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Identification of OmpR Protein and its Role in the Invasion Properties of *Yersinia enterocolitica*

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Abstract

Yersinia enterocolitica is a human pathogen that causes gastroenteric infections. Various environmental signals control the expression of the virulence factors in pathogenic *Y. enterocolitica* strains. OmpR, a global transcriptional regulator controls the expression of a wide spectrum of genes, some of which are required for virulence. In this study, we amplified, cloned and sequenced a *Y. enterocolitica* Ye9 *ompR* gene. Deduced amino acid sequence has been shown to have 98% homology to the *Y. enterocolitica* O:8, *Y. pestis*, *S. typhi* and *S. enterica* serovar Typhimurium OmpR proteins. Additional cell culture experiments was performed to investigate whether OmpR takes part in the virulence of *Y. enterocolittica*. We found that the *Y. enterocolitica ompR* mutant was unable to invade HeLa cells. In conclusion, we have shown that OmpR is a very highly conserved protein among enteric bacterial pathogens which plays an important role in the *Y. enterocolitica* virulence.

Key words: *Y. enterocolitica*, invasion, OmpR

Introduction

Yersinia enterocolitica is a gram-negative intestinal rod that causes human diseases generally termed yersinioses. It is a facultative pathogen that is capable of infection and propagation in the host but is also able to grow as a saprophyte. Based on biochemical and genetical differences the *Y. enterocolitica* species has been divided into 40 serotypes and six biogroups. Two types of pathogenic strains of *Y. enterocolitica* have been defined, so called New World strains (serotypes O:8, O:4, O:20, O:13) and Old World strains (O:9, O:3). With respect to mouse virulence and ecology *Y. enterocolitica* New World strains are a high pathogenicity group (LD_{50} 10^2–10^3 compared to10^5–10^6 for European strains, Bottone, 1997). According to16SrRNA sequence analysis these strains belong to two distinct phylogenetic groups. Pathogenesis determinants of *Y. enterocolitica* are coded chromosomally and by plasmid pYV (*Yersinia* virulence) (Cornelis *et al*., 1987; Cornelis *et al*., 1998). In early phases of infection chromosomal proteins responsible for the colonization and invasion of epithelial cells of the intestine are synthesized. These include flagellin, invasin, adhesin, proteins involved in iron metabolism and also toxin Yst that is produced by some strains (Pepe and Miller, 1993).

Pathogenic bacteria residing in various ecological niches are constantly threatened by changes of physical and chemical environmental factors, such as osmolarity, pH, accessibility of nutrients, light intensity, viscosity of medium, *etc.* (Straley and Perry, 1995). The adaptation of bacteria to new conditions involves both rapid changes, *e.g.* of cell motility, as well as prolonged, global reorganization of gene expression. The mechanisms of molecular responses to signals from the outer environment are complex and depend, among others, on two-component regulatory systems (Albright *et al*., 1989; Barrett and Hoch, 1998).

In two-component regulatory systems transmission of the signal occurs through a pair of proteins that communicate with each other *via* the conserved mechanism of phosphorylation (Stock *et al*., 1989; Bourret *et al*., 1991). The regulatory system OmpR-EnvZ participates in the bacterial response to changes in the

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osmolarity of the external environment (Russo and Silhavy, 1991). It has best been studied in *Escherichia coli*, but is also found in other pathogens such as *Salmonella* or *Shigella* (Miller *et al*., 1989). The *ompR* and *envZ* genes of *E. coli* form part of the *ompB* operon. They code for the regulatory protein OmpR and the sensor protein EnvZ, respectively. They are involved in the osmoregulation of the transcription of the porin proteins OmpF and OmpC (Hall and Silhavy, 1981). The functions of protein OmpR include both the positive and negative regulation of the transcription of both proteins. OmpR of *E. coli* is a cytoplasmic protein (~28 kDa) consisting of 239 amino acid residues. A modulator domain in the N-terminal part of the protein and a DNA-binding effector domain, located in the C-terminal part, can be distinguished. The DNA-binding domain is capable of interacting with the promotor regions of genes, whereas the regulator domain is responsible for interactions with EnvZ and RNA polymerase (Itou and Tanaka, 2001).

