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# Pathogenicity of Yersinia: A Strategy for Extracellular Survival and Multiplication

## Abstract

*Yersinia enterocolitica* and *Y. pseudotuberculosis* are enteropathogenic for humans and rodents. After oral uptake, yersiniae invade the Peyer's patches followed by rapid extracellular multiplication and dissemination presumably via blood and lymphatic vessels into spleen, liver, lung and mesenteric lymph nodes.

Plasmid- and chromosomally-encoded factors have been identified enabling yersiniae to resist the primary unspecific host response. The plasmid-encoded yersinia adhesin YadA mediates binding to extracellular matrix proteins, inhibition of serum complement activation and cell adherence. The protein-tyrosine phosphatase YopH suppresses the generation of oxygen radicals by professional phagocytes. To compete with the high-affinity iron binding proteins of the host, *Yersinia* has evolved diverse pathways for ferric iron uptake. The most efficient of those is the yersiniabactin system. For eradication of the pathogen the host has to activate a specific cellular and humoral immune response including IFN-producing specific T-cells and YadA-specific antibodies.

## Introduction

The impact of foodborne intestinal infectious diseases all over the world is well established. Improvement of the hygiene standards and efficient vaccination strategies are required to eradicate enteric pathogens in the environment and to protect humans, children in particular. However such measures demand exact knowledge of the pathogenicity, epidemiology and ecology of these pathogens. Up to now there are only few bacterial microorganisms of which infectious strategies have been at least partially dissected on a molecular level. Amongst these are the three pathogenic species of the genus *Yersinia*, namely *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*. These species are gramnegative rods and belong to the family of *Enterobacteriaceae*.

In 1980 it was recognized that the pathogenicity of yersiniae is controlled by a 70-kilobase (kb) plasmid which is now called pYV (*p*lasmid of *Yersinia v*irulence) (Zink *et al.*, 1980). This virulence plasmid is present in all three pathogenic *Yersinia spp.* and encodes diverse pathogenic factors which will be

discussed later. The sequence similarities between pYV plasmids of the three species are very high (70–90%) (Heesemann *et al.*, 1983; Cornelis, 1994). Interestingly, the agent of bubonic plague, *Y. pestis*, harbours two further plasmids in addition to pYV: pPCP is a 10 kb plasmid encoding pesticin (a bacteriocin) as well as a plasminogen activator which enhances bacterial dissemination after transmission by a flea bite; and pTOX , a 100 kb plasmid encoding a mouse toxin and fraction 1 protein (capsule-forming protein) which is believed to protect *Y. pestis* against phagocytosis.

Chromosomal determinants with proven or putative virulence functions have also been identified in yersiniae. Two proteins with cell adherence and invasion functions have been characterized (Isberg, 1989; Miller *et al.*, 1988). The first one named Invasin (Inv) is an outer membrane protein of about 100 kilodalton (kDa) which is well expressed below 30°C by pathogenic *Y. pseudotuberculosis* and *Y. enterocolitica* strains. Inv triggers the internalization of the attached pathogen after interaction with $\beta$1 integrins on the surface of mammalian cells. The second invasin called Ail, (encoded by the *a*ttachment *i*nvasin *l*ocus) is restricted to enteropathogenic *Y. enterocolitica* strains (Miller *et al.*, 1988). This 20 kDa outer membrane protein has cell adherence functions and enables yersiniae to survive in liquid medium supplemented with 5%–10% human serum. However, there are presently only weak evidences that these two adhesin factors are absolutely required for entry, survival and multiplication in the hosts such as mice.

It is commonly established that enterotoxins produced by enterics are directly involved in the pathogenesis of diarrhea. Accordingly, *Y. enterocolitica* produces a heat-stable enterotoxin (Yst) which is closely related to the known heat-stable enterotoxin of *E. coli* (ST). Yst-negative mutants of *Y. enterocolitica* generated by insertional mutagenesis of the chromosomally located gene *yst* turned out to be attenuated in causing diarrhea in the young rabbit oral infection model (Delor and Cornelis, 1992). Strikingly, the invasiveness of *yst* mutant was hardly affected in this model indicating that other virulence genes control this phenotype. In contrast to *Y. enterocolitica*, *Y. pseudotuberculosis* lacks the *yst* gene. This may explain why *Y. pseudotuberculosis* infections are rarely associated with watery of bloody diarrhea. Instead, the typical clinical manifestation of *Y. pseudotuberculosis* infection is known as mesenteric lymphadenitis.

