Expression *in vivo* of additional plasmid-mediated proteins during intestinal infection with *Yersinia enterocolitica* serotype O8

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Summary. The decisive aspect of *Yersinia enterocolitica* virulence *in vivo* is the ability of the plasmid-bearing bacteria to resist the immune response within the host tissue. The expression of plasmid-mediated virulence proteins was investigated in the intestinal lumen and in the Peyer's patches of infected mice. Three novel plasmid-mediated outer-membrane proteins have been identified with antisera raised against bacteria grown *in vivo*. When the bacteria were grown in the intestinal lumen, all plasmid-coded proteins known to be expressed *in vitro*, except the 26-Kda protein were expressed. Additionally, a novel outer-membrane protein of 23 Kda was synthesised. After penetration into the Peyer's patches, two further proteins of 240 and 210 Kda were expressed. None of these three proteins was detected in the outer membrane of bacteria grown *in vitro*. By contrast, plasmid-coded released proteins, which are abundantly synthesised in Ca^{2+} -deficient media *in vitro*, were not detectable in the ileal lumen nor in the tissue of infected Peyer's patches, which suggests that they are not required for *Y. enterocolitica* pathogenesis.

Introduction

Enteropathogenic *Yersinia enterocolitica* strains, as well as virulent strains of *Y. pestis* and *Y. pseudotuberculosis*, harbour a 42–46-Mda plasmid¹ coding for several outer-membrane proteins (YOPs), which are considered to be potential virulence factors.² The plasmid also codes for growth inhibition in Ca^{2+} -deficient medium³ and concomitant release of several proteins—released proteins (RPs) and V antigen^{4,5}—into the medium. Some of these proteins are structurally and immunologically related to YOPs.^{6,7} Experiments with *Yersinia* spp. carrying recombinant plasmid DNA indicate the relevance of some YOPs for pathogenesis. The absence of the proteins YOP20, YOP25 or YOP48 of *Y. enterocolitica* serotype O9 reduced markedly the virulence of this species in mice.^{8,9} Similarly, the elimination of YOP2b or YOP5 from the outer membrane of *Y. pseudotuberculosis*^{10,11} or YOPE, YOPK and YOPL from *Y. pestis*¹² by transposon mutagenesis decreased the virulence of these species in mice.

Most published work on plasmid-coded yersinia outer-membrane proteins has been done with organisms grown *in vitro* in synthetic media. However, environmental factors such as various nutrients, temperature, oxygen and carbon dioxide content can influence the macromolecular composition of *Y. enterocolitica*¹³ and possibly also the YOPs. Recent

evidence obtained by immunohistochemistry, and transmission and scanning electronmicroscopy indicates that the decisive aspect of *Y. enterocolitica* virulence *in vivo* is the ability of these bacteria to survive within the intestinal tissue,^{14–16} i.e., the proteins expressed *in vivo* may be crucial for virulence.

There is little information on the composition of the outer membrane of *Yersinia* spp. in the environment found in the host during infection. Skurnik and Poikonen¹⁷ examined by immunohistochemistry the expression of plasmid-coded outer-membrane proteins of *Y. enterocolitica* serotype O3 in the rat intestinal lumen and tissue. However, neither the expression of single YOPs nor of RPs *in vivo* has been studied in detail. The aim of the present work was to investigate whether proteins are released *in vivo* during *Y. enterocolitica* infection. Furthermore, the expression of plasmid-coded proteins in the lumen of the intestine or in the Peyer's patches was compared with that in bacteria grown *in vitro*.

Materials and methods

Bacterial strains and in-vitro growth conditions

A plasmid-bearing *Y. enterocolitica* strain (NCTC 10938, denoted O8⁺) and an isogenic plasmid-free strain (NCTC 10598, denoted O8⁻) of serotype O8, were obtained from the National Collection of Type Cultures, Colindale Avenue, London. For optimal

expression of RPs and YOPs *in vitro*, *Y. enterocolitica* was grown, in Brain-Heart Infusion Broth (BHB) (Oxoid) overnight at 24°C. This culture was then diluted 1 in 15 with fresh BHB, incubated with shaking at 37°C for 2 h, supplemented with edetic acid and incubated for a further 2 h.