It has been proven that the two-component system OmpR/EnvZ plays an important role in controlling of the virulence of such enteric pathogens as *Shigella* and *Salmonella.* It has been found that in *S. flexneri* the deletion of a operon *ompB* that codes for the proteins OmpR-EnvZ, significantly reduces the ability of this bacterium to proliferate in epithelial cells and destroy them (Bernardini *et al*., 1990). It is also known that in *Salmonella* there is also a dependence between virulence and the two-component system OmpR-EnvZ (Dorman *et al*., 1989). Mutations in the regulator gene *ompR* have been shown to alter the pathogenicity of *S. enterica* serovar Typhimurium. Mutants of the virulent strain were attenuated *in vivo*. Moreover, it has been proven that *S. enterica* with mutations in the gene *ompR* are not able to infect murine cells and to induce the apoptosis of macrophages *in vitro* (Lindgren *et al*., 1996).

We have previously demonstrated that the *Y. enterocolitica* Ye9 OmpR protein is involved in controlling the production of Yop virulence proteins and in the adaptation of the bacteria to multiple stresses. Furthermore, a *ompR* deletion mutant was impaired in survival and replication within macrophages (Brzostek *et al*., 2003).

In this study, we performed a HeLa cell virulence assay and analyzed the predicted amino acid sequence of the *Y. enterocolitica* Ye9 OmpR protein.

Experimental

Material and Methods

Bacterial strains, plasmids and growth conditions. *Y. enterocolitica* Ye9 is a serotype O:9 strain from the collection of the State Institute of Hygiene, Warsaw. The bacterial strains and plasmids used in this study are listed in Tables I and II.

Bacteria were routinely grown in brain heart infusion (BHI) and Luria Bertani (LB) medium. Strains of *Y. enterocolitica* were cultivated with shaking at 25°C whereas strains of *E. coli* were grown at 37°C. The antibiotics used for the selection procedures were ampicillin (Ap, 200 µg/ml) and kanamycin (Km, 50 µg/ml).

DNA manipulation and PCR conditions. The entire open reading frame of *ompR* gene of *Y. enterocolitica* Ye9 (0.720 kb) was obtained by the PCR amplification which was performed in an automated thermal cycler (MJ Research, Inc.) with *Taq*I

Table I
Bacterial strains used in this study

Strain	Comments	Source or Reference
Y. enterocolitica Ye9	serotype O:9 wild-type strain, pYVO9$^+$	State Institute of Hygiene, Warsaw
Y. enterocolitica AR4	serotype O:9 *ompR* mutant, pYVO9$^+$, Km^R	(Brzostek *et al*., 2003)
E. coli Top10F’	F’:{*lacI*^{q} Tn10 (Tet^R)}, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), Φ80Δ*lacZ*Δ*M15*, Δ*lacX74*, *deoR*, *recA1*, *araD139*, Δ(*ara-leu*), 7697*galU*, *galK*, *rpsL* (Str^R), *endA1*, *nupG*	Invitrogen

Table II
Plasmids used in this study

Plasmid	Comments	Source or Reference
pBluescript II SK(+)	cloning vector, Ap^R	Stratagene
pYR9	pBluescript II SK(+) with 0.720 kb *ompR* gene of *Y. enterocolitica* Ye9 (from start to stop codon of translation)	This study

DNA polymerase (Qiagen). The initial denaturation step (94°C, 5 min) was then followed by 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 2 min). The oligonucleotide primers used for PCR, forward pR1Bam (5'-CGCGGATCCATGCAAGAGAATCACAAGATTC-3') and reverse pR2Sma (5'-TCCCCCGGGTCATGCTTTACTGCCGTCCGG-3'), were based on the known *ompR* sequence data for *Y. enterocolitica* serotype O:8. The *ompR* PCR product (0.720 kb) was cloned into the pBluescript II SK(+) vector in the *Bam*HI/*Sma*I sites. The nucleotide sequence of *ompR* gene was determined using the universal primers of pBluescript II SK(+) (T3 and T7) and the ABI Prism BigDye terminator Cycle Sequencing System (Perkin-Elmer). This was subsequently read on an ABI Prism 377 DNA Sequencer (DNA Sequencing and Oligonucleotide Synthesis Laboratory, IBB PAS). The *ompR* sequence was analyzed using the Wisconsin Sequence Analysis Package (GCG, Madison, Wis.). The OmpR alignments were generated with program BESTFIT and PILEUP.