The pathogenicity of *Y. enterocolitica* was in question for many years because of inconsistencies in mouse virulence. First, it was observed that *Y. enterocolitica* strains of biotype IB (so-called American serotypes 08, 013, 020, 021) lost their mouse virulence potential after repeated subcultivation in the laboratory (Carter, 1975). This phenomenon is ascribed to the selective growth advantage of plasmid-cured derivatives at 37°C. Second, *Y. enterocolitica* strains of European origin, which are of biotype 2 (serotype 09) or of biotype 4 (serotype 03) are not mouse virulent in spite of the presence of pYV (Heesemann *et al.*, 1984). However, if mice were pretreated with ferric iron or the iron chelator desferrioxamine, European serotypes of *Y. enterocolitica* likewise turned out to be mouse virulent (Robins-Browne and Prpic, 1985; Autenrieth *et al.*, 1994;

Boelaert *et al.*, 1987). Obviously, non-biotype 1 B strains of *Y. enterocolitica* lack a sufficient iron-utilization system required for multiplication in host tissue and therefore are of moderate pathogenicity for mice. (This issue will be discussed in the section on iron uptake and virulence of yersiniae).

## Orogastric infection of mice

The mouse virulent *Y. enterocolitica* strains of serotype 08 have been used by several research groups to study the infection process after orogastric challenge of mice (Carter, 1975; Grutzkau *et al.*, 1990; Hanski *et al.*, 1991; Hanski *et al.*, 1989). Within hours after challenge yersiniae preferentially enter the Peyer's patches (PPs) of the small bowel. There are evidences that M-cells are involved in translocation of yersiniae. Carefully performed kinetic studies by Hanski *et al.* revealed that the efficiency of the initial translocation process was similar for both, plasmid-harbouring strains and their plasmidless derivatives (Hanski *et al.*, 1991). However, survival, multiplication and dissemination of translocated yersiniae were controlled by the virulence plasmid pYV. While plasmid-positive strains survived and disseminated into mesenteric lymph nodes, spleen, liver and lung (probably via blood and lymphatic vessels), resulting in the formation of granuloma-like lesions and abscesses, plasmidless yersiniae were rapidly eradicated within the PPs. Carefully performed eletronmicroscopical and immunohistological investigations of infected tissues revealed that yersiniae are located exclusively in the extracellular matrix, surrounded by polymorphonuclear leukocytes, macrophages and lymphocytes (Hanski *et al.*, 1989; Heesemann *et al.*, 1993; Simonet *et al.*, 1990). From these results we conclude that enteropathogenic yersiniae must be well equipped for extracellular survival and multiplication in host tissue.

## Plasmid-encoded virulence functions

The virulence plasmid pYV encodes for outer membrane proteins (e.g. *y*ersinia *a*dhesin, YadA), secreted proteins (*y*ersinia *o*uter *p*roteins: YopE, YopD, YopH, YopM and others), gene regulator proteins (e.g. VirF), proteins involved in the Yop-secretion (lcrD and Ysc-proteins) and a translocation machinery (e.g. YopD). For further details concerning Yop secretion and Yop-gene regulation the reader is referred to the recent review of G. Cornelis, 1994).

Recently it was demonstrated that YopE is translocated into epithelial cells after attachment of yersiniae to the host cell membrane (Rosqvist *et al.*, 1990; Rosqvist *et al.*, 1994). Moreover, there is accumulating evidence that YopD functions as Yop-protein translocator at the bacterial host cell contact point. Presumably, also YopH, YopO and YopM are processed via this polarized translocation pathway. After YopE is translocated into the cytoplasm of the

target cell, cell-rounding and detachment is observed and accompanied by aggregation of actin fibrillae and destruction of the cytoskeleton (cytotoxic effect).

