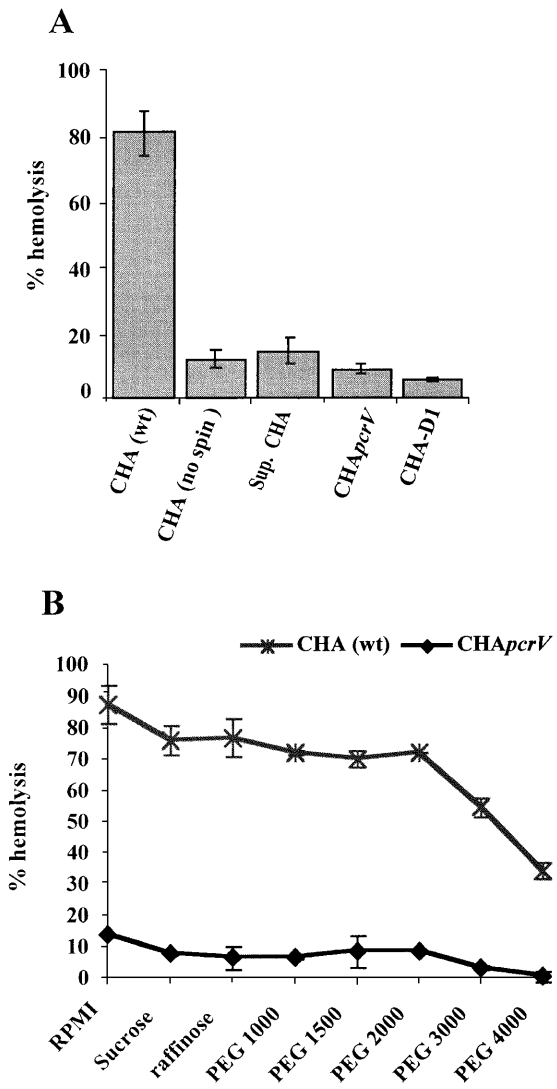

Fig. 4. Characterization of *P. aeruginosa*-induced contact-dependent haemolysis.
A. Haemolysis of RBCs incubated with CHA, CHA-D1 and CHA*pcrV* at an MOI of 1. Bacterial culture supernatants (CHA sup., 100 μl) were added to RBCs, and incubations were performed in parallel with bacterial infections.
B. Protection of RBCs from CHA-induced haemolysis by 30 mM osmoprotectants. All experiments were performed at least three times in triplicate. Standard deviation is shown.

Lysis of RBCs by bacterial pore-forming toxins occurs through osmotic shock (Menestrina *et al.*, 1994a, b) and could be prevented by osmoprotectants present in the

medium at a concentration of 30 mM. To estimate the size of the pore inserted into the membrane of RBCs, haemolysis experiments were performed by incubation with different-sized molecules (Fig. 4B). Although sucrose, raffinose and PEG1000 had no significant effect on the efficacy of haemolysis, PEGs larger than PEG2000 afforded protection against CHA-induced haemolysis. This protection increased with the size of the molecule and reaches ≈ 60% protection with PEG4000. PEG6000 protected RBCs from haemolysis completely, but this result was not taken into account, as the cells started to fuse in the presence of PEG6000 and were difficult to manipulate (data not shown). Taken together, the osmoprotection of macrophages and RBCs from CHA-provoked lysis with molecules larger than PEG2000 allowed us to estimate the size of the pore incorporated into eukaryotic cell membranes to between 2.8 and 3.5 nm.

Time-lapse uptake of the vital dye Yo-Pro-1 into CHA-infected macrophages

The bacterial chemotactic response towards infected macrophages, together with the results of osmoprotection experiments, indicated strongly that *P. aeruginosa* strain CHA inserts pores into macrophage and RBC membranes via type III-secreted proteins. To follow the kinetics of pore formation and macrophage cell death, the uptake of Yo-Pro-1, a membrane-impermeant molecule, into cells was followed at a single-cell level during infection. Yo-Pro-1 was selected because it is a small (630 Da) fluorescent marker with a high affinity for DNA and RNA, enabling uptake to be detected even in the cell cytoplasm. Macrophages were infected with CHA (MOI of 1) in medium containing Yo-Pro-1, incubated for 10 min at 37°C and observed by fluorescence and phase-contrast microscopy simultaneously. One cell around which we could observe the beginning of pack swarm formation was selected for monitoring for change in fluorescence as a function of time. As can be seen on the selected micrographs (Fig. 5), the selected cell started Yo-Pro-1 uptake at a point at which no obvious changes in membrane or cell morphology could be observed (compare images A and A′). The progressive uptake of the dye occurred over ≈ 8 min and then reached a plateau. At this time point, the selected cell showed maximal dye uptake and the morphological changes typical of oncosis (Fig. 5, images B and B′). The time course of Yo-Pro-1 uptake, followed by cellular and nuclear swelling characteristic of oncosis, further confirmed the presence of transmembrane pores in CHA-infected macrophages, the activity that precedes macrophage cell death.