Growth and recovery of Y. enterocolitica from the intestinal lumen

The CD-1 mice (each weighing *c.* 30 g) were starved for 18 h before they were anaesthetised with 150 µl of Ketanest (50 mg/ml) (Parke, Davis and Co., Munich, Germany). The technique of Wolf *et al.*¹⁸ was used for ligation of the ileum. The small intestine was made accessible by midventral laparotomy and a 10-cm loop was prepared in the ileum with string ligature. Ligation was performed without interrupting the blood supply or motility within the ligated part of the small intestine.

Bacteria were grown overnight in BHB at 24°C, pelleted by centrifugation and resuspended in BHB. The suspension (250 µl) containing *c.* 10⁸ organisms, was injected into the ligated ileal segments. The animals were killed after 4 h, and the fluid from the loops was obtained by extrusion and subsequent lavage with 5 ml of sterile saline. The fluid was first centrifuged at 800 *g* for 5 min to remove epithelial cells. Bacteria were then pelleted from the supernate by centrifugation at 6000 *g* for 20 min.

Oral infection of mice

Infection of mice was performed as described previously.¹⁵ For each of four experiments, five male CD-1 mice (age 8 weeks; weight 30 g) were deprived of water for 18 h and then each mouse was given 5 ml of a suspension containing *c.* 5 × 10⁸ bacteria/ml to drink.

Recovery of bacteria from infected Peyer's patches

The mice were killed by cervical dislocation 72–84 h after infection. Peyer's patches from the small intestine were excised, washed twice in sterile saline and homogenised as described previously.¹⁹ The number of bacteria was determined in dilutions of the homogenate by counting the colonies formed on Endo agar. The homogenates of Peyer's patches obtained from five mice contained *c.* 10⁸ cfu. The bacteria were recovered from homogenates by differential centrifugation. After centrifugation at 800 *g* for 5 min at 4°C, intact murine cells and large cell fragments were pelleted. The supernate was centrifuged at 6000 *g* for 20 min and yielded predominantly bacterial sediment.

Preparations of outer membranes and soluble protein fractions

Supernates from the 6000 *g* centrifugation were sterilised by membrane filtration and then centrifuged

at 100 000 *g* for 1 h at 4°C. The soluble protein fraction was precipitated with trichloroacetic acid (TCA, final concentration 10%).

To obtain outer membranes, the bacterial suspension was exposed to five cycles, each of 15 s, of ultrasonication in 15 ml of buffer containing 10 mM Tris-HCl, pH 7.8, 1 mM mercaptoethanol and 5 mM edetic acid, at full power with a Vibra cell (Sonics and Materials Inc., Danbury, USA) as described by Bölin *et al.*²⁰ After centrifugation at 6000 *g* for 30 min at 4°C, membranes remaining in the supernate were sedimented by ultracentrifugation at 100 000 *g* for 1 h at 4°C. The inner bacterial membranes in the pellet were solubilised for 30 min at 4°C with sodium N-lauroylsarcosinate 0.5% and 1 mM mercaptoethanol. After centrifugation at 100 000 *g* for 1 h at 4°C, the sedimented outer membranes were resuspended in distilled water and centrifuged again. Outer membranes and TCA-precipitated soluble proteins were dissolved in sample buffer²¹ and boiled for 5 min.

Preparation of antisera

Rabbit anti-RP antisera and monospecific antisera against single RPs were induced as described previously.⁷ For pre-adsorption of the orally induced (p.o.) rabbit antisera against O8⁺ or O8[−] strains, the antisera were diluted 1 in 50 in phosphate-buffered saline (PBS) and incubated with formalin-fixed, washed bacteria of the O8[−] strain as reported previously.¹⁹ The pre-adsorbed rabbit p.o. antisera reacted with all the plasmid-coded yersinia proteins known to be expressed *in vitro*.¹⁹

Iodination of IgG

Rabbit immunoglobulin G against the whole RP fraction of strain O8⁺ was affinity-purified on Protein A-Sepharose and radio-iodinated by the lactoperoxidase method as described previously.⁷

Autoradiography

For detection of virulence-associated antigens, the solubilised membranes were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on polyacrylamide 11.5% gels and transferred on to PVDF-Immobilon membrane (Millipore). After blocking with bovine serum albumin (BSA) 4% and gelatin 0.1% in PBS for 15 min at 37°C, the membrane was washed with washing buffer (BSA 0.1%, gelatin 0.1% in PBS) and incubated overnight at 4°C with 5 ml of the same buffer containing ¹²⁵I-labelled anti-RP-IgG (*c.* 9.6 × 10⁵ dpm). The membrane was then washed three times with washing buffer, dried, and autoradiographs were produced by overnight exposure, at 70°C, to Hyperfilm®-MP (Amersham Buchler, Braunschweig, Germany) with an X-Omatic intensifying screen (Eastman Kodak Co., Rochester, NY, USA).

