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SHORT COMMUNICATION

Characterisation of Yersinia Secretion Apparatus – Pathogenicity Island (Ysa-PI) of *Yersinia enterocolitica* 1B/O8 in Poland: an Idle Ysa is a Specific Hallmark of the Epidemic Sensu Stricto Strain

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Abstract

Yersinia secretion apparatus (Ysa), the chromosomal type three secretion system (T3SS) is considered to contribute to virulence of high-pathogenicity Yersina *enterocolitica* biovar 1B. DNA-sequence of Ysa pathogenicity island was determined for clinical isolate DM0110 of *Y. enterocolitica* 1B/O8 with origin in Poland. We found a premature stop-codon in the regulatory gene *ysrR* (mutation at position 269). Altered *ysrR* was detected in all tested 78 isolates of *Y. enterocolitica* 1B/O8 collected from clinical samples in Poland from 2004 to 2013. Since aberrations in YsrR are considered to inactivate Ysa, our findings may suggest Ysa is not indispensable for *Y. enterocolitica* 1B/O8 to infect humans.

Key words: *Y. enterocolitica* 1B/O8, T3SS, virulence, Ysa, Ysa-PI

Yersina enterocolitica is a causative agent of gastrointestinal disorders in humans with a variety of clinical manifestations, including reactive arthritis and erythema nodosum (Bottone, 1997). Pathogenic strains of *Y. enterocolitica* harbour a suit of virulence factors encoded on virulence plasmid pYV and the chromosome. Strains of bioserotype 1B/O8 are considered lethal to mouse and highly pathogenic to humans (Aulisio *et al*., 1983). *Y. enterocolitica* 1B/O8 was originally reported in northern America in the 70s and 80s of the past century. In the early 90s bioserotype 1B/O8 disappeared in America and emerged in Japan (Ichinohe *et al*., 1991). The first case of human *Y. enterocolitica* 1B/O8 infection in Europe was reported in Germany (Schubert *et al*., 2003). However, this bacterium is nowadays most numerously isolated in Poland, where 224 human clinical isolates were collected from 2004 to 2013. *Y. enterocolitica* 1B/O8 isolates collected were tightly clonal (Gierczyński *et al*., 2009; Zacharczuk, 2012). The bacterium is an important causative agent of yersiniosis in Poland as shown by serological studies (Rastawicki *et al*., 2013).

High pathogenicity of *Y. enterocolitica* 1B/O8 is attributed to virulence factors encoded in the chromosome. Chromosomal type III protein secretion system (T3SS) named Yersinia secretion apparatus (Ysa) is considered a potential virulence factor of the bacterium. Ysa was found to have specific induction conditions – nutrient rich medium supplemented with a high salt concentration (190–400 mM NaCl or KCl) at a moderate temperature (26–28°C) (Haller *et al*., 2000, Venecia and Young, 2005). Therefore, Ysa activity was initially considered as limited to the gastrointestinal tissues. The T3SS was shown to translocate some specific proteins named Yersinia secreted proteins (Ysps) (Haller *et al*., 2000, Venecia and Young 2005). Moreover, Ysa was found to secrete some of pYV-encoded Yersinia outer proteins (Yops) (Venecia and Young 2005). In recent studies, Ysa was proposed to act also at the systemic phase of the disease (Bent *et al*., 2013). All the aforementioned findings were derived from investigations conducted on laboratory reference strainsof *Y. enterocolitica* 1B/O8 and their mutants. However, little is known about Ysa activity in clinical isolates. Therefore we decided to investigate the Ysa activity in clinical isolates of *Y. enterocolitica* 1B/O8 collected in Poland.

Clinical strain DM0110 of *Y. enterocolitica* 1B/O8 originated in Poland in 2005 and was selected for DNA-sequencing of the complete Ysa pathogenicity-island (Ysa-PI). DNA-sequencing was conducted by Sanger method using a BigDye Terminator v3.1 (Applied Biosystem). A library of 1.5–2.5 kbp fragments of Ysa-PI

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Table I
Y. enterocolitica 1B/O8 reference strains and clinical isolates tested in this study

