**Yersinia enterocolitica** induces apoptosis in macrophages by a process requiring functional type III secretion and translocation mechanisms and involving YopP, presumably acting as an effector protein

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**ABSTRACT** *Yersinia*, causative agents of plague and gastrointestinal diseases, secrete and translocate Yop effector proteins into the cytosol of macrophages, leading to disruption of host defense mechanisms. It is shown in this report that *Yersinia enterocolitica* induces apoptosis in macrophages and that this effect depends on YopP. Functional secretion and translocation mechanisms are required for YopP, and strongly suggesting that this protein exerts its effect intracellularly, after translocation into the macrophages. YopP shows a high level of sequence similarity with AvrRxv, an avirulence protein from *Xanthomonas campestris*, a plant pathogen that induces programmed cell death in plant cells. This indicates possible similarities between the strategies used by pathogenic bacteria to elicit programmed cell death in both plant and animal hosts.

**Yersinia** spp. pathogenic to humans (*Y. pestis, Y. enterocolitica,* and *Y. pseudotuberculosis*) all harbor a highly conserved 70-kb plasmid (*pYV*) that is essential for virulence (1). This plasmid contains ~50 virulence genes encoding an elaborate type III secretion system (*usc*) and several proteins called Yops that are secreted upon contact with eukaryotic host cells. Some of the Yops (including YopE, YopH, YopO, and YopM) are delivered into the host cell cytosol where they damage the cytoskeleton and disrupt the signaling network (2–6). These Yop effectors are translocated across the eukaryotic cell membrane by a specialized apparatus made up of several other Yops including YopB and YopD (2, 6, 7). Secretion of Yop effectors and translocators requires specific bacterial chaperones, called Sye proteins (8). Related type III secretion systems have been encountered in various other animal pathogenic bacteria such as *Pseudomonas aeruginosa* (Exs/Pse), enteropathogenic *Escherichia coli* (Sep), *Shigella* spp., *Mxi/Spa*, and *Salmonella* spp. (Inv/Spa), as well as plant pathogenic bacteria such as *Xanthomonas campestris* (Hrp), *Pseudomonas syringae* (Hrp), and *Erwinia* spp. (Hrp) (9, 10). In all of these bacteria, type III secretion of virulence proteins is required for successful host–pathogen interactions.

In this paper, we show that *Y. enterocolitica* induces apoptosis in the mouse monocyte–macrophage cell line J774A.1 and that this effect depends on YopP, which is presumably translocated by the pathogen into the target cell.

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**MATERIALS AND METHODS**

**Bacterial Strains and Methods.** *Y. enterocolitica* O:9 E40 (pYY40) (4) and nonpolar knockout mutants thereof (construction described below) were used throughout this study. *Y. enterocolitica* W22703 (pYY227) and transposon mutants *yopE* (*pGC1256*), *yopH* (*pGC1152*), *yopM* (*pBM15*), *yopOP* (*pGC559*), *yopP* (*pGB107*), and *yopBD* (*pGC133*) (11) as well as nonpolar *yscN* (*pSW2276*) (12) and *ycE* (*pPW2254*) (13) mutants were used to support data obtained with strain E40. Bacterial growth, conjugations, temperature induction of the yop virulon in a Ca**2⁺**-deficient medium, and Yop protein analysis were as described (3).

**yop Mutant Constructions, Plasmids, and Nucleotide Sequencing.** Nonpolar mutations in the following genes in strain E40 virulence plasmid (*pYY40*) have been described previously: *yseN* (*pMSL41*) (4), *yopB* (*pPW401*) (6), and *yseP* (*pAB41*) (4). Additional mutations in *yop* genes of the virulence plasmid *pYY40* were constructed as follows: The *yopD* mutant E40 (pMSL44) was made by an in-frame deletion of codons 121–165. The *yopE* mutant E40 (pAB4052) was constructed using the *yopE* mutator pPW52 (13). The *yopH* mutant E40 (pSI4008) was constructed by deleting the first 352 codons. The *yopM* mutant E40 (pAB408) has a stop codon after 23 codons of *yopM*. The *yopO* mutant E40 (pAB4068) was made by an in-frame deletion of codons 65–558. The *yopP* mutant E40 (pMSK41) contains a deletion of a central 514-bp region of *yopP* between two BamHI sites. All mutants were obtained by allelic exchange (14).

To overexpress YopP in *Y. enterocolitica*, yopP was PCR amplified using oligonucleotide primers MIPA 495 (5′-CCTGAATAGGATAAAACATAGATTGGGCCA) and MIPA 494 (5′-CCACTTGAGCAAGTTTCCAAGTACATTA) and cloned downstream of the strong *yopE* promoter in pCNR26 vector to make pMSK13. Plasmid pCNR26 is a mobilizable derivative of pTZ19R, containing the promoter of gene *yopE* and an optimized Shine Dalgarno sequence (C. Neyt and G.R.C., unpublished work).