Immunoblotting

Immunoblot analysis of virulence-associated proteins separated by SDS-PAGE with 11.5% gels was performed by the method of Towbin *et al.*²² PVDF-membranes with transblotted antigens were blocked as described for autoradiography. Then the membranes were agitated overnight at 4°C with the appropriate dilutions of antisera in BSA 0.1% and gelatin 0.1% in PBS. After washing in PBS-BSA-gelatin, the antibodies were detected with immunogold-labelled goat anti-rabbit immunoglobulin G (diluted 1 in 400) (Auro Probe One, Janssen, Biotech, Olen, Belgium) followed by signal enhancement with silver salt solution (IntenseBL, Janssen, Biotech), as recommended by the manufacturer.

Results

Expression of plasmid-mediated proteins in bacteria grown in the intestinal lumen

The initial interaction between an enteropathogenic bacterium and the host immune system takes place in the intestinal lumen, where the bacteria are suspended in the intestinal fluid or adhere to the mucus layer. The pre-adsorbed, p.o. rabbit antisera reacted specifically with plasmid-coded yersinia proteins known to be expressed *in vitro*.¹⁹ Immunoblotting of outer membranes from bacteria grown *in vivo* with these antisera showed that after growth for 4 h in this environment, yersiniae exhibit distinct alterations of their surface composition. The detection of the YOPs was performed with a pre-absorbed, p.o. antiserum against strain O8⁺. The 26-Kda YOP expressed *in vitro* is repressed during growth in the intestinal lumen. By contrast, a 24-Kda YOP was produced in greater amounts and a further novel YOP of 23 Kda was expressed in the lumen (fig. 1). The 24-Kda polypeptide was not a proteolytic product of the 26-Kda peptide; indeed the monospecific rabbit antiserum against the 24-Kda protein did not cross-react with the 26-Kda peptide.⁷ Prominent protein bands of 110 and 116 Kda present in the plasmid-containing strain grown *in vivo* were also detected in the plasmid-free strain grown under the same conditions i.e. they were chromosomally-coded.

In the yersiniae-free fluid obtained from intestinal loops containing $> 10^8$ bacteria, no RPs were detected after TCA-precipitation and immunoblotting of precipitates. This result indicates that protein release does not occur at all in the intestinal lumen or is largely repressed, because this number of bacteria would produce large amounts of RPs under favourable conditions *in vitro*. The absence of detectable amounts of RPs was not due to adsorption to the tissue nor to complete proteolysis; the RPs are resistant to luminal or tissue proteases and their adsorption to tissue components in homogenates was negligible.

Fig. 1. Immunoblot of the outer-membrane proteins from *Y. enterocolitica* serotype O8 from cultures grown in BHB (1, 2) or in mice intestinal loops (3, 4): —, outer membranes from plasmid-free bacteria; +, outer membranes from plasmid-bearing bacteria. The arrows indicate differences in expression of the plasmid-coded proteins *in vitro* (1, 2) and *in vivo* (3, 4). The positions of corresponding M_r markers are shown on the left of the figure.

Expression of plasmid-mediated membrane proteins in Peyer's patches

The YOPs expressed *in vivo* are the likely components responsible for resistance of yersiniae to the immune reaction of the host. Therefore, outer membranes from bacteria recovered from Peyer's patches were examined. As shown in fig. 2 (track 2) the main plasmid-coded YOPs present *in vitro* were also expressed by the bacteria grown in the tissue of Peyer's patches. As described previously,⁷ the monospecific anti-RP antisera against RP24 and RP56 cross-reacted with the corresponding YOPs (fig. 2, tracks 3 and 5). The monospecific antiserum against RP34 detected an additional 36-Kda YOP on the bacterial membrane (fig. 2, track 4). This protein was not detected on the membranes obtained from the bacteria grown *in vitro* or in the intestinal lumen (fig. 1, tracks 2 and 3), probably because of the large amount of the porin protein in this M_r range.²³ Immunoblotting of the membranes with a pre-adsorbed antiserum against O8⁺ bacteria revealed that, besides the YOPs present in the bacteria grown *in vitro* and the 23-Kda protein expressed in the intestinal lumen, two further novel proteins of 240 and 210 Kda were expressed in bacteria grown within the Peyer's patches (fig. 3, track 3). Because of the inaccessibility of some integral membrane proteins of intact bacteria, the pre-adsorption was never complete. To ensure that the detected

