УДК 616.9

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DIFFERENT SIDEROPHORES CONTRIBUTE TO THE HIGH-PATHOGENICITY PHENOTYPE IN *YERSINIA*

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For successful proliferation in the hostile mammalian environments, the highly pathogenic *Yersiniae* (*Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* subsp. *enterocolitica*) have to sequester ferric iron from the host iron storage molecules. Siderophores, low molecular weight molecules, that demonstrate high affinity for the iron, are responsible for Fe^{3+} capture and transport in bacteria. At least one endogenous siderophore system, yersiniabactin, is known to be involved in iron acquisition in highly virulent *Yersiniae*. Its inactivation in *Y. pestis* and *Y. enterocolitica* subsp. *enterocolitica* results in significant attenuation of virulence. However, the yersiniabactin is not present in all highly virulent *Yersiniae.* Indeed, the large group of *Y. pseudotuberculosis* serotypes O2, O3, O4, O5 as well as serotype O1 Far East Scarlet like fever (FESLF) strains carry an alternative, iron acquisition system, pseudochelin, encoded by the *Yersinia* non-ribosomal peptide *ynp* locus. Thus, the yersiniabactin activity is not the only one associated with the high-pathogenicity phenotype of the human pathogenic *Yersiniae*.

Key words: pathogenic *Yersiniae,* high-pathogenicity phenotype, siderophores

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Различные сидерофоры обусловливают фенотип высокой патогенности иерсиний

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Высоковирулентным *Yersiniae* (*Y. pestis*, *Y. pseudotuberculosis* и *Y. enterocolitica* subsp. *enterocolitica*) для успешного размножения в неблагоприятных условиях хозяйского организма необходимо трехвалентное железо, которое связано железосодержащими молекулами хозяина. Низкомолекулярные молекулы, сидерофоры, обладающие высоким сродством к железу, отвечают за эффективный захват и транспорт Fe^{3+} в бактериальную клетку. По крайней мере одна сидерофорная система, а именно, система синтеза и транспорта йерсиниобактина, отвечает за активный транспорт железа у йерсиний. Инактивация этой системы приводит к значительному снижению вирулентности у *Y. pestis* и *Y. enterocolitica* subsp. *enterocolitica.* Однако, йерсиниобактин присутствует далеко не у всех представителей рода йерсиний. Так, *Y. pseudotuberculosis* серотипов O2, O3, O4, O5, а также штаммы O1, вызывающие Дальневосточную скарлитиноподобную лихорадку, содержат альтернативную систему транспорта железа, псевдохелин, которая кодируется локусом *ynp* (нерибосомный синтез пептидов). Следовательно, как минимум две системы снабжения железом ассоциированы с фенотипом высокой патогенности у йерсиний, а именно, системы йерсинибактина и псевдохелина.

Ключевые слова: патогенные иерсинии, фенотип высокой патогенности, сидерофоры.

The highly-pathogenic *Yersiniae* are defined as bacteria, which are able to kill mice in low infectious doses. *Yersinia pestis, Y. pseudotuberculosis* and *Y. enterocolitica* subsp. *enterocolitica* belong to this highly virulent group. Presence of the so-called High-Pathogenicity Island (HPI), encoding a siderophore yersiniabactin iron acquisition system (Ybt), is a main prerequisite for the high-pathogenicity phenotype in *Yersinia*. Loss or inactivation of this system results in significant virulence attenuation of *Y. pestis* and *Y. enterocolitica* subsp. *enterocolitica* [3, 5, 7, 11, 14, 15]. Thus, the yersiniabactin-mediated ferric iron uptake system is accepted as one of the main virulence-associated determinants in human pathogenic *Yersiniae*. However, although all highly pathogenic *Y. pseudotuberculosis* serotypes were initially suspected to possess the *ybt* cluster this is not the case. It has been shown that only strains of serotype O1 possess the complete yersiniabactin gene cluster [15] while other highly virulent strains of serotypes O2, O4, O5 lack *ybt*. The situation becomes even more intriguing with strains of serotype O3, which partly contain the *ybt* genes and partly are devoid of *ybt*. Moreover, the *ybt* cluster is not functional in the O3*ybt* group, due to a partial truncation of the *ybt* genes including the *fyuA* gene encoding the yersiniabactin receptor [9, 15]. Accordingly, the O3*ybt* strains are unable to produce the yersiniabactin, while the other O3 strains demonstrate siderophore activity on the chrome azurol S siderophore detection CAS agar [15, 19]. The O3*ybt* strains show also reduced virulence in animals compared to their siderophore positive counterpart O3 group [9]. Moreover, the strains of other highly virulent, human pathogenic *Y. pseudotuberculosis* serotypes, O2, O4, and O5, also produce siderophores in the absence of the yersiniabactin genes. Previously we have applied suppression subtractive hybridization to two serotype O3 strains of both groups (O3*ybt* and O3), the siderophore-negative Yp346 with a truncated *ybt* and the prototype highly virulent siderophore-positive, but *ybt*-negative YPIII strain, widely used in *Yersinia* pathogenicity research [17]. Several gene sequences with high similarities to iron siderophore biosynthetic genes were uncovered in the YPIII genome (e.g. YPO0776, according to *Y. pestis* CO92 annotation). These data strongly support the presence of an alternative iron siderophore system