Nucleotide sequencing of *yopP* was performed by the dyeoxy-nucleotide chain-termination method (15) using a Taq cycle sequencing kit (Amersham) and an automated sequencer (Li-Cor, Lincoln, NE). Sequence homology searches were performed using BLASTp (16).

**Abbreviations:** TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling; yop*P*+, yop*P* (pMSK13).

**Data deposition:** The sequence reported in this paper has been deposited in the GenBank database [accession no. AF023202 (yopP)].
Cell Culture and Macrophage Infection. Mouse monocyte–
macrophage J774A.1 cells (ATCC TIB 67) were grown in
RPMI 1640 medium supplemented with 10% fetal bovine
serum (GIBCO) at 37°C under 8% CO2. Unless otherwise
indicated, macrophages were seeded at 5 × 105 cells/12-mm
glass coverslip in 24-well tissue culture plates 15 h in advance
of infection. Macrophages were infected with 10 bacteria per
cell with Y. enterocolitica strains grown under conditions for
moderate Yop induction at 37°C (17). After a 30-minute
infection period, cells were incubated with gentamicin at 30
µg/ml to kill extracellular bacteria. Where indicated, cytochalas
D (Sigma), at a final concentration of 2.5 µg/ml, was added
to the J774A.1 cells 30 minutes before infection and was
maintained throughout the experiment.

Assessment of Apoptosis by Terminal Deoxyribonucleotidyl
Transferase-Mediated dUTP-Digoxigenin Nick End-Labeling
(TUNEL) Reaction and Epifluorescence Microscopy. Immunofluo-
rescence detection of fragmented genomic DNA (characteristic
of apoptosis) was accomplished using the TUNEL reaction
followed by the addition of fluorescein isothiocyanate-conju-
 gated anti-digoxigenin antibodies. Y. enterocolitica-infected or unin-
fected J774A.1 cells were fixed for 20 minutes in 2.5% parafor-
maldehyde, extracted with 2:1 ethanol:acetic acid for 5 minutes at
−20°C, and processed for epifluorescence microscopy using the
ApopTag In Situ Apoptosis Detection Kit (Oncor Apoptag S7110-
KIT) according to the manufacturer’s instructions. Processed
cells were evaluated by phase contrast and epifluorescence (480
nm) microscopy of three random fields of view (100–175 cells per
field) to determine the percentage of TUNEL-positive nuclei at
×400 magnification. We confirmed that 95–100% of the cells had
bacteria associated with them as determined by indirect immu-
nofluorescent staining of Y. enterocolitica using rabbit anti-O:9 Y.
enterocolitica antiserum (gift of G. Wauters) followed by the
addition of Texas Red-conjugated goat anti-rabbit antibodies
(Molecular Probes).

Analysis of DNA Fragmentation. Oligonucleosomal length
DNA fragmentation in Y. enterocolitica-infected or uninfected
J774A.1 cells was detected by agarose gel electrophoresis as
described (18). In brief, 107 J774A.1 cells were seeded into
100-mm tissue culture dishes and infected using a multiplicity
of infection of either 10 or 100 bacteria per cell as described
above. J774A.1 DNA was isolated from cells 2 h after infection
in 5 ml of lysate buffer (8 mM EDTA/10 mM Tris, pH 7.2/0.2%
Triton X-100/15 µg/ml proteinase K) as described (18).

Electron Microscopy. J774A.1 cells (20 × 105) were seeded
into 100-mm tissue culture dishes and infected as described
above. Cells were fixed with 1% glutaraldehyde and processed
for electron microscopy in pellets as described (19). Semi-thin
sections were stained with toluidine blue and examined by light
microscopy.

RESULTS

Y. enterocolitica Induces Apoptosis in Infected Macrophages.
In preliminary experiments, we have found by phase contrast
microscopy that the wild-type Y. enterocolitica strain E40
causes cytotoxicity in J774A.1 cells whereas the type III
secretion mutant, yscN, has no such effect. These results
indicated that a secreted Yop was likely to be involved in
macrophage cytotoxicity. However, a mutant unable to secrete
cytotoxin YopE was even more cytotoxic than the wild-type,
indicating that YopE was not involved. Infected macrophages
displayed general features of apoptosis such as membrane
blebbing (apoptotic body formation) and nuclear and cellular
shrinkage (data not shown). Results presented in Fig. 1A show
that DNA fragmentation occurred in a significant number of
macrophage nuclei, as revealed by the TUNEL reaction (20).
Morphological observations confirming these findings are
shown in Fig. 1B.