One of the best characterized Yops is YopH. By amino acid sequence comparisonYopH was identified as protein-tyrosine phosphatase (PTPase) (Guan and Dixon, 1990). The PTPase activity of YopH was found to be optimal at about pH 5.0. After infection of the murine macrophage-like cell line J774A.1 with plasmid-positive yersiniae two tyrosine-dephosphorylated proteins of 55 kDa and 120 kDa could be identified (Bliska *et al.*, 1992). We compared the chemiluminescence signal of PMNLs induced by in vitro infection with plasmidless (strain WA-C), plasmid-positive (strain WA-314) and YopH-mutant (strain WA-C (pYV *yopH*)) *Y. enterocolitica* strains, respectively (Ruckdeschel *et al.*, in preparation). Within the first 60 min. after infection WA-C generated a high and WA-314 a low chemiluminescence signal, whereas the signal of YopH mutant was moderate. After two hours of infection the PMNLs were restimulated with opsonized zymosan. A high oxidative burst was observed in case of WA-C- and WA-C (pYV *yopH*), whereas WA-314 infected PMNLs did not respond. These results demonstrate that YopH has the capacity to turn down or to block the generation of superoxide anion radicals. Moreover, the PTPase activity of YopH may also be responsible for blocking the phagocytic capacity of PMNLs and macrophages (Bliska *et al.*, 1992; Rosqvist *et al.*, 1988). There is strong evidence that besides YopH YadA also favours the extracellular location of yersiniae (Heesemann and Laufs, 1985; Heesemann J. and Grüter L. 1987). First, YadA prevents activation of complement by binding of the C3-convertase-inhibitor factor H. Second, YadA covers the surface of the bacterial cell and thus blocks the interaction between Inv and cellular $\beta$1-integrins. Whether YopO (also called YpkA), which has protein-kinase activity,

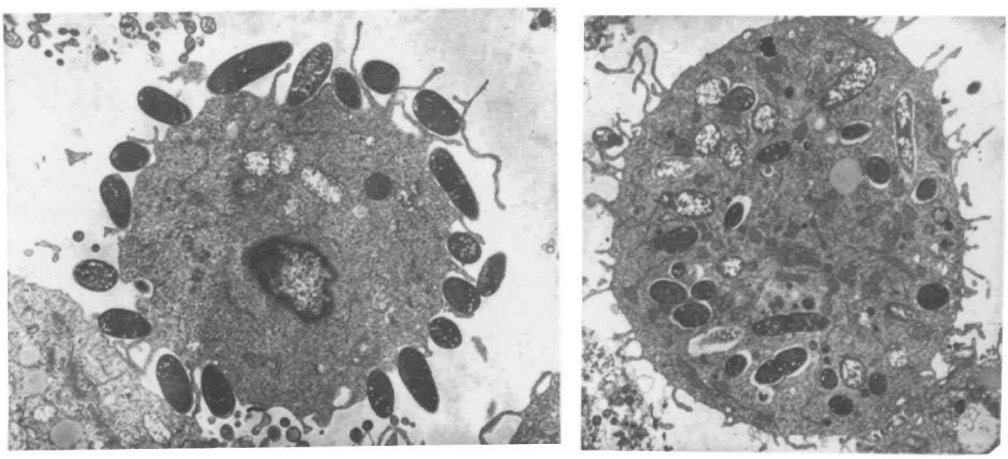

Fig. 1. Transmission electronmicrograph of mouse peritoneal macrophages infected with plasmid-positive *Y. enterocolitica* strain WA-314 (left) and plasmid-negative derivative WA-C (right), respectively.

does also contribute to inhibition of phagocytosis is not yet known (Galyov *et al.*, 1993). Probably, several plasmid-encoded proteins are involved in suppression of phagocytosis (Lian *et al.*, 1987). In Fig. 1 mouse macrophages infected with plasmid-positive and plasmid-negative *Y. enterocolitica*, respectively, are shown to demonstrate the different properties of an isogenic pair.

Finally YopM which seems to be involved in blood coagulation process should be mentioned. Short stretches of YopM exhibit amino acid sequence identity to the platelet receptor GP1bα. Therefore, it is not surprising that YopM binds thrombin and inhibits thrombin-induced platelet aggregation (Leung *et al.*, 1990; Reisner and Straley, 1992). Although the *in vivo* function of YopM is not known, testing of YopM mutant of *Y. pestis* in the mouse model turned out that YopM is required for full virulence expression.