in O3, a system that is able to substitute the Ybt and to support the high-virulent phenotype of *Y. pseudotuberculosis*. Indeed, sequence of the YPIII strain (http://www.ncbi.nlm.nih.gov/genome/510?project_id=59151) demonstrated the presence of siderophore iron acquisition systems in the O3 group and the complete absence of the *ybt* cluster. The functionality of at least some of these systems is supported by the ability of O3 strains to synthesize and produce a siderophore. In contrast, one of the potential alternative siderophore clusters, initially designated as HPI-2 (High-Pathogenicity Island-2, due to its similarity with the *ybt* cluster located on a mobile HPI structure) is evidently non-functional in *Y. pestis,* due to insertion of the IS*100* element, a frameshift disrupting the biosynthetic ORF and / or its disruption into two separated parts [8]. However, the HPI-2 cluster, designated *ynp* (*Yersinia* non-ribosomal peptide locus) is evidently intact in *Y. pseudotuberculosis* but entirely absent in *Y. pestis* Pestoides F strain. In *Y. pseudotuberculosis* serotype O1 IP32953 strain, the *ynp* locus (YPTB3290-3298) contains putative siderophore assembly genes (YPTB3296-3297) coding for the mixed nonribosomal peptide synthetase (NRPS) / polyketide synthase (PKS) pathway and transport genes (YPTB3290-3291) and TonB-dependent outer membrane receptor (YPTB3298) (Fig. 1). The *ynp* locus does not contain a phosphopantetheinyl (P-pant) transferase, which is necessary for the assembly of the NRPS-PKS complex. Therefore, one of the two P-pants encoded within the *Yersinia* genomes is necessary for the siderophore biosynthesis (Bobrov *et al.,* 2002).

Here we aimed to look for distribution of the *Yersinia* pseudochelin Ynp system in *Y. pseudotuberculosis* with special impact on serotype O3 strains and address its possible implication in siderophore synthesis and iron uptake.

Materials and methods

Bacterial strains were obtained from the collection of the Max von Pettenkofer-Institute, LMU, Munich, Germany.

Siderophore production was demonstrated in a colorimetric chrome azurol S assay using a solidified liquid CAS agar developed by Schwyn and Neilands (1987).

Conditions of iron deprivation were achieved in NBD medium (Nutrient Broth, 5 g of NaCl with addition of 0,1 mM 2-2'-dipyridyl).

Primer walking was used to close the deletion gap in the *ybt* cluster sequence in siderophore-negative but *irp2*-positive *Y. enterocolitica* O3 genomes (*O3ybt* group).

A genome wide hybridization microarray [12] was applied to compare gene contents of the selected *Yersinia* genomes.

High-quality *Y. pseudotuberculosis* genome sequences were obtained from the publicly accessible databases http://www.ncbi.nlm.nih.gov and draft genome sequences of *Y. pseudotuberculosis* serotype O4 and O5 strains were obtained in cooperation with BGI-Hongkong Co. (Hong Kong) and annotated with the RAST server [2]. Circular representation of the genome comparisons was performed with BRIG [1].