Y. enterocolitica-Induced Apoptosis Requires Secretion and
Translocation of One or More Effector Proteins. The yscN
secretion mutant and the yopB, yopD, and yscD translocation
mutants (4, 6) failed to elicit macrophage apoptosis (Fig. 1 A
and B). It thus appeared that apoptosis induction requires
functional secretion and translocation systems. These findings
suggest strongly that apoptosis induction is mediated by one or
more bacterial proteins translocated into the cytosol of the
target cell. Similar results were obtained with a different
macrophage cell line, PUS.5–1–8 (ATCC TIB 61)(data not
shown).

The YopP Protein Is Required for Apoptosis Induction. To
identify the Yop effector protein(s) required for apoptosis,
nonpolar mutations were engineered in the known effector
genes yopE, yopH, yopO, and yopM, as well as in the gene yopP,
which codes for a 30-kDa secreted Yop protein of unknown
function (11) (Fig. 2A). These mutants were used to infect
J774A.1 macrophages. A series of eight mutants constructed
previously in Y. enterocolitica W22703 (11–13) also was in-
cluded in this study.

It is shown in Fig. 2B that yopP is the only E40 mutant that
failed to induce apoptosis. A similar result was obtained with
the W22703 mutants (data not shown). Gene yopP from Y.
enterocolitica then was cloned downstream from the strong
yopE promoter (plasmid pMSK13) and introduced in trans into
the yopP mutant. After infection of macrophages with the
complemented strain (yopP⊥), the percentage of apoptotic
nuclei was 4-fold higher than that observed after infection with
the wild-type strain (Fig. 2 C and D), showing that yopP is the
gene required to cause apoptosis in macrophages that is
missing in the yopP mutant.

The role of YopP is unlikely to be in internalization of Yop
effectors because the yopP mutant is as efficient as the
wild-type in translocating proteins YopE, YopM, and YopO (data not shown). On the other hand, because functional translocation is necessary to obtain the effect (Fig. 1 for strain E40 and not shown for W22703), it is likely that YopP must be translocated to be active and, therefore, is a new effector protein.

In both strains, the yopE mutant was more efficient in inducing apoptosis than the wild-type pathogen. The explanation for this may be that the YopE protein competes physically with YopP for internalization into host cells. The Y. enterocolitica wild-type E40 strain and the yopP111 strain did not induce nuclear DNA fragmentation in HeLa cells, even at a multiplicity of infection up to 500 bacteria per cell (data not shown). Thus, Yersinia-induced apoptosis presents some cell type specificity. However, the wild-type strain and the yopP mutant were cytotoxic to HeLa cells, as previously reported for Y. pseudotuberculosis (21, 22), which indicates that apoptosis is different from YopE-mediated cytotoxicity.

Extracellular Y. enterocolitica Induces Apoptosis. To know whether the induction of apoptosis in macrophages requires internalization of the bacteria, we infected J774A.1 cells with E40 and some mutants in the presence or absence of 2.5 μg/ml cytochalasin D (inhibitor of phagocytic bacterial uptake). According to the gentamicin protection assay (23), cytochalasin D prevented uptake of all of the mutants tested, including the yscN mutant, which is unable to secrete the antiphagocytic YopH protein. Wild-type E40 and its yopE mutant induced nuclear DNA fragmentation equally well in the absence (Fig. 3A) and in the presence of cytochalasin D whereas secretion and translocation mutants did not (Fig. 3B). Thus, the bacteria need not be internalized to induce apoptosis.

Size of DNA Fragments Isolated from Y. enterocolitica-Infected J774A.1 Cells. DNA was prepared from macrophages infected with wild-type E40, yopP mutant, or yopP+++ bacteria and analyzed by agarose gel electrophoresis. DNA fragmentation was seen in samples from macrophages infected with wild-type and yopP+++ bacteria but not in samples from uninfected macrophages or macrophages infected with yopP mutant bacteria. Fragmented DNA formed ladders of ~200 bp

FIG. 2. YopP, a new Y. enterocolitica Yop effector, is required to induce apoptosis in macrophages. (A) 12% SDS-PAGE of secreted proteins from isogenic nonpolar yop mutants and overexpression of YopP in the yopP mutant. Lanes: 1, wild-type E40; 2, yopE; 3, yopH; 4, yopO; 5, yopM; 6, yopP; and 7, yopP+++ (yopP mutant overexpressing YopP). Arrowheads indicate the position of the deleted protein. Asterisk (*) denotes overexpressed YopP. To clearly show the secretion profile of the yopO mutant, samples were overloaded with supernatants of (lane 8) wild-type E40 and (lane 9) yopO mutant cultures. Loading was based on equivalent numbers of bacteria. Molecular masses are shown at left, and positions of Yops are shown at right. (B) Percentage of TUNEL-positive nuclei in J774A.1 cells infected with wild-type strain E40 or isogenic nonpolar Yop effector mutants (4 h postinfection). n values refer to the number of total cells evaluated per treatment. All strains were tested a minimum of three times. (C) Percentage of TUNEL-positive nuclei in J774A.1 cells infected with wild-type (WT) E40, yopP mutant, or yopP+++. Error bars represent the SEM for 1 field of view (~100–150 cells) from three separate coverslips. (D) Phase contrast (left) and fluorescence (right) images depicting the results of the infection (cell morphology) and TUNEL reaction (nuclear morphology), respectively in J774A.1 cells infected with (A)yopP mutant and (B) yopP+++.