Fig. 2. Immunoblot of outer membranes of *Y. enterocolitica* serotype O8 recovered from infected Peyer's patches with rabbit anti-RP antisera. Tracks: 1, separated RPs from bacteria grown *in vitro* (the lane was overloaded to make the 79-Kda RP visible); 2, outer membranes immunoblotted with the rabbit antiserum against all RPs; 3, 4 and 5, outer membranes immunoblotted with monospecific antisera induced against single RPs of 24, 34 and 56 Kda.

proteins were not the result of incomplete pre-adsorption, the same membranes were immunoblotted with a pre-adsorbed antiserum against O8⁻ bacteria. As shown in fig. 3 (track 4), none of the plasmid-coded proteins recognised by the O8⁺ antiserum, was detected. The 160 Kda protein band in track 3 of fig. 3 was the proteolytic product of the P1 protein.

Investigation of released proteins *in vitro* and *in vivo*

To ensure high sensitivity of detection, ¹²⁵I-labelled anti-RP IgG was used for immunoblotting and autoradiography. The TCA-precipitate of soluble proteins released from 10⁶ bacteria grown *in vitro* and, in parallel samples, the precipitate of the soluble fraction of the Peyer's patches homogenate containing 10⁷ bacteria, were investigated. As shown in fig. 4, large amounts of proteins released *in vitro* were detected (track 1), whereas there was no evidence for protein release *in vivo* (track 2).

Discussion

The present work defines the alterations of *Y. enterocolitica* outer-membrane proteins during growth

of bacteria in the intestinal lumen or the intestinal tissue. These alterations are of particular interest because the effects on the bacteria of growth under different conditions *in vivo* may have profound effects on the host-parasite interaction. As reported recently, yersiniae preferentially enter the intestinal tissue via M cells in the epithelium of Peyer's patches and proliferate beneath the epithelium, from where they spread into the lamina propria.^{14,15}

The molecular mechanisms by which *Y. enterocolitica* attaches to, penetrates, and survives within tissue are unknown. The expression of plasmid-coded outer-membrane proteins plays an important but as yet incompletely defined, role in yersinia infection.^{10,11,24,25} These antigens could be expressed at different stages of infection and thus contribute to bacterial survival in different environments. Indeed, the data presented here indicate an additional expression of three, hitherto undetected, plasmid-mediated proteins, suggesting that the conditions *in vivo* modulate bacterial gene expression. Within 4 h of intraluminal growth, *Y. enterocolitica* expressed a novel 23-Kda protein on its outer membrane. Moreover, the expression of the 24-Kda YOP was strongly enhanced and the expression of the 26-Kda YOP was suppressed. After passage into the Peyer's patches tissue, two additional YOPs of 240 and 210 Kda were expressed. Immunoblot analysis of yersiniae grown in the peritoneal cavity confirmed this result; in the membranes of the bacteria grown in the peritoneal cavity, the same YOPs as those identified in the membrane from bacteria grown in the Peyer's patches were present.²⁶ The 24-Kda protein, which is conserved in all three pathogenic *Yersinia* spp.,²⁷ inhibits the primary host defence.²⁸ Another protein of 36 Kda was detected in membranes of bacteria grown *in vivo* in the tissue of Peyer's patches but not in the intestinal lumen nor *in vitro*. However, it appears that this protein is also present in cells grown *in vitro*, but its detection is prevented by the large amount of the porin protein present in the same M_r range.²³ During growth of bacteria in Peyer's patches, expression of the porin and the "heat-modifiable" protein OmpA is suppressed, thus allowing the detection of the 36 Kda-YOP. The loss of the porin and OmpA proteins in *Y. enterocolitica* serotype O9 under certain conditions *in vitro* was previously described by Cornelis *et al.*²⁹ They showed also that transposon-mutagenised bacteria deficient in YOP40.8 and YOP37.4 express the porin protein and OmpA, which indicates that one or both YOPs have a modifying influence on expression of these proteins. All these alterations are detectable with orally induced pre-adsorbed rabbit antiserum against the O8⁺ strain. None of the three YOPs expressed *in vivo* reacted with the pre-adsorbed O8⁻ antiserum, thus excluding the possibility that they are chromosomally coded, or that they are of host rather than bacterial origin.