Results

We have compared the strains of serotype O3 available in our collection (Table 1) for the presence of the yersiniabactin biosynthetic *irp2* gene and the ability to produce a siderophore on the CAS agar, to grow in iron-deficient NBD medium and to support growth of *E. coli* H1884 *entD,F* (siderophore enterochelin -negative mutant unable to grow in iron depleted NBD medium). Only strains of the siderophore-positive O3 group, but not of the O3*ybt* group, were able to grow in iron depleted

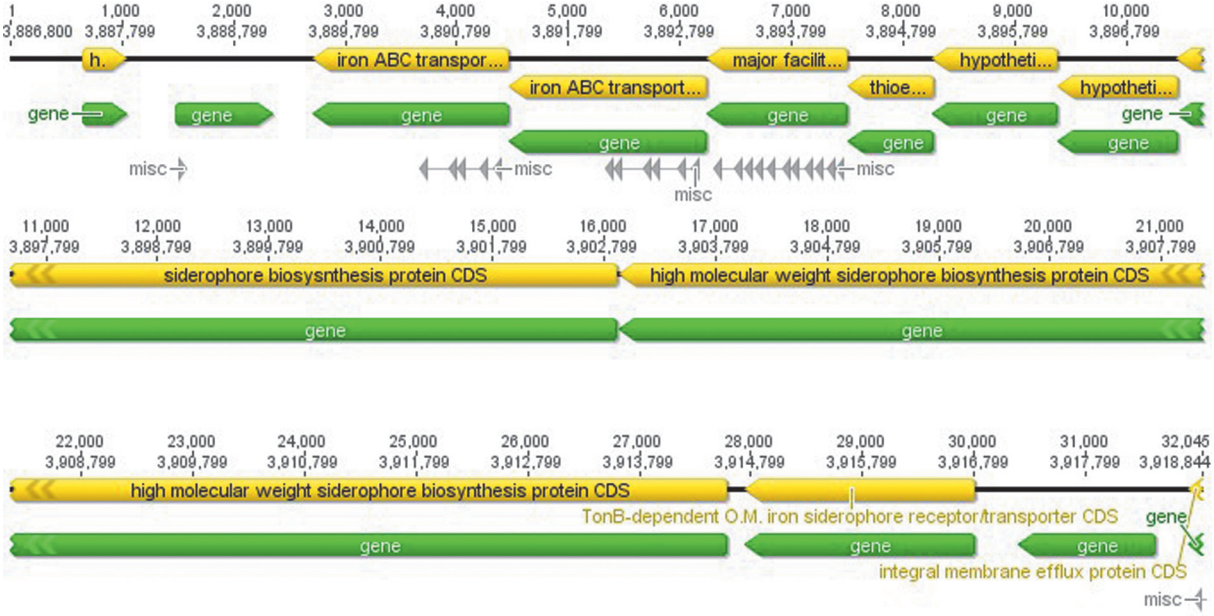

Fig. 1. The pseudochelin iron uptake operon (*Yersinia* non-ribosomal peptide locus *ynp,* YPTB3296-3297) in *Y. pseudotuberculosis* IP32953 strain (NCBI Reference Sequence: NC_006155.1). The picture is generated using Geneious program 6.1.5

Table 1

Comparison of *Y. pseudotuberculosis* O3 strains according to their CAS activity, presence of the *ybt* genes and ability to grow and support growth in iron-depleted media

Strain, isolation place	CAS activity	Presence of the irp2 gene	Growth in NBD, support of H1884 *entD,F* growth
1216/93, Norway	No	No	
14240, Turmenistan	No	No	
200/90, Germany	No	No	No
298/89, Australia	No	No	
1134/90, Japan	No	No	
2887, Argentina	No	No	
Turku, Finland	No	No	
Act-2, Russia	No	No	
307, New Zealand	No	No	No
346, Denmark	No	No	No
201/90, Germany	Yes	No	
201, Denmark	Yes	No	
1401, Sweden	Yes	No	
YPIII, U.S.A.	Yes	No	
146, Germany	Yes	No	Yes
445/73, Russia	Yes	No	Yes
483/71, Russia	Yes	No	Yes
714, Japan	Yes	No	

medium as well as to support the growth of the H1884 *entD,F* mutant.