## *Yersinia* adhesin YadA - a multifunctional protein

The first gene of the virulence plasmid pYV which has been cloned and sequenced was *yadA* (Skurnik and Wolf Watz, 1989). *yadA* encodes a protein (formerly called as P1 or Yop-1) which forms multimeric fibrillae on the surface of enteropathogenic yersiniae (Kapperud *et al.*, 1987). The apparent molecular weight as determined by SDS-PAGE depends on the solublization conditions: extraction of YadA from outer membranes at 30°C results in a single broad band of 120 kDa (presumably the native form); extraction at 100°C leads to two bands one oligomeric form of about 200 kDa and one monomeric form of about 50 kDa (as predicted from yadA sequence); treatment of outer membranes with 6 M urea results in the exclusive appearance of the monomeric form (Skurnik and Wolf Watz, 1989; Mack *et al.*, 1994; Tamm *et al.*, 1993). Expression of YadA depends on temperature (37°C) and VirF (Cornelis, 1994).

*yadA* genes of enteropathogenic *Yersinia species* comprise a gene family with common structural elements, conserved domains and strain-specific domains which can be distinguished by polyclonal and monoclonal antibodies (Skurnik and Wolf Watz, 1989; Sory *et al.*, 1990). YadA knock-out mutants of *Y. enterocolitica* are of reduced virulence for mice (increase of the 50% mouse lethal dose, $LD_{50}$) and YadA can thus be considered as a pathogenic factor of *Y. enterocolitica* (Kapperud *et al.*, 1987; Tamm *et al.*, 1993; Roggenkamp *et al.*, 1995). Diverse functions have been ascribed to YadA: (i) YadA mediates autoagglutination of yersiniae at 37°C (Skurnik and Wolf Watz, 1989). This feature may favour formation of microcolonies in the host. (ii) YadA competes with Inv for cell adherence. According to the inverse temperature regulation of *yadA* (37°C) and *inv* (<30°C), and the surface properties of YadA (masking of Inv), YadA appears to be the dominant cell adhesin *in vivo* (Kapperud *et al.*, 1987; Cornelis, 1994; Heesemann J. and Grüter L. 1987). (iii) YadA turned out to have extroordinary capacities to bind various extracellular matrix (ECM) proteins such as collagen type I, II, III, IV, V, IX, XI, cellular fibronectin, and laminin-1 (Emody *et al.*, 1989; Flügel *et al.*, 1994; Schulze Koops *et al.*, 1992;

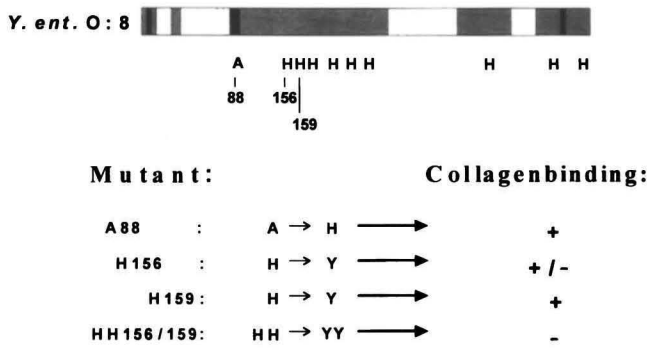


Fig. 2. Schematic drawing of YadA domains and histidyl residues (H); black: hydrophobic domains; halftone: conserved domains; white: variable domains.

Schulze Koops *et al.*, 1993). (iv) Plasmid-positive *Y. enterocolitica* strains are able to survive in 50% human serum (Heesemann *et al.*, 1983; Pilz *et al.*, 1992). It could be demonstrated that YadA inhibits formation of the complement attack complex on the bacterial surface, presumably by binding of the C3-convertase inhibitor factor H (Cornelis, 1994). (V) There are evidences that interaction of YadA with the surface of epithelial cells bypasses the phagocytosis trigger which is mediated by Inv- 1 integrin interaction (Ruckdeschel *et al.*, in preparation).

