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Molecular analysis of a novel Toll/interleukin-1 receptor (TIR)-domain containing virulence protein of *Y. pseudotuberculosis* among Far East scarlet-like fever serotype I strains

Dominik Nörenberg, Andreas Wieser, Giuseppe Magistro, Christiane Hoffmann, Christian Meyer, Maxim Messerer, Sören Schubert*

Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, Marchioninistr. 17, D-81377 München, Germany

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ABSTRACT

Pathogenicity of *Yersinia pseudotuberculosis* is determined by an arsenal of virulence factors. Particularly, the *Yersinia* outer proteins (Yops) and the Type III secretion system (T3SS) encoded on the pYV virulence plasmid are required for *Yersinia* pathogenicity. A specific group of *Y. pseudotuberculosis*, responsible for the clinical syndrome described as Far East scarlet-like fever (FESLF), is known to have an altered virulence gene cluster. Far East strains cause unique clinical symptoms for which the pYV virulence plasmid plays apparently a rather secondary role. Here, we characterize a previously unknown protein of *Y. pseudotuberculosis* serotype I strains (TcpYI) which can be found particularly among the FESLF strain group. The TcpYI protein shares considerable sequence homology to members of the Toll/IL-1 receptor family. Bacterial TIR domain containing proteins (Tcps) interact with the innate immune system by TIR-TIR interactions and subvert host defenses via individual, multifaceted mechanisms. In terms of virulence, it appears that the TcpYI protein of *Y. pseudotuberculosis* displays its own virulence phenotype compared to the previously characterized bacterial Tcps. Our results clearly demonstrate that TcpYI increases the intracellular survival of the respective strains in vitro. Furthermore, we show here that the intracellular survival benefit of the wild-type strain correlates with an increase in *tcpYI* gene expression inside murine macrophages. In support of this, we found that TcpYI enhances the survival inside the spleens of mice in a mouse model of peritonitis. Our results may point toward involvement of the TcpYI protein in inhibition of phagocytosis, particularly in distinct *Y. pseudotuberculosis* strains of the FESLF strain group where the pYV virulence plasmid is absent.

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Introduction

The genus *Yersinia* contains three causative agents of human disease which differ in virulence and individual infection pathways. *Y. pestis*, the causative agent of plague, is transmitted through the bite of an infected flea, whereas *Y. pseudotuberculosis* and *Y. enterocolitica* cause lymphadenitis, septicemia, and gastrointestinal syndromes as food-born pathogens by fecal-oral transmission (Cornelis et al., 1998). Nevertheless, all three *Yersinia* spp. survive and multiply in lymphoid tissues and pathogenicity is connected to interaction with the host immune system (Cornelis and Wolf Watz, 1997). Subversion of innate immune signaling of *Yersiniae*, as a facultative intracellular pathogen, is linked to their ability to survive and replicate in innate immune cells and leads to increased pathogenicity (Pujol and Bliska, 2003, 2005). In general, *Yersinia* strains produce several chromosomal encoded virulence factors such as invasin, which is important for attachment and penetration of the intestinal barrier (Isberg and Falkow, 1985; Isberg and Leong, 1988; Miller et al., 1988). Additionally, important virulence factors contributing to *Yersinia* pathogenicity such as YadA, the type III secretion system and *Yersinia* outer proteins (Yops) are encoded on the pYV virulence plasmid (Cornelis and Wolf Watz, 1997; Biot and Cornelis, 1988). Furthermore, it is known that most of the pYV-related virulence factors are secreted extracellularly or transmitted via the type III secretion apparatus directly into the cytoplasm of innate immune cells (Heesemann, 1990; Simonet et al., 1990; Hanski et al., 1991). Several studies have shown that the pYV encoded "Yop regulon" is responsible for immune evasive mechanisms and inhibition of phagocytosis (Cornelis and Wolf Watz, 1997; Rosqvist et al., 1988). Notably, subsets of *Y. pseudotuberculosis* strains cause a clinical syndrome known as the so-called Far East scarlet-like fever (FESLF) (Somov, 1976). FESLF is characterized by high fever, gastrointestinal symptoms, erythematous skin lesions, a toxic shock-like syndrome and accompanied eponymously by a

* Corresponding author. Tel.: +49 89 2180 78 202; fax: +49 89 2180 78 294.
E-mail address: schubert@med.uni-muenchen.de (S. Schubert).