Using the whole genome hybridization microarray [12] we tested the presence of the siderophore-associated sequences (*ybt* and *ynp*) in the O3 strains. It turns out that, while the strains of the O3*ybt* group contain the *int, ybtS-ybtP, ybtA* and *irp2-irp1* genes (YPO1911 – YPO1916, according to *Y. pestis* CO92 annotation [13], they completely lack the *ynp* genes (clustered in two groups, YPO0770 – YPO0778 and YPO1011 – YPO1012 in *Y. pestis* CO92, or YPTB3290-YPTB3298, according to IP32953 annotation). Alternatively, the O3 group siderophore-positive strains possess the complete *ynp* gene clusters but none of the *ybt* associated genes.

Using primer walking we have defined the deletion end point in the *ybt* cluster in O3*ybt* strains (Fig. 2). The deletion includes *ybtU-fyuA* sequences and terminates 30 bp downstream the *irp1* stop codon.

Several completed and draft *Yersinia* genomes are now available in the public databases (http://www.ncbi.nlm.nih.gov/genome). For genome comparison we have included two draft genome sequences obtained from *Y. pseudotuberculosis* serotype O4 and O5 strains in cooperation with BGI, China. This allows analysis and comparison of the corresponding human-pathogenic *Y. pseudotuberculosis* for the presence of the siderophore iron uptake systems on the whole genome scale (Figure 3).

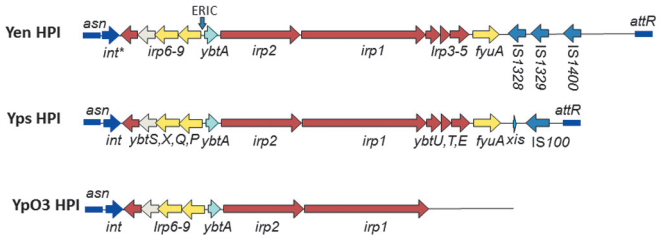

Fig.2. Structure of the yersiniabactin mobile gene cluster (HPI) in different *Yersiniae*. Note different designations of the *ybt* genes in *Y. pestis* /*Y. pseudotuberculosis*. Yps HPI stands for *Y. pestis* HPI and YpO3 HPI – for *Y. pseudotuberculosis* O3*ybt* HPI and Yen HPI – for *Y. enterocolitica* subsp. *enterocolitica* HPI

The partial deletion of the yersiniabactin *ybt* gene locus is evident in serotype O3*ybt* strains B6796_O3, B6862_O3, B6863O3 and B6864_O3 while the complete *ybt* cluster is missing in strain YPIII serotype O3; strains 66, O4 and 68, O5 and, to our surprise, also in the serotype O1 Far East Scarlet like fever (FESLF) strain IP31758 [6, 10]. Further sequencing of additional eight FESLF strains supports the absence of the *ybt* genes in this group of serotype O1 strains (data not shown). In contrast, the *ynp* cluster was absent in all O3*ybt* isolates but present in the strains of the other serotypes under comparison. Moreover, another potentially active siderophore iron acquisition system, aerobactin (*iuc*), is absent in the O3*ybt* strains. Thus, none of the three siderophores possibly supporting iron acquisition, the yersiniabactin, the pseudochelin (*ynp*) and the aerobactin (*iuc*) is present or functional in the O3*ybt* group. Interestingly, both iron uptake systems, *ynp* and *iuc*, are located in the integration hot spot that might represent another Plasticity Zone (PZ2) in *Y. pseudotuberculosis* genome. In contrast, sporadic *Y. pseudotuberculosis* O1 strains carry all three siderophore ferric iron acquisition systems. However, the functionality of these systems has to be demonstrated [8].