HeLa cell infection. HeLa cells were cultured in 24-mm-diameter plastic wells containing minimal Eagle's medium (MEM, Gibco BRL) supplemented with 5% heat-inactivated fetal bovine serum and 2 mM glutamine. HeLa cells were cultured until almost confluent and then were infected with the bacterial suspension at a multiplicity of 10. Cells were incubated at 37°C in a 5% CO_2 atmosphere with saturated humidity for 1 h. After this infection period, 100 µg of gentamicin per ml was added and then the plates were incubated for 2 h under the same conditions to kill adherent extracellular bacteria. After this period gentamicin was removed by two washes with PBS. Tissue culture cells were then lysed with 0.2 ml of 0.1% Triton X-100 to release intracellular bacteria. After 5 min, 0.8 ml of LB medium was added. The suspension was then diluted and plated on LB agar to determine viable counts. Viable counts of the initial bacterial culture were also determined.

Results and Discussion

A variety of studies indicate that OmpR protein is required in *E. coli* for the osmodependent transcriptional regulation of *ompC* and *ompF* gene expression (Hall and Silhavy, 1981) but also plays an essential role in controlling flagellar expression (Shin and Park, 1995), cell division (Pruss, 1998), fatty acid transport (Higashitani *et al*., 1993) and acid tolerance response (Bang *et al*., 2002). It needs to be emphasized that OmpR protein is involved in the regulation of genes associated with the virulence of pathogenic bacteria like *Salmonella* or *Shigella* (Dorman *et al*., 1989; Bernardini *et al*., 1990; Chatfield *et al*., 1991; Lindgren *et al*., 1996; Lee *et al*., 2000). In view of these findings, the general regulator OmpR protein may contribute to the virulence of *Y. enterocolitica* Ye9. In this study, we contributed to knowledge about the role of OmpR in *Y. enterocolitica* Ye9 pathogenesis.

The *Y. enterocolitica* Ye9 *ompR* gene was amplified by PCR with oligonucleotides pR1Bam and pR2Sma, designed from the *ompR* sequence of *Y. enterocolitica* serotype O:8. The primers pR1Bam and pR2Sma corresponded to nucleotides 1–22 of the sense strand and 720–699 of the antisense strand, respectively. This PCR product was then purified from the primers used for amplification, cloned into the pBluescript II SK (+) and sequenced. Sequence analysis using the Wisconsin Sequence Analysis package (GCG, Madison, Wis) indicated that the *ompR* gene encodes a protein of 239 amino acid residues. The predicted amino acid sequence was very similar to that of OmpR from other pathogens. OmpR has two distinctive domains: the receiver domain at the N-terminal portion (residue 1–125), and the C-terminal DNA-binding domain (residues 137–239). Analysis of OmpR predicted amino acid sequence has shown that observed 10 amino acid substitution in the protein sequence among chosen enteric bacterial pathogens did not affect function of OmpR (Fig. 1). The deduced amino acid sequence was 98% identical to OmpR of *Y. enterocolitica* O:8, *Y. pestis*, *S. typhi* and *S. enterica* serovar Typhimurium. This comparison revealed extensive amino acid identity, which confirmed the highly conserved structure of the OmpR regulator protein.

The nucleotide sequence of the *Y. enterocolitica* Ye9 *ompR* gene has been submitted to the Gene Bank NCBI data base under accession number AY210888.