Discussion

Pseudomonas aeruginosa clinical isolates intoxicate eukaryotic cells through the activity of the type III secretion system. Some strains are able to modify the

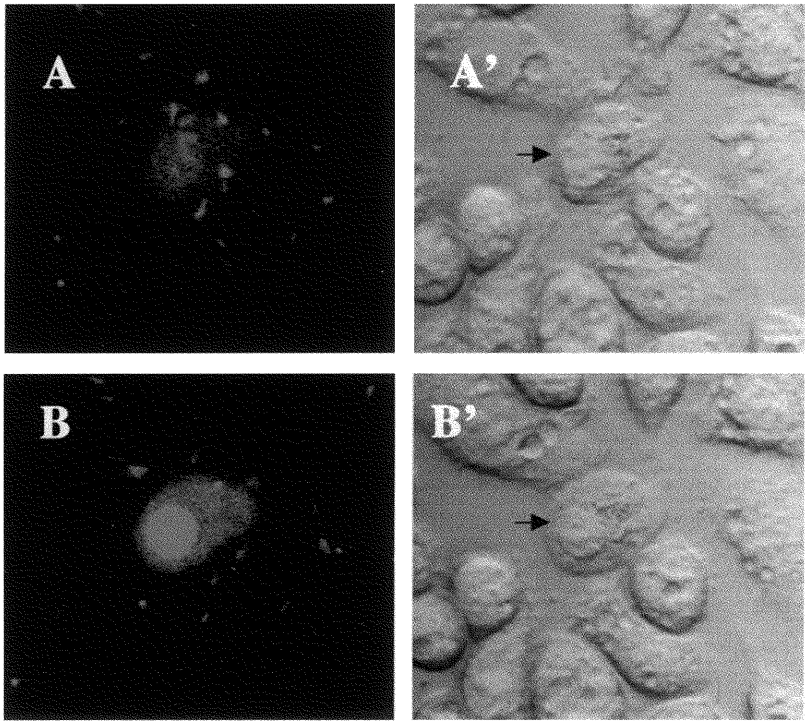

Fig. 5. Uptake of Yo-Pro-1 into CHA-infected macrophages. Cells were infected with CHA expressing GFP (MOI of 1) in DMEM in the presence of Yo-Pro-1 (1 μM). Incubation was performed at 37°C for 5–10 min. When pack swarming was detected, a selected cell was followed for 8 min by observations in time lapse (xyt scan) under a confocal microscope. Images were saved each minute. Selected micrographs A and B correspond to the beginning and end, respectively, of the time lapse. A′ and B′ are corresponding phase-contrast images. Arrows indicate the selected cell.

cellular actin cytoskeleton by ExoS-exerted GAP activity (Pederson *et al.*, 1999) and ADP-ribosylation activity (McGuffie *et al.*, 1998) directed towards GTP-binding proteins of the Ras and Rho families. ExoT, which is sequence and activity related to ExoS, is required for antiphagocytic activity (Cowell *et al.*, 2000), and ExoY, an adenylate cyclase, changes cAMP levels in eukaryotic cells (Yahr *et al.*, 1998). All these three cytotoxins have been shown to modify cell morphology significantly, with only slight effects on cell viability in *ex vivo* infection models (Vallis *et al.*, 1999). In contrast, the ExoU toxin, which is expressed by some isolates, is responsible for non-apoptotic cell death of macrophages and epithelial cells (Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998; Sawa *et al.*, 1999). In previous studies, we have analysed several CF isolates, including the CHA strain, for their cytotoxicity towards eukaryotic cells, phagocytes and epithelial cells. Epithelial cells, upon infection with CHA, round up and detach from the dish surface without losing their membrane integrity. This phenotype has been associated with the expression of effectors ExoS, T and Y (Vallis *et al.*, 1999). In contrast, in low-multiplicity infections, CHA induces rapid oncotic death of phagocytes that was dependent on a functional type III secretion system, but independent of the ExoU toxin (Dacheux *et al.*, 1999; 2000).