Strain ID (no. isolates)	Source[a]	Year of isolation	Ysps secretion[b]	*ysrR* type[c]
WA-314	Laboratory, MPI	NA	+	Wild
1105	Laboratory, IP	NA	+	Wild
13804	Laboratory, IP	NA	+	Wild
17451	Laboratory, IP	NA	+	Wild
20167	Laboratory, IP	NA	+	Wild
20169	Laboratory, IP	NA	+	Wild
20175	Laboratory, IP	NA	+	Wild
20176	Laboratory, IP	NA	+	Wild
20178	Laboratory, IP	NA	+	Wild
20189	Laboratory, IP	NA	+	Wild
20232	Laboratory, IP	NA	+	Wild
DM0099	Clinical sample	2008	–	Altered
DM0102	Clinical sample	2008	–	Altered
DM0110	Clinical sample	2005	–	Altered
DM0147	Clinical sample	2006	–	Altered
DM0149	Clinical sample	2005	–	Altered
DM0150	Clinical sample	2004	–	Altered
DM0209	Clinical sample	2009	–	Altered
DM0249	Clinical sample	2009	–	Altered
(n = 70)	Clinical samples	2004–2013	Not tested	Altered

a MPI (Max Von Pettenkofer Institut, Munich, Germany); IP (Pasteur Institute, Paris, France); b (+) for Ysps secretion (–) for no Ysps secretion; c result of PCR-RFLP assay for position 269 in the *ysrR* gene; NA – not applicable

of DM0110 was cloned in plasmid pJet1.2/blunt Cloning Vector (Thermo Scientific, Lithuania) prior to DNA sequencing. Sequencing results were read on automated capillary sequencers in a biotechnological company (Genomed, Poland). Reviewed complete 30379 bp – long YSA-PI cluster was deposited in GenBank (www.ncbi.nlm.nih.gov) database under no. KC784374.1.

Further examinations were performed on a number of 78 clinical isolates and 11 reference strains (Table I). PFGE typing (data not shown) showed that all the isolates were tightly-clonal and belonged to the previously described *Xba*I genotype II (Gierczyński *et al*., 2009). Ysa and Ysps proteins were visualised by SDS-PAGE assay. The assay was performed in accordance with the procedure described by Matsumoto and Young (2006). Eight *Y. enterocolitica* 1B/O8 clinical isolates originating in Poland were tested by SDS-PAGE together with DM0110 and the 11 reference strains listed in Table I. Bands specific for Ysps and Ysa proteins were detected for the reference strains only. In contrast to the reference strain WA-314 no Ysps could be detected for clinical isolate DM0110 in duplicate experiments (Fig. 1A). Ysps were also lacking in seven randomly selected clinical isolates (Fig. 1B).

The Ysa-PI sequence of DM0110 was aligned to the homologous loci deposited in commonly accessible databases using CLC Sequence Viewer 7 (CLC Inc, Denmark). Comparison of nucleotide and deduced amino-acid sequences of Ysa-PI of *Y. enterocolitica* 1B/O8 reference strains: 8081 (NC_008800.1), A127/90 (AF369954.1 and AY100449.2) and WA314 (AKKR01000083) was conducted to trace amino-acid substitutions and other alterations.

DM0110 revealed 99% homology to Ysa-PI of reference strains 8081, A127/90 and WA314. No structural aberrations were observed. The only significant mutation specific for tested DM0110 was found in *ysrR* gene that is part of the YsrRST phosphorelay system (Venecia and Young 2005, Walker *et al*., 2010). Single nucleotide substitution (C to A) at position 269 of *ysrR* resulted in a premature stop-codon that terminates translation of YsrR at position 90. No such mutation was found in the reference *Y. enterocolitica* 1B/O8 strains examined in this study.

To verify whether the stop-codon prematurely terminates the expression of *ysrR*in DM0110, the complete *ysrR* gene (711 bp length) from DM0110 was cloned to expressing vector and examined in *Escherichia coli*. Expression system pET-30 Ek/LIC Vector Kit (Novagen, USA) was used according to the manufacturer's instruction. The unaltered *ysrR* gene from strain WA-314 was used as a reference. Recombinant proteins of both