A recent study has shown that the pleiotropic regulator OmpR controlling the coordinate expression of virulence determinants is required for bacteria to survive in the foreign host. It has been previously reported that *ompR* mutants of *S. enterica* serovar Typhimurium were avirulent in a mouse model and did not kill macrophages *in vitro* (Dorman *et al*., 1989, Chatfield *et al*., 1991; Lindgren *et al*., 1996). In *S. typhi* OmpR-EnvZ regulatory system was involved in the regulation of biosynthesis of the Vi polysaccharide capsular antigen which is associated with the virulence (Pickard *et al*., 1994). Furthermore, virulence of the *S. flexneri ompR* mutant was significantly decreased as a result of its inability to invade ephitelial cells (Bernardini *et al*., 1990).

The ability of pathogenic *Y. enterocolitica* strains to invade HeLa cells is an important correlate of virulence (Lee *et al*., 1977). The pattern of invasion of the *Y. enterocolitica* Ye9 was compared with the AR4 strain, *ompR* mutant, which was constructed in the previous study by allelic exchange (Brzostek *et al*., 2003). The data have shown that the *Y. enterocolitica ompR* mutant invaded HeLa cells relatively poorly, at the levels of about 0.02% of the original recovered inoculum (Fig. 2). In contrast, the control strain, Ye9,

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      1                                                    50
 St   mqen y kilvvdddmrlrallerylteqgfqvrsva n aeqmdrlltresfh
Stm   mqen y kilvvdddmrlrallerylteqgfqvrsva n aeqmdrlltresfh
 Yp   mqen h kilvvdddmrlrallerylteqgfqvrsva n aeqmdrlltresfh
Ye8   MQEN H KILVVDDDMRLRALLERYLTEQGFQVRSVA N AEQMDRLLTRESFH
Ye9   MQEN H KILVVDDDMRLRALLERYLTEQGFQVRSVA D AEQMDRLLTRESFH
      .... * .............................. * ..............

      51                                                100
 St   lmvldlmlpgedglsicrrlrsqsnpmpiimvtakgeevdrivgleigad
Stm   lmvldlmlpgedglsicrrlrsqsnpmpiimvtakgeevdrivgleigad
 Yp   lmvldlmlpgedglsicrrlrsqsnpmpiimvtakgeevdrivgleigad
Ye8   LMVLDLMLPGEDGLSICRRLRSQSNPMPIIMVTAKGEEVDRIVGLEIGAD
Ye9   LMVLDLMLPGEDGLSICRRLRSQSNPMPIIMVTAKGEEVDRIVGLEIGAD
      ..................................................

      101                                                150
 St   dyipkpfnprellarir a vlrrqanelpgapsqee av iafgkfklnlgtr
Stm   dyipkpfnprellarir p vlrrqanelpgapsqee av iafgkfklnlgtr
 Yp   dyipkpfnprellarir a vlrrqanelpgapsqee ai iafgkfklnlgtr
Ye8   DYIPKPFNPRELLARIR A VLRRQANELPGAPSQEE TV IAFGKFKLNLGTR
Ye9   DYIPKPFNPRELLARIR A VLRRQANELPGAPSQEE AV IAFGKFKLNLGTR
      ................. * ................. ** .............

      151                                                     200
 St   em f redepmpltsgefavlkalvshpreplsrd n l m nlargreysamers
Stm   em f redepmpltsgefavlkalvshpreplsrd k l m nlargreysamers
 Yp   em f redepmpltsgefavlkalvshpreplsrd k l m nlargreysamers
Ye8   EM F REDEPMPLTSGEFAVLKALVSHPREPLSRD K L M NLARGREYSAMERS
Ye9   EM S REDEPMPLTSGEFAVLKALVSHPREPLSRD K L I NLARGREYSAMERS
      .. * ............................. * . * ..............