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scarlet-like rash (Eppinger et al., 2007; Somov, 1976). In terms of virulence, Far East strains are known to have an altered gene cluster, especially lacking the *ybt* gene operon in a large number of isolates (Rakin et al., 2012). Moreover, Far East strains possess an additional virulence plasmid called pVM82 which is responsible for the unique Far East scarlet-like fever symptoms (Shubin et al., 1985). Interestingly, Far East strains are partially known to lack the pYV virulence plasmid which does not seem to be mandatory in these strains, yet they show a highly virulent phenotype and unique clinical manifestations (Eppinger et al., 2007).

Recently, certain bacterial virulence factors among various bacterial species shifted into the center of interest. A number of studies have shown that immune evasion is triggered in host cells in response to infection by bacterial strains expressing TIR domain containing proteins (Tcps) which belong to the Toll/IL-1 receptor family (O'Neill, 2008a,b). Particularly, these virulence factors affect the host immune response and subvert innate immune signaling each in a different manner. Rana et al. summarized recently characterized bacterial TIR proteins and their different strategies to interact with the innate immune system (Rana et al., 2013). Innate immune evasion via bacterial Tcps is caused by a variety of mechanisms including inhibition of host cell proteins which are important in TLR signaling. On this occasion, the TIR domain containing proteins of *E. coli* (TcpC) (Cirl et al., 2008), tlpA of *S. enterica* (Newman et al., 2006), YpTdp of *Y. pestis* (Rana et al., 2011) and PdTLP of *P. denitrificans* (Low et al., 2007) are sufficient to impair MyD88 signaling via their individual, multifaceted TIR-TIR interactions. In contrast, TcpB of *B. melitensis* impairs TLR signaling by targeting the human Toll/IL-1 receptor family adaptor protein MAL (Radhakrishnan et al., 2009), thus also undergoing innate immune signaling (Cirl et al., 2008). Even though all bacterial TIR proteins share homologies in their amino acid sequence, it appears that there is no uniform mechanism of how bacterial TIR proteins subvert host defenses and how they interact with the innate immune system in general (Rana et al., 2013). Furthermore, it is known that TIR domain containing proteins take over manifold functions via protein–protein interactions beyond the TLR signaling cascade in a variety of bacteria, fungi, archaea and viruses (Spear et al., 2009).

In this study, we demonstrate that a previously uncharacterized TIR domain containing protein of the *Y. pseudotuberculosis* serotype I strain 488, namely TcpYI, promotes *Yersinia* pathogenicity in vitro and in vivo. The TcpYI protein was identified within the serotype I strain group among isolates of patients suffering from Far East scarlet like fever. Our data proves that TcpYI contributes to virulence specifically without involvement of the pYV virulence plasmid since it is absent in our strains. In murine J774.1 macrophages, the respective *Y. pseudotuberculosis* serotype I 488 wild-type strain shows clear manifestations of an improved intracellular survival and furthermore, an increased survival in the spleens of mice in vivo. By contrast, the *Y. pseudotuberculosis* serotype I 488 mutant strain, that is deficient for the full-length TcpYI protein, shows a reduced virulence phenotype in infected macrophages and in the mouse model of peritonitis, respectively. The ability to survive in macrophages and within the spleens of mice is restored in this mutant by expressing TcpYI on a low-copy plasmid under the control of its native promoter.