P-pant transferases are necessary for assembly of the NRP/PKS complex and synthesis of the pseudochelin. Indeed, all analyzed *Y. pseudotuberculosis* strains analyzed have both unaltered genes encoding P-pant transferases, *ybtD*, which initiates the YBT system and *acpP*, required for the fatty acid synthesis. Thus, the two O3 groups do not differ by their ability to support the synthesis of the Ynp siderophore due to the absence of the supporting P-pants.

Discussion

The so-called High-Pathogenicity Island supplies bacteria with the ability to synthesize the yersiniabactin (Ybt), a siderophore sequestering bound ferric iron from the host iron sources. The HPI represents an archetypical genetic mobile structure, a genomic island that transposes to any available free *asn*-tRNA genes in the host genome using a site-specific recombination. The HPI originally described in *Yersinia* is widely disseminated in *Enterobacteriaceae* (20) due to its mobile nature. However, the Ybt system is not present in all highly pathogenic *Yersiniae* (as it was initially suspected) and it is restricted to *Y. pestis* and *Y. enterocolitica* subsp. *enterocolitica*, both of which demonstrate their absolute dependence on Ybt for *in vivo* survival and virulence [3, 5, 7, 11, 14].

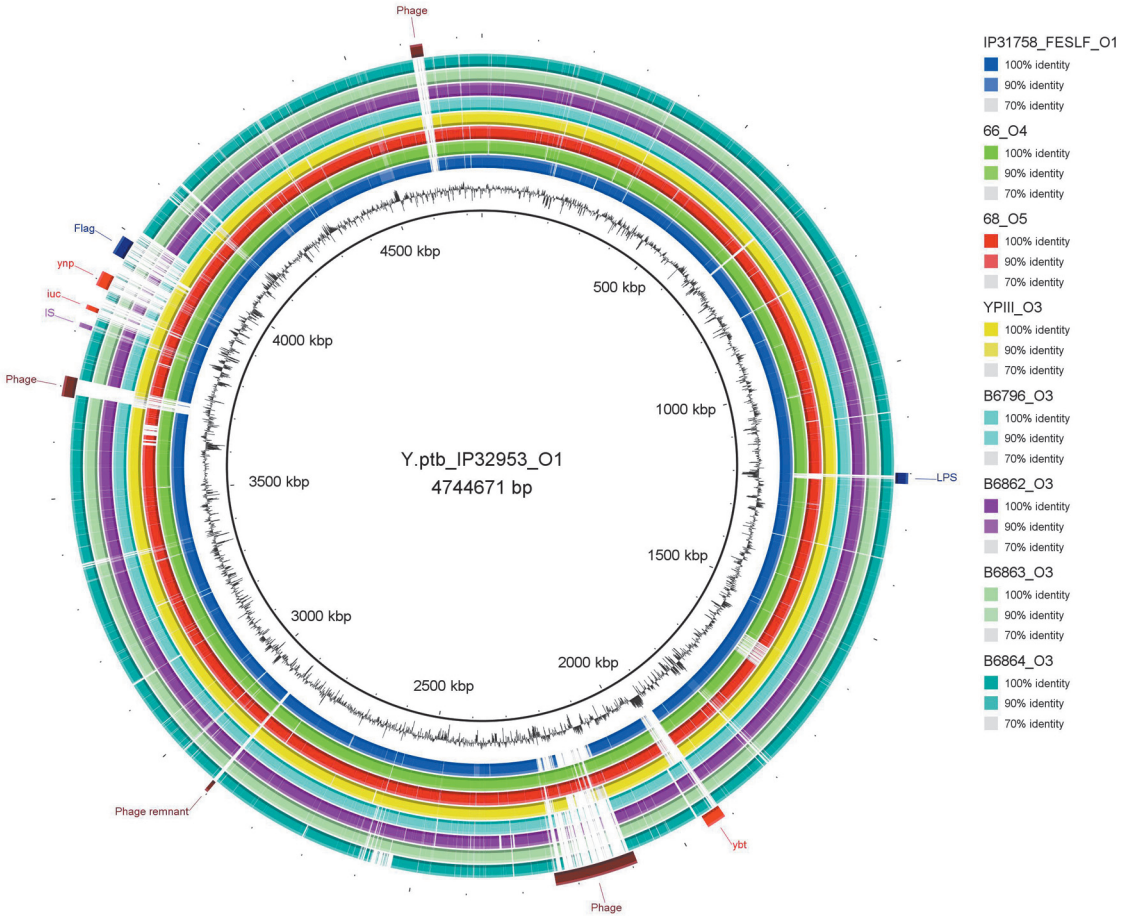

Fig. 3. Whole genome alignment of *Y. pseudotuberculosis* strains. Significant differentiating gene regions are represented. The white color indicates regions present in strain IP32953 but absent from the other strains

In contrast, most representatives of another group of highly virulent *Yersinia*, *Y. pseudotuberculosis*, do not possess the Ybt iron supply system. Only sporadic *Y. pseudotuberculosis* O1 strains demonstrate the presence of the *ybt* gene cluster while strains of the other highly virulent serotypes, O2, O4, O5, as well as epidemic FESLF O1 strains, lack the *ybt* genes. They also carry the pseudochelin Fe^{3+} acquisition system (Ynp) as an alternative to the yersiniabactin [18]. Moreover, serotype O3 strains likely exhibit competition between the Ybt and the Ynp ferric iron systems; they possess either of the systems, but not both of them. The similarity of the Ybt and Ynp systems and their potential biochemical and genetic cross talk might explain such competition of the two iron scavenging systems. Simultaneous presence and expression of these two related non-ribosomal peptide synthesized siderophore systems in sporadic O1 strains awaits its explanation.