These multifunctional features of YadA prompted us to dissect functional domains. Using site-directed mutagenesis we succeeded in abolishing the binding activity of YadA for diverse ECM proteins by substitution in YadA of two histidyl residues (His 156/His 159) by tyrosine (Fig. 2). This *yadA* mutant was also unable to adhere to Hep-2 or Hela cells and turned out to be attenuated in mouse virulence, whereas resistance to human serum (complement) and autoagglutination were not affected (Roggenkamp *et al.*, 1995). These results are in contrast to those of Tamm *et al.* who constructed a *yadA* mutant by complete deletion of a hydrophobic domain of 20 amino acids of YadA (Tamm *et al.*, 1993): in this mutant loss of collagen-binding was associated with loss of autoagglutination.

## Iron-uptake and virulence of yersiniae

Transfer of the pYV plasmid from mouse-virulent 08 strain WA-314 (biotype 1 B) to non-mouse virulent 03 or 09 strains (biotype 4 and 3, respectively) did not result in concomitant transfer of the mouse virulent phenotype (Heesemann *et al.*, 1984). This result prompted us to look for chromosomal

virulence determinants. Using the siderophore indicator agar (chrome azurol S agar, CAS) we were able to demonstrate that biotype 1 B *Y. enterocolitica* strains produce siderophores (CAS-positive) in contrast to biotyp 1 A, 2 and 4 strains which were CAS-negative (Heesemann, 1987). Moreover, CAS-positive strains turned out to be highly susceptible to pesticin, a bacteriocin which is produced by *Y. pestis* harbouring pPCP (Heesemann *et al.*, 1993). Previously, it had been described that *Y. enterocolitica* serotype 08 and *Y. pseudotuberculosis* serotype 1 lost their mouse virulence phenotype after selection for pesticin resistance (Une and Brubaker, 1984). From these observations we concluded a link between the pesticin receptor and a putative mouse virulence factor, presumably a siderophor receptor. To verify this hypothesis we introduced a cosmid library of *Y. enterocolitica* serotype 08 into a pesticin-resistant mutant of *Y. enterocolitica 08* and selected the obtained transconjugants for mouse virulence by intraperitoneal infection of mice. By this procedure we obtained transconjugants from mouse tissues which harboured a unique cosmid and regained the pesticin-sensitive phenotype (Rakin *et al.*, 1994). Subcloning and sequencing revealed a Fur-regulated (iron-repressible) open reading frame of 2022 bp which encodes an outer membrane protein of 71 kDa with high homologies to other iron regulated Ton B-dependent outer membrane proteins with siderophore receptor functions. Moreover, we were able to demonstrate that the 71 kDa protein functioned as a receptor both for pesticin and Yersiniabactin and was required for full mouse virulence. Because of this *f*erric *y*ersiniabactin *u*ptake function the 71 kDa protein was called FyuA. Moreover we could demonstrate that pesticin-susceptible bacteria such as *Y. enterocolitica* of serotypes 013, 020 and 021, *Y. pseudotuberculosis* serotype 1 and *Y. pestis* express FyuA. Sequence comparison of *fyuA* genes of diverse *Yersinia spp.* revealed more than 99% sequence homology and two evolutionary lineages of highly pathogenic yersiniae: (i) *Y. enterocolitica* biotype 1 B and (ii) *Y. pseudotuberculosis* serotype 1 and *Y. pestis* (including pesticin-sensitive *E. coli*) (Rakin *et al.*, 1995). Functional FyuA could not be detected in *Y. enterocolitica* of biotype 1 A, 2 and 4 and *Y. pseudotuberculosis* of serotypes 2 and 3 which is consistent with their pesticin-resistant phenotype but not with the mouse-virulence phenotpye of *Y. pseudotuberculosis.* However, we have evidences that certain strains of *Y. pseudotuberculosis*, serotype 3 produce a siderophore distinct from yersiniabactin which mediates mouse virulence (unpublished results).