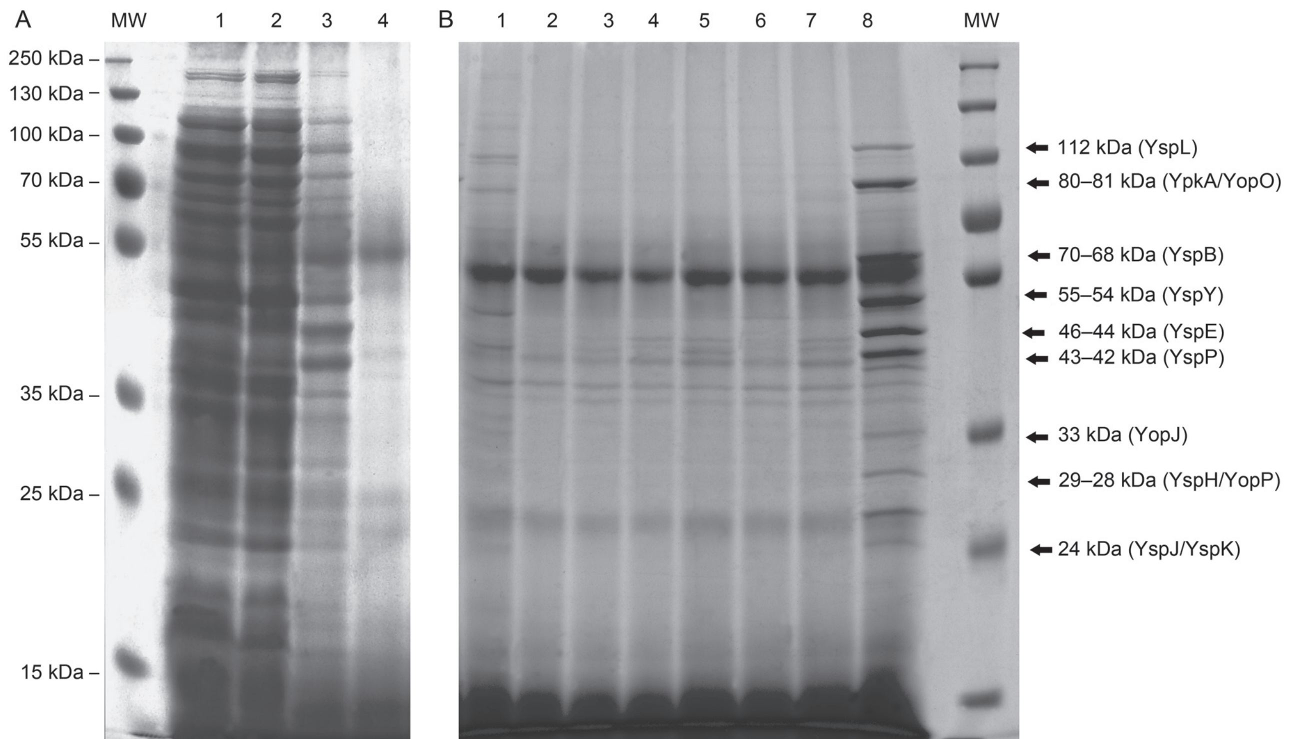

Fig. 1. SDS-PAGE profiles of secreted proteins Ysa-Ysp of *Y. enterocolitica* 1B/O8 reference strains and clinical isolates from Poland
A: lines 1 and 2 – strain WA-314; 3 – strain 20175; 4 – clinical isolate DM0110. B: lines 1 to 7 – clinical isolates: DM0150, DM0147, DM0099, DM0102, DM0149, DM0249, DM0209 respectively; line 8 – strain WA-314; MW – molecular weight standard

DM0110 and WA-314 were purified on column with Ni^{2+}-ISA His-Binding Resin (Novagen, USA). Molecular weight of the expressed recombinant proteins was determined by SDS-PAGE according to Laemmli (1970).The recombinant YsrR protein of DM0110 was about 10 kDa, while the YsrR recombinant of the unaltered *ysrR* gene from WA-314 was 27 kDa. These results confirmed that the premature stop-codon in *ysrR* is active *in vitro*. Further analysis *in silico* performed with Domain Enhanced Lookup Time Accelerated BLAST (DELTA-BLAST) algorithm showed that YsrR protein REC domain is only half-expressed in DM0110 while the LuxR-C-like domain is lacking.