To shed light on the mechanism of macrophage intoxication by CF isolates, we followed the macrophage infection by time-lapse microscopy. This allowed us to observe that macrophage cell death, in an *ex vivo* model, is a stochastic event accompanied by unusual bacterial behaviour. Motile bacteria are directed towards cells and accumulate rapidly around oncotic macrophages, a phenomenon that we have termed pack swarming. By constructing and analysing an isogenic, non-chemotactic mutant, CHA*cheZ*, we showed that the phenomenon of pack swarming is a bacterial chemotactic response towards molecule(s) released by infected macrophages, which, however, does not play an important role in the overall kinetics of CHA-induced cytotoxicity. Non-cytotoxic mutants lacking the type III secretion system proteins were not able to swarm during infection, although they were fully chemotactic on soft agar plates. Indeed, when the activity of the intact type III secretion system was provided by the parental strain in the co-infection experiments, pack swarming of the non-cytotoxic mutants was indistinguishable from that of the parental strain. As the bacterial chemotactic response requires the establishment of concentration gradients of chemoattractant, we postulated that type III secreted proteins are able to form small pores in macrophage membranes. Using two prototypes of pore-forming toxins in infection experiments with non-cytotoxic mutants, we showed that streptolysin O treatment provides the same chemotactic signal as the action of type III secreted proteins PcrV, PopB and PopD (absent in the mutant CHA*pcrV*). Curiously, treatment of macrophages with α-toxin, which forms transmembrane pores of about 1 nm, did not provoke a chemotactic response by mutants, suggesting that the chemoattractant(s) released from cells is/are too large to pass through α-toxin-induced pores.

Treatment of macrophages with streptolysin O, which can form large pores up to 30 nm, resulted in cells that had a flattened appearance resembling 'fried eggs' and detached rapidly from the dish surface. This appearance of dying cells is also characteristic of CHA-infected macrophages (see Fig. 1; Dacheux *et al.*, 2000). Interestingly, the same phenotype was reported with macrophages infected with mutant strains of *Yersinia enterocolitica* that were deficient in all type III secreted effectors, but had an intact 'translocator' composed of YopB and YopD (Neyt and Cornelis, 1999). The authors suggested that YopB and YopD in mutated *Yersinia* insert into the macrophage membranes and form pores, leading to cell flattening. In contrast to the wild-type *P. aeruginosa* strains, the wild-type *Yersinia* strain does not provoke oncosis but, rather, rounding up of macrophages as a result of the action of the cytotoxic effectors YopH and/or YopE (Rosqvist *et al.*, 1995; Cornelis *et al.*, 1998). It was suggested by the authors that 'translocator' pores are filled by secreted Yops that are passing into the cell cytosol, thus preventing cell flattening (Hakansson *et al.*, 1996; Neyt and Cornelis, 1999). Indeed, YopB and YopD are able to interact with artificial liposomes to form protein channels (Tardy *et al.*, 1999), and YopB was also shown to be haemolytic and to have a membrane-disrupting activity on artificial lipid bilayers (Hakansson *et al.*, 1996).

The osmoprotection experiment, together with time course of Yo-Pro-1 uptake, confirmed that the CHA-induced oncosis of macrophages is preceded by pore formation, an activity that requires the secretion of PcrV, PopB and PopD. The fact that YopB/D are able to insert into eukaryotic membranes suggests that it is possible that PopB alone, or together with PopD and/or PcrV, could be inserted into macrophage membranes and, by forming small pores, allows leakage of the cytoplasm, which later results in cell lysis. Indeed, PopB of *P. aeruginosa*, expressed in a *Yersinia* host, was able to induce RBC lysis as a result of a 2- to 3-nm-sized pore (Frithz-Lindsten *et al.*, 1998). Based on osmoprotection experiments, the size of the pore inserted into macrophage and RBC membranes by *P. aeruginosa* CHA (estimated to be 2.8–3.5 nm) is similar to that of YopB-dependent pores (1.2–3.5 nm) (Hakansson *et al.*, 1996). Using dye exclusion experiments, Neyt and Cornelis (1999) estimated the size of YopB/D pores to be 1.6–2.3 nm. Taking into account these similarities, we propose that the *P. aeruginosa* 'translocator' pore is not sufficiently filled with Exo effectors, and that it may have, specifically when interacting with macrophages, the role