      201                                   239
 St   idvqisrlrrmveed p ahpryiqtvwglgyvfvpdg s ka
Stm   idvqisrlrrmveed p ahpryiqtvwglgyvfvpdg s ka
 Yp   idvqisrlrrmveed p ahpryiqtvwglgyvfvpdg n ka
Ye8   IDVQISRLRRMVEED T AHPRYIQTVWGLGYVFVPDG S KA
Ye9   IDVQISRLRRMVEED P AHPRYIQTVWGLGYVFVPDG S KA
      ............... * .................... * ..
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Fig. 1. Amino acid sequence alignment of the *Y. enterocolitica* Ye9 OmpR with *Y. enterocolitica* serotype O:8 (Ye8), *Y. pestis* (Yp), *S. enterica* serovar Typhimurium (Stm), *S. typhi* (St) homologs.
Protein sequences were aligned by the PILEUP program (Genetics Computer Group).
Changes in amino acids are indicated in frames and by stars.

exhibited levels of invasion of 1%. All results are expressed as % invasion = 100 x (number of bacteria resistant to gentamicin/number of bacteria added).

These experiments indicate that the AR4 strain (the *ompR* mutant) is weakly infective in comparison to wild type strain. It has been previously reported that *ompB* mutant of *S. flexneri* was impaired in intercellular spread and multiplied within the initially invaded HeLa cells (Bernardini *et al.*, 1993). In addition other authors demonstrated that mutation in *ompR* and *envZ* genes of *S. enterica* serovar Typhimurium rendered these strains highly reduced for the induction of Sif formation during infection of HeLa cells (Mills *et al.*, 1998). Sifs are tubular structures, which are somehow involved in bacteria's ability to acquire nutrients and to replicate intracellularly. With regard to the absence of these specific structures in other known enteropathogenic bacteria, including *Yersinia*, we suppose that the global regulator, OmpR, could be involved in coordinating gene expression upon entry into the host cells in *Y. enterocolitca* Ye9. We also cannot exclude that genes submitted to OmpR regulation like *ompC* and *ompF* are involved in *Y. enterocolitica* virulence. It has been previously reported in *Escherichia*, *Shigella* and *Salmonella* that in the absence of OmpR protein neither porin is expressed (Russo and Silhavy, 1991; Bernardini *et al.*, 1990; Dorman *et al.*, 1989). It is possible that the lack of the major outer membrane proteins OmpC or OmpF in *Y. enterocolitica ompR* mutant (AR4) could cause a reduction of virulence. For example, in *S. enterica*

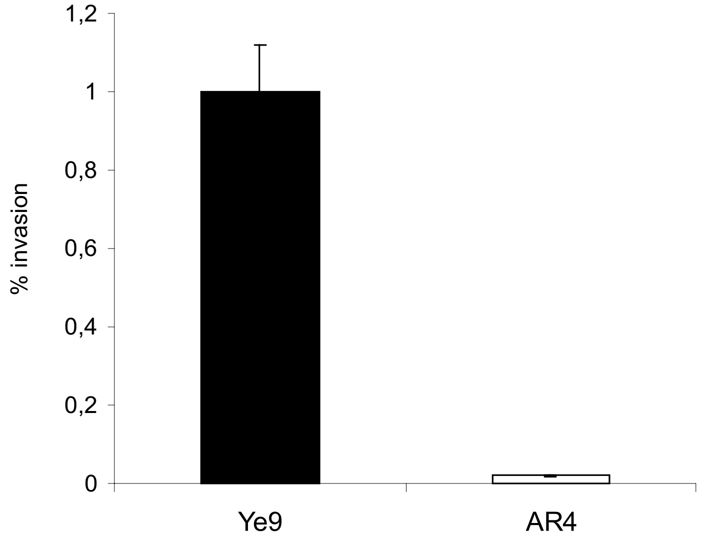

Fig. 2. Invasion of the wild-type strain Ye9 and the *ompR* mutant AR4 of *Y. enterocolitica* in HeLa cells.
Results shown are the means of duplicate determinations in two separate experiments with standard errors. ■ – Ye9 (wild type strain), □ – AR4 (the *ompR* mutant).

serovar Typhimurium OmpC was a candidate ligand that potentially participates in host-cell recognition of bacteria by phagocytic cells (Negm and Pistole, 1999). On the contrary, in *S. flexneri* OmpC was involved in spreading from cell to cell and killing epithelial cells during infection (Bernardini *et al*., 1993).

To help to clarify the nature of the *Y. enterocolitica* virulence network that is under the control of *ompR* gene we have yet to carry out further studies.

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