Taken together, the alternative non-ribosomal peptide synthesized siderophore iron supply system Ynp (designated as "pseudochelin" due to its preferential distribution in *Y. pseudotuberculosis* [18]), is also responsible for the ferric iron siderophore mediated acquisition and, thus, for the highly-virulent phenotype of the vast majority of *Y. pseudotuberculosis* strains and serotypes. Indeed, inactivation of the siderophore biosynthetic gene YPBT3297 by homologous recombination with a chloramphenicol resistance cassette containing DNA fragment in YPIII, serotype O3 resulted in its inability to produce the siderophore (data not shown).

The ferric iron supply is not directly bound to pathogenicity of bacteria but it is a prerequisite for their successful multiplication in host and virulence potential. The Fur-dependent regulation of both iron supply and virulence supports a cross link between these traits. Bacteria, including pathogenic ones, possess different siderophore iron systems. Some of them have been acquired through a horizontal gene transfer and might be exposed in the available global gene pool and, thus, become available for import by other microorganisms to exploit alternative pathways.

Summary

1. The yersiniabactin ferric iron acquisition system, traditionally supposed to be responsible for the high-pathogenicity phenotype of *Yersinia,* is restricted to *Y. pestis* and *Y. enterocolitica* subsp. *enterocolitica* strains.

2. Human highly pathogenic *Y. pseudotuberculosis* O2, O4, O5, O3*ynp* and epidemic FESLF O1 demonstrate alternative siderophore activity in the absence of

the yersiniabactin system.

3. The alternative iron acquisition system designated as pseudochelin (originally annotated as *Yersinia* non-ribosomal peptide locus, *ynp*) is responsible for the production of the siderophore in *Y. pseudotuberculosis* O2, O4, 05, O3*ynp* and FESLF O1 non-Ybt group.

4. Strains of two groups of *Y. pseudotuberculosis* O3, namely O3*ybt* and O3, demonstrate differences in their siderophore activity. Strains of the O3*ybt* group do not exhibit any siderophore activity and are of reduced virulence due to the absence of functional iron acquisition systems (the pseudochelin, *ynp*; the aerobactin, *iuc* and truncation of the yersiniabactin locus, *ybt*). In turn, the O3*ynp* strains are siderophore pseudochelin positive but lack the yersiniabactin locus, *ybt.*

5. The present designation of the *ybt* genomic island as the High-pathogenicity Island [4] as the only one associated with the high-pathogenicity phenotype in *Yersinia* is misleading and must be withdrawn.

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Поступила 05.08.13.