In cell lysates of *Y. enterocolitica* serotype O3 grown in peritoneal capsules, Skurnik³⁰ detected several

Fig. 3. Immunoblot of outer membranes of *Y. enterocolitica* serotype O8 from bacteria grown *in vitro* (1, 2, 5, 6) or recovered from infected Peyer's patches (3, 4). The electrophoresed and blotted proteins were detected with different antisera: tracks 1-3, p.o. antiserum against O8⁺ strain, pre-adsorbed with O8⁻ bacteria; 4-6 p.o. antiserum against O8⁻ strain, pre-adsorbed with O8⁻ bacteria; -, outer membranes from plasmid-free bacteria; +, outer membranes from plasmid-bearing bacteria. The arrows indicate the differences in expression of plasmid-coded proteins expressed *in vitro* and *in vivo*. The positions of corresponding M_r markers are shown on the left of the figure.

additional proteins. This was the first indication that gene expression in yersiniae is modulated by the growth conditions *in vivo*. Furthermore, in the intestinal lumen and intestinal tissue the expression of protein P1 was shown by immunohistochemistry with a monoclonal antibody.¹⁷ The present work extends these data by showing the expression of the hitherto known and, additionally, novel outer-membrane proteins in the intestine (summarised in the table). Recently Carniel *et al.*³¹ found several new chromosomally-coded outer-membrane proteins in *Y. enterocolitica* serotype O8 strain 8081 grown in iron-deficient media. The proteins expressed in the intestine and described in the present work are plasmid-mediated, i.e., are different from the proteins detected by Carniel *et al.*³¹.

RPs or V antigen could be detected neither in the intestinal lumen nor in the tissue of the infected Peyer's patches. It is concluded that the release of these antigens is suppressed *in vivo* probably because

of the high Ca²⁺ concentration.¹⁹ Price and Straley³² described recently an avirulent mutant of *Y. pestis* that showed growth restriction and V-antigen secretion also in the presence of calcium. This indicates the importance of proliferation during pathogenesis. Since growth restriction and protein release appear to be strictly correlated, protein release in the Ca²⁺-rich extracellular fluid may impair the survival of the bacteria.

It is possible that the RPs and V antigen could be released within macrophages, where the Ca²⁺-concentration is very low.³³ However, the number of phagocytosed bacteria is small (Hanski *et al.*, unpublished results) and the RPs, if released, were not detectable. This result corresponds to the data of Rosqvist *et al.*,²⁸ who found no expression of plasmid-coded proteins by *Y. pestis* grown intracellularly in HeLa cells.

The antigens identified as unique to bacteria grown *in vivo* might be useful markers for growth conditions

Fig. 4. Autoradiographic detection of TCA-precipitated proteins from soluble fractions of bacteria grown *in vitro* in Ca^{2+} -deficient medium (track 1) or from homogenates of infected Peyer's patches (2). The precipitate applied to track 1 corresponds to 10^6 bacteria, and that in track 2 to 10^7 bacteria.

which adequately mimic the *in-vivo* situation. Our findings that growth of *Y. enterocolitica* in the intestine of mice resulted in the disappearance of some outer-membrane proteins and, at the same time, the appearance of others, indicate that the intestinal milieu determines, at least in part, the composition of the outer membrane. The function of the plasmid-mediated proteins induced *in vivo* and the factors that induce their expression are not known. Also the significance of these novel antigens for bacterial survival *in vivo* remains to be defined.

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Table. Relative molecular masses of the YOPs of *Y. enterocolitica* serotype O8 grown *in vitro* or *in vivo* determined by SDS-PAGE

Protein	M_r (Kda) of proteins from bacteria grown		
	<i>in vitro</i>	<i>in vivo</i>	
		luminal	Peyer's patches
YOP240	ND	ND	240
YOP210	ND	ND	210
P1	180	180	180
YOP79	79	79	79
YOP56	56	56	56
YOP47	47	47	47
YOP45*	45	45	45
YOP42	42	42	42
YOP36	ND	ND	36
YOP34	34	34	34
YOP26	26	ND	ND
YOP24	24	24	24
YOP23	ND	23	23

ND, not detected.

*Subunit of protein P1.

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