FIG. 3. Extracellular Y. enterocolitica induce apoptosis. J774A.1 cells were infected in the presence or absence of 2.5 μg/ml cytochalasin D and processed for the TUNEL reaction at 4 h postinfection. n values indicate the total number of cells evaluated per treatment. Wild-type strain E40 and the yopE mutant were able to induce apoptosis as efficiently in the absence (A) or presence (B) of cytochalasin D (inhibitor of phagocytic bacterial uptake). Uninfected cells, and yscN, yopB, and yopP mutant-infected cells, were negative for the TUNEL reaction. All strains were tested a minimum of three times.
DISCUSSION

Yersinia secretes and injects into the cytosol of eukaryotic target cells an arsenal of Yop effectors that undermine the host cellular immune response (1). For example, delivery of tyrosine phosphatase YopH into macrophages leads to the inhibition of phagocytic bacterial uptake (28) and oxidative burst (29). In this report, we demonstrate that type III secretion also induces apoptosis in infected macrophages and that a newly identified Yop effector, YopP, is involved in this phenomenon. There was some cell type specificity: Y. enterocolitica did not induce apoptosis in an epithelial cell line. These observations, which suggest that Yersinia can eliminate macrophages without inducing an inflammatory response, complete our understanding of the mechanisms by which Yersinia can proliferate in lymphoid tissues.

There have been reports of Shigella- and Salmonella-induced cytotoxicity in macrophages in which type III secretion is required (18, 30, 31). In the case of Salmonella, no specific effector protein has been identified (30). However for Shigella, it has been shown that IpaB, a secreted protein that is required for invasion of nonphagocytic cells, induces macrophage apoptosis by binding directly to interleukin 1B-converting enzyme (32). To induce apoptosis, Shigella must be internalized by macrophages and have access to the host cell cytoplasm (18). The mechanism by which Yersinia induces apoptosis appears to be different. First, Yersinia induces apoptosis from outside the host cell. Second, the proteins involved are very different. Indeed, IpaB from Shigella shares some similarity to the Yersinia YopB but not to the effector protein YopP (32). In the
case of *Yersinia*, YopB also was required for apoptosis but presumably only indirectly because of its translocating role. Thus, the mechanism by which *Yersinia* induces macrophage apoptosis appears to be quite different from that used by *Shigella*. In contrast, it evokes that used by cytotoxic T lymphocytes, which deliver granzyme B into the cytosol of their target cells thereby inducing apoptosis (33). One of the virulence functions of *Yersinia* appears thus to mimic a physiological process of their host.

Not surprisingly, sequence analysis revealed that YopP is nearly identical to YopJ, its homologue from *Y. pseudotuberculosis* (25). More interesting, YopP (and YopJ) shares a high level of similarity with AvrRxv from *X. campestris*. AvrRxv is one of many avirulence proteins identified in plant pathogenic bacteria that mediates the hypersensitive response, a process that is likely to result from the activation of a programmed cell death pathway (26, 34). It recently has been proposed that AvrBS3, also from *X. campestris*, is translocated into plant cells via the *Xanthomonas* Hrp type III secretion system (35). Additional recent studies have shown that transient expression of avrPto (from plant pathogen *Pseudomonas syringae*) in plant leaf tissue also results in hypersensitive response, suggesting that AvrPto may also be a translocated effector (36, 37). Given that *Yersinia* and *Xanthomonas* share related type III secretion systems and that type III secretion is required for delivery of their respective cell death-inducing proteins, it is likely that the homology between YopP and AvrRxv has functional relevance. Thus animal and plant pathogens not only use related secretion systems to deliver effectors into the cytosol of target cells but the similarity between YopP and AvrRxv indicates that they also deliver related effectors to induce similar answers. AvrBS3 has been shown to require functional nuclear localization signals for eliciting the hypersensitive reaction, suggesting that nuclear factors are involved in AvrBS3 recognition by the plant (35). No such potential nuclear localization motifs were detected in YopP.

In conclusion, we have shown that *Y. enteroxolitica* specifically induces apoptosis in macrophages and that this phenomenon requires type III secretion, Yop translocators, and YopP, which appears to be a novel effector. Most important, YopP shares significant homology with AvrRxv from *X. campestris*. Animal and plant pathogenic bacteria thus share a type III secretion-dependent effector to elicit programmed cell death (apoptosis) in their respective hosts.

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