Table 2. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
E. coli DH5α		Gibco BRL
P. aeruginosa		
CHA	Mucoid CF isolate	Toussaint *et al.* (1993)
CHA-D1	CHA *exsA*::Gm	Dacheux *et al.* (1999)
CHA*cheZ*	CHA *cheZ*::Gm	This work
CHA*pcrV*	Tn*5*Tc insertion in *pcrV*	D. Dacheux (unpublished)
Plasmids		
pUCGm	Ap^r, Gm^r	Schweizer (1993)
pUCP20	Ap^r	Schweizer (1991)
pEX100T	Ap^r	Schweizer and Hoang (1995)
pUCP20-*gfp*mut3	Ap^r	This work

Ap^r: ampicillin resistance, Gm^r: gentamicin resistance.

of a pore-forming toxin, the activity of which leads to rapid cell oncosis.

Several other bacterial pathogens express proteins that share the same organization of functional domains with PopB/D and YopB/D. These include EspD of enteropathogenic *Escherichia coli*, IpaB of *Shigella* and SipB of *Salmonella*. It has been shown recently that the induction of necrosis in neutrophils by *Shigella flexneri* requires type III secretion and 'translocators' IpaB and IpaC (Francois *et al.*, 2000), putative functional homologues of PopB/D. Almost nothing is known about the action and localization of PcrV and PopB/D in *P. aeruginosa*. Whether and how these proteins insert into eukaryotic cell membranes to act as effector 'translocators' and the mechanism of pore formation are the challenge of our ongoing studies.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The strains used in this study are listed in Table 2. *Pseudomonas aeruginosa* CHA, referred to here as the wild type, is a mucoid CF isolate. All mutants used in this study were isogenic of the CHA strain. CHA-D1 has the *exsA* gene inactivated by the insertion of the gentamicin (Gm) resistance cassette. CHA*pcrV* contains the transposon Tn*5*Tc insertion in the *pcrV* gene (D. Dacheux, unpublished). To obtain the CHA*cheZ* mutant, part of the *cheYZ* operon was isolated from CHA by polymerase chain reaction (PCR) amplification. Sense (5′-GCCTCGACCGCAGCTTGTAAC) and antisense (5′-GTCCTGGTAATCCTGCGCC) primers were synthesized according to the sequence obtained from the *Pseudomonas* genome project database (http://www.pseudomonas.com). The PCR product of 1.025 kb was cloned in pEX100T (Schweizer and Hoang, 1995) and inactivated by the insertion of the Gm cassette from pUCGm (Schweizer, 1993) into the single *Eco*RI site, located 35 nucleotides downstream from the *cheZ* translation start codon. The resulting plasmid was transferred to CHA by triparental conjugation, using pRK2013 as helper plasmid (Schweizer and Hoang, 1995). The correct double recombination event at the *cheZ* locus was verified by Southern blot and PCR analysis. *P. aeruginosa* strains were grown on *Pseudomonas* isolation agar (Difco) plates or in liquid Luria–Bertani (LB) medium at 37°C with agitation. The antibiotics used were carbenicillin (300 μg ml^{-1}), tetracycline (100 μg ml^{-1}) and gentamicin (200 μg ml^{-1}). For time-lapse microscopy experiments, *P. aeruginosa* strains were rendered fluorescent using plasmids expressing GFP. The promoterless *gfp*mut3 (Cormack *et al.*, 1996) was cloned as a *Xba*I–*Pst*I fragment into pUCP20 (Schweizer, 1991). The resulting plasmid, pUCP20-*gfp*mut3, was introduced into bacteria by electroporation (Enderle and Farwell, 1998).

Cell culture and infection conditions

The macrophage cell line J774 (ATCC) was grown in DMEM (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco). The cells were seeded in 24-well culture plates at 3×10^5 cells $well^{-1}$ 20 h before infection. The bacterial strains were grown over night in LB, diluted to an optical density of 0.1 at 600 nm (OD_{600}) and grown further for ≈ 3 h to an OD_{600} of between 1.0 and 1.5. Macrophages were infected with bacteria resuspended in 100 μl of DMEM−10% FCS to give an MOI of 1–10, as indicated. Incubation was performed in 300 μl of DMEM−10%FCS in a CO_2 (5%) incubator at 37°C. In osmoprotection experiments, infections were performed in 30 mM solutions of sucrose, raffinose, PEG2000, PEG3350 or PEG6000 made up in DMEM−10%FCS and filter sterilized. Cytotoxicity was assessed by determination of LDH release into infected supernatants using a cytotoxicity detection kit (Roche) as described previously (Dacheux *et al.*, 2000).