To determine whether other clinical isolates of *Y. enterocolitica* 1B/O8 in Poland carry unaltered or prematurely-terminated *ysrR*, PCR-RFLP assay targeting the unaltered (wild-type) *ysrR* gene was developed. Primers (ysrR-F 5'ATGACACAAACGAAAACGCTCAAT and ysrR-R 5'TTATAGAGAAATTTCATGAGCAT) were used to amplify the 711 bp PCR-product of the *ysrR* gene. PCR was conducted as described previously (Wołkowicz *et al.*, 2014). The *ysrR* PCR-product was further digested by *Bts*I endonuclease (New England BioLabs) as recommended by the manufacturer. The PCR-product of the unaltered, wild-type *ysrR* gene yielded two fragments of expected size 268 and 445 bp. In the case of mutation in position 269 of *ysrR*, the PCR-product remained undigested. In each PCR-RFLP experiment the reference strain WA-314 was used as a control for the wild-type *ysrR*. DM0110 and all the tested clinical isolates listed in Table I were shown by the PCR-RFLP assay to bear *ysrR* gene altered in position specific for the premature stop-codon. No alterations were detected in ten reference strains of *Y. enterocolitica* 1B/O8 (Table I) previously found to secrete Ysps (Rokosz-Chudziak *et al.*, 2013).

In the past two decades *Y. enterocolitica* 1B/O8 emerged in Japan, Germany and Poland. Since 2004 this bacterium has been isolated in Poland from humans with clinically confirmed yersiniosis. Noteworthy, all clinical isolates of *Y. enterocolitica* 1B/O8 in Poland were found to be tightly clonal (Gierczyński *et al.*, 2009, Zacharczuk, 2013). *Y. enterocolitica* 1B/O8 was isolated from a variety of clinical samples including stool and blood. We, therefore, assumed our collections of clinical isolates constitute an excellent material to study Ysa activity in human yersiniosis.

The results obtained in this study are, however, surprising when compared with other reports on Ysa T3SS published to date (Venecia and Young 2005, Matsumoto and Young 2006, Mildiner-Earley *et al.*, 2007, Bent *et al.*, 2013). First of all we showed that clinical isolates of *Y. enterocolitica* 1B/O8 collected in Poland from 2004 to 2009 were unable to secrete Ysps. Moreover, clinical isolate DM0110 was unable to produce Ysps under a variety of inducible conditions described elsewhere (Mildiner-Earley *et al.*, 2007, Witkowski *et al.*, 2008) (data not shown). These findings together have

prompted us to search Ysa-PI of DM0110 for possible reasons for Ysa inactivation. One particularly interesting mutation in the *ysrR* gene of YsrRST phosphorelay system was detected. This mutation triggers the premature stop-codon that early terminates the translation of regulatory gene *ysrR*.

The YsrRST phosphorelay system is considered the major regulatory system for YSA-PI (Venecia and Young, 2005, Walker *et al.*, 2010). Consequently, the premature stop-codon in *ysrR* may play a key role in Ysa T3SS silencing in *Y. enterocolitica* 1B/O8 circulating in Poland. Noteworthy, disruption of the *ysrR* gene has been already reported to inactivate Ysps secretion by Ysa T3SS. Similarly to strain DM0110, a *ysrR* mutant of *Y. enterocolitica* 1B/O8 strain JB580v, constructed by Venecia and Young (2005), was also found unable to secrete Ysps.

Our results showed that expression of *ysrR* in the clinical strain of *Y. enterocolitica* 1B/O8 is terminated at one third of the functional protein. Important REC and LuxR-C-like domains of the functional YsrR are, therefore, lacking or partially expressed in *Y. enterocolitica* 1B/O8 circulating in Poland. Interestingly, PCR-RFLP test showed that all investigated clinical isolates from Poland bear the altered *ysrR* gene. This finding corresponds with the strong clonality of clinical isolates of *Y. enterocolitica* 1B/O8 in Poland revealed by PFGE. Therefore, we conclude that the premature stop-codon in the *ysrR* gene may serve as a hallmark of the epidemic sensu stricto strain of *Y. enterocolitica* 1B/O8 in Poland.

To the best of our knowledge, dysfunctional YSA-PI has not been yet reported in *Y. enterocolitica* 1B/O8 from patients with clinically confirmed yersiniosis. Our findings together may, however, suggest that Ysa T3SS is not indispensable for *Y. enterocolitica* 1B/O8 to cause infection in human.

Preliminary results of this study were, in part, presented during the 11th International Symposium on Yersinia, Suzhou, China, 24–28 June 2013 (Rastawicki W., Szych J., Rokosz N., Zacharczuk K., Wołkowicz T. and Gierczyński R. The emergence of high-pathogenicity Yersinia enterocolitica bioserotype 1B/O8 infections in Poland. Final Program *Yersinia* 11th, pp. 74).

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