Presently, there are only few data concerning the structure of yersiniabactin and the determinants for synthesis (Haag *et al.*, 1993; Heesemann *et al.*, 1993). Since mutations in *aroA* and *irp2* (*irp2* is homologous to *entF* of *E. coli*) result in abrogation of yersiniabactin synthesis, we assume a enterochelin-like structure of yersiniabactin (Heesemann *et al.*, 1993; Guilvout *et al.*, 1993).

In conclusion, mouse virulence of *Y. enterocolitica* is closely associated with efficient ferric iron provision mediated by the yersiniabactin determinant *fyu.* However, yersiniae are able to express also other iron uptake systems which may be important for iron provision in diverse ecological niches (Fig. 3). Human

# *Yersinia enterocolitica* biotype 1 B strains

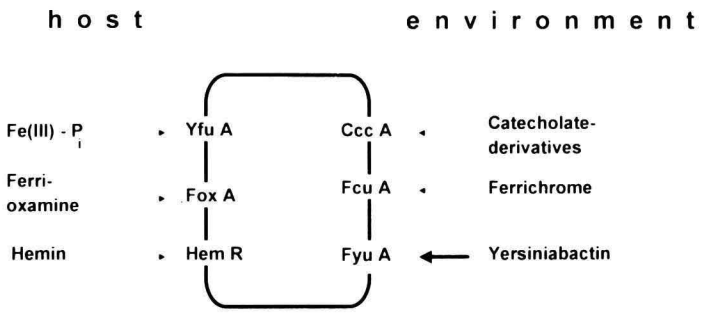


Fig. 3. Iron-uptake systems identified in *Y. enterocolitica* of serotype 08, biotype 1B (mouse-virulent strain). Non-biotype 1B strains are lacking yersiniabactin uptake determinant (fyu).

pathogenic *Y. enterocolitica* express TonB-dependent specific receptors for uptake of ferrichrome (FcuA), ferrioxamines (FoxA) and hemin (HemR) (Bäumler and Hantke, 1992; Bäumler *et al.*, 1993; Stojiljkovic and Hantke, 1992). Moreover, ferric iron bound to polyphosphates can be utilized by the TonB-independent transport system *yfu* (Saken *et al.*, in prepraration). The impact of these transport systems for virulence depends on the ability of *Y. enterocolitica* to use the yersiniabactin uptake system. In case of *fyu*-negative strains (biotype 2 and 4) disruption of *hemR* or *yfuB* results in virulence attenuation whereas corresponding mutants of *fyu*-positive strains (e.g. *Y. enterocolitica 08*) are not affected in virulence.

## The host response against *Y. enterocolitica*

Mice and rats are suitable animal models to analyze the host defence mechanisms against *Yersinia enterocolitica* (Heesemann *et al.*, 1993). During the early phase of infection (up to three to five days after challenge) the unspecific host defence (e.g. complement attack, polymorphonuclear leukocytes) fail to eliminate the invading yersiniae (Autenrieth *et al.*, 1993; Autenrieth *et al.*, 1993). C57Bl/6 mice which are able to recruit sufficiently IFN-g-producing *Yersinia*-specific T-cells are able to eradicate yersiniae efficiently from spleen and liver (*Yersinia*-resistant mice). In contrast, the *Yersinia*-specific T-cell response in BALB/c mice is delayed, weak and IFN-g-production could not be detected before three weeks after infection (*Yersinia*-susceptible mice) (Autenrieth *et al.*, 1994; Autenrieth and Heesemann, 1992; Autenrieth *et al.*, 1993). Recently, the heat shock protein HSP60 could be identified as protective T-cell specific antigen of *Y. enterocolitica 08* (Noll *et al.*, 1994). Besides T-cells, a specific

humoral antibody response can be protective as has been demonstrated by pretreatment of mice with YadA-specific rabbit antiserum (Vogel *et al.*, 1993). Interestingly, YadA-specific antiserum was not protective for strains expressing heterologous YadA (no cross-protection against *Y. enterocolitica* of different serotypes). In summary, *Y. enterocolitica* appears to be well equipped for extracellular survival in host lymphatic tissue and able to resist the unspecific primary host defence reaction. Whether yersiniae can be totally eradicated by an efficient cell-mediated immune response of the host or are still able to evade into protected niches (causing latent persisting infection) has to be elucidated.

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