Materials and methods

Bioinformatics, software and statistical analysis

The open reading frame YpsIP31758_3704 of the *Y. pseudotuberculosis* serotype I strain IP 31758 (Eppinger et al., 2007) was designated as *tcpYI* gene. The ORF YPK_3142 was found in the *Y. pseudotuberculosis* serotype III strain (YPIII) and was named *tcpYIII*. Both open reading frames encode for the putative TIR domain containing proteins TcpYI and TcpYIII. Identification of both TIR proteins was based on sequence alignments of the amino acid sequence of TcpC as described by Cirl and Miethke (2010). An internal nucleotide database of whole genome sequenced Far East scarlet-like fever (FESLF) isolates of *Y. pseudotuberculosis* serotype I strains (Rakin et al., 2012) was screened for the presence of the *tcpYI* gene. The isolated TIR domain of the TcpYI protein (TIR-TcpYI) was used for a cascade of BLASTp searches on NCBI (http://ncbi.nig.gov). Here, TIR-TcpYI homologous bacterial TIR-domain containing proteins (Tcps) were identified. The phylogenetic relationship of TIR-TcpYI, multiple sequence alignment of various bacterial and human TIR proteins and the map of the putative TIR island of the *Y. pseudotuberculosis* serotype I 488 strain were created with DNASTAR Lasergene Core Suite Software 8.0 (DNASTAR, Inc., Madison, USA) including SeqBuilder, MegAlign, Protean and EditSeq. The data of this study was collectively analyzed with the help of the statistics program SigmaStat Version 3.5 (Systat Software, Inc. Richmond, CA). All experiments were performed at least 3 independent times. Statistical analysis was carried out using paired Student's t test and $P < 0.05$ was considered significant.

Bacterial strains and growth curves

Bacterial strains and plasmids used in the present work are listed in Table 1. All *Y. pseudotuberculosis* strains used in this study were obtained as clinical isolates from patients who suffered from Far East scarlet-like fever (FESLF) and do not carry the pYV virulence plasmid. The *Y. pseudotuberculosis* serotype I strain 488 as wild-type strain, *tcpYI* deletion mutant strain and plasmidal recomplemented strain harboring pWKS30-pTcpYI were used for infection experiments. The *Escherichia coli* BL-21 strain (Stratagene) was used for subsequent cloning, amplification of the ligated vectors and recombinant protein expression. *Yersinia* strains were grown at 30 °C and *E. coli* strains at 37 °C under aerobic conditions in standard Luria-Bertani (LB) broth unless otherwise indicated. Antibiotics were supplemented as necessary (ampicillin 100 μg/ml, chloramphenicol 20 μg/ml). For growth curves, *Yersinia* strains were grown to stationary phase overnight, sub-cultured to a defined OD_{600nm} of 0.01 in LB broth and incubated aerobically at 30 °C with mild agitation (175 rpm). The incubated samples were subject to analysis every 30 min up to 9 h by measuring the OD_{600nm}. For infection experiments, *Yersinia* strains were grown to an OD_{600nm} of 0.5 and washed twice in phosphate buffered saline (PBS). The absence of the pYV virulence plasmid was confirmed by PCR for the pYV encoded *yadA* gene (el Tahir and Skurnik, 2001).

PCR screening, construction of isogenic deletion mutants and recomplementation of tcpYI

Standard genetic methods were performed mainly as described by Sambrook and Russell (Sambrook et al., 1989). Primers used for the present work are listed in Table 3. Enzymes for cloning experiments were purchased from Fermentas (Fermentas, St. Leon-Rot, Germany) and were used according to the manufacturer's recommendations. The genetic knockout mutant of *tcpYI* was generated by using the lambda red recombinase model described by Datsenko and Wanner (2000). The *tcpYI* gene was deleted and replaced by a chloramphenicol resistance gene (Cm). Briefly, primers were designed consisting of 40-nt homology extensions to the 5′ and 3′ regions of the gene of interest and 20-nt priming sequences for the template plasmid pKD3 carrying the resistance cassette flanked by FRT recognition target sites. The resulting PCR product was gel purified and electroporated into the *Y. pseudotuberculosis* serotype I 488 wild-type strain carrying the heat labile helper

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