Phenotypic characterization

P. aeruginosa strains used in this study were characterized for their ability to secrete type III system proteins *in vitro*. Strains were grown in LB supplemented with appropriate antibiotics for 16 h at 37°C with agitation. Cultures were then diluted to an OD_{600} of 0.1 in calcium-depleted medium (induction condition) containing 5 mM EGTA and 20 mM $MgCl_2$ and grown for 4 h. After centrifugation, 40 μl of culture supernatants was analysed directly by 0.1% SDS−12%

PAGE. Motility was assessed by phase-contrast microscopy of bacteria grown in liquid LB to an OD_{600} of 1–2. Swimming and chemotaxis were tested on motility plates in several independent experiments. Freshly grown cells were transferred by a sterile toothpick to a 0.3% agar LB plate and incubated at room temperature for 8 h.

Observation of pack swarming

Macrophages were infected with bacteria at an MOI of 1–10, incubated for 15 min in a CO_2 incubator at 37°C and taken for observation. Bacterial pack swarming and changes in macrophage morphology during infection were followed by observation under a Zeiss inverted microscope (objective 32×) in more than five independent experiments. Streptolysin O (Sigma) was diluted in sterile PBS to 50 U μl^{-1} and aliquoted. Before use, it was treated with 10 mM dithiothreitol (DTT) for 5 min at room temperature. Between 2 and 10 U μl^{-1} was added to infected medium. Staphylococcal α-toxin (Sigma) was added to macrophages at 25–50 U μl^{-1}. The permeabilization of macrophages by toxins was checked using Yo-Pro-1 dye (Molecular Probes) at 1 μM.

Time-lapse in vivo *microscopy*

The CHA strain, expressing GFP, was used to infect J774 macrophages in Lab-Tek I chambers (Nunc). Infection was carried out for 5–10 min at 37°C, and the chambers were then taken for observation. When indicated, the infected medium was supplemented with 500 ng ml^{-1} EtBr or 1 μM Yo-Pro-1 dye. Infection was followed using an inverted Zeiss (LSM 410) confocal microscope (in xyt scan) interfaced with a mixed-gas helium–argon laser. Phase-contrast and fluorescent images were collected simultaneously every 15 s and processed using ADOBE PHOTOSHOP 5.0 software.

Haemolysis assay

Haemolysis was performed essentially as described by Blocker *et al.* (1999). Sheep RBCs, obtained from BioMérieux (France), were washed three times in PBS, pH 7.4 (150 mM NaCl), and resuspended in RPMI-1640 medium (Sigma) at 5×10^8 RBCs ml^{-1} at 4°C. Bacteria were grown in LB to an OD_{600} of 1.0–1.5, centrifuged and resuspended in RPMI-1640 at 5×10^8 bacteria ml^{-1}. Haemolysis assays were started by mixing 100 μl of RBCs and 100 μl of bacteria in round-bottomed, 96-well plates, which were than centrifuged at 1500 *g* for 10 min and incubated at 37°C for 1 h. The release of haemoglobin was measured at 540 nm, after centrifugation, in 100 μl of cell supernatants. The percentage (%) of total lysis was calculated as follows: $\% = [(X-B)/(T-B)] \times 100$. B (baseline) was a negative control, corresponding to RBCs incubated with 100 μl of RPMI-1640, and T was a positive control, corresponding to total lysis obtained by incubating the cells with 0.1% SDS. X is the OD value of the analysed sample. When indicated, RBCs were resuspended in 60 mM sterile solutions of osmoprotectants, made up in RPMI-1640, giving a final concentration of molecules to 30 mM. All experiments were performed at least three times in triplicate.

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Supplementary material

The following material is available from http://www.blackwell-science.com/products/journals/suppmat/mmi/2368/mmi2368sm.htm

Time-lapse movie showing J774 macrophages surrounded by motile *Pseudomonas aeruginosa*.

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