Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie
Ludwig -Maximilians-Universität
München
Direktor: Prof. Dr. Dr. J. Heesemann

Factors and Mechanisms of Mobility of the High Pathogenicity Island of *Yersinia*

Dissertation
zur
Erlangung des Doktorgrades der
Fakultät für Biologie der
Ludwig-Maximilians-Universität
München

von

Uladzimir Antonenka

aus

Gomel, Belarus

München 2007

Dissertation eingereicht am: 13. Juni 2007
Vorsitzender: Prof. Dr. Elizabeth Weiss
Gutachter: Prof. Dr. Anton Hartmann
Gutachter: Prof. Dr. Heinrich Jung
Protokoll: Prof. Dr. Makoto Hayashi
Sondervotum: Prof. Dr. Dr. Jürgen Heesemann
Tag der mündlichen Prüfung: 17. Dezember 2007

A. INRODUCTION

1. General characteristics of *Yersinia* species

The genus *Yersinia* is composed of Gram-negative coccobacilli belonging to the family of *Enterobacteriaceae*. Members of the *Yersinia* genus are facultative non-sporulating anaerobes with optimal growth at 27-30 °C. According to biochemical and metabolic characteristics, DNA-DNA hybridization, and 16S rRNA sequencing results, the genus *Yersinia* comprises 11 different species. The G+C content of the DNA of the genus is 46 to 50 mol% (Bercovier and Mollaret, 1984). DNA hybridization studies revealed more than 90% intra- and interspecies relatedness between *Y. pestis* and *Y. pseudotuberculosis* and 20 to 55% between *Y. pseudotuberculosis* and the other *Yersinia* species (Perry and Fetherston, 1997). It was found that the 16S rDNA sequence of *Y. pseudotuberculosis* is identical to that of *Y. pestis* (Trebesius *et al.*, 1998).

Y. pestis, *Y. pseudotuberculosis*, and *Y. enterocolitica* are pathogens for humans and other mammals, birds. *Y. ruckeri* is known as a fish pathogen (Bottone, 1997). *Y. pestis*, the bacterial agent of bubonic plague, has been responsible for devastating epidemics throughout human history. This pathogen persists among certain wild rodent populations in many parts of the world (except Australia) and is transmitted by the bite of infected fleas. The blockage of the proventriculae of fleas by *Y. pestis* forces infected fleas to bite and subsequently regurgitate the infected blood meal into the bite site of a new host. The subsequent bacteremia in rodents completes the rodent-flea-rodent cycle which is essential for *Y. pestis* spread. The ecology, pathogenicity, and host range of *Y. pseudotuberculosis* and *Y. enterocolitica* differ fundamentally from those of *Y. pestis*. Both species are transmitted perorally by contaminated food or drinking water and subsequently invade Peyer's patches of the small bowel and multiply extracellularly. In the murine infection model bacteria disseminate to mesenteric lymph nodes and occasionally via the bloodstream to the spleen, liver, and lungs, causing septicemic plague-like infections. Normally, infections with *Y. enterocolitica* or *Y. pseudotuberculosis* (yersiniosis) are self-limiting and benign. *Y. pseudotuberculosis* is widely distributed in nature in aquatic and animal reservoirs (rodents, cattle, swine, deer, and birds). Although the three pathogenic *Yersinia* species differ greatly in their lifestyle, they have evolved common strategies of pathogenesis, e.g., tropism for lymphatic tissue and extracellular multiplication. *Yersiniae* carry multiple sets of diverse pathogenicity- and transmission-related genes localized on the chromosome and on plasmids (Finlay and Falkow, 1997; Hinnebusch, 1997). There are several genes for cell adhesion (*inv*, *ail*,

myfA, *psa*, and *yadA*), invasion (*invA*), evasion of the host immune response (virulence plasmid pYV, shared by all three *Yersinia* species), and plague pathogenesis and transmission (*hms*, *pla*, *ymt*, and *caf*).

The high-pathogenicity group of *Yersinia* species, which are highly pathogenic for mice carries genes for the biosynthesis and uptake of the ferric iron-chelating substance (siderophore) yersiniabactin, located on a specific genomic element, so called "**H**igh **P**athogenicity **I**sland" (HPI) (Rakin *et al.*, 1999b).

2. The concept of "Pathogenicity Island" (PAI)

The concept of PAI was founded in the late 1980s by Jörg Hacker and colleagues in Werner Goebel's group at the University of Würzburg, Germany, who were investigating the genetic basis of virulence of uropathogenic (UPEC) *E. coli* strains 536 and J96 (Hacker *et al.*, 1990; Knapp *et al.*, 1986). The group observed a genetic linkage of determinants encoding P fimbriae, P-related fimbriae, and hemolysins in these strains and could also detect a codeletion of these linked genes (Hacker *et al.*, 1990).

PAIs could be characterized by the following features:

- Their G+C content usually differs from that of the bacterial core chromosome and PAIs-sequences also show a different codon usage. It is considered that the horizontally acquired PAI still has the base composition of the donor species (Dobrindt *et al.*, 2004).
- PAI carry one or more virulence genes; genomic elements with characteristics similar to PAI but lacking virulence genes are referred to as genomic islands. Accordingly to their function they can be denoted e.g. fitness islands, or metabolic islands.
- PAIs are present mostly in the genomes of pathogenic bacteria but absent from the genomes of a non-pathogenic representative of the same species or a closely related species. Nevertheless, HPI could be detected in some facultative-pathogenic *E. coli* strains (Karch *et al.*, 1999; Schubert *et al.*, 2000).
- PAIs occupy relatively large genomic regions. The majority of PAI are in the range of 10 to 200 kb.
- PAIs are frequently located adjacent to tRNA genes. The frequent insertion at tRNA loci may be explained by the observation that genes encoding tRNAs are highly conserved between various bacterial species. tRNA genes may represent specific anchor points for the integration of foreign DNA (Williams, 2002).

- PAIs are often flanked by direct repeats (DR). DR are defined as DNA sequences of 16 to 20 bp (up to 130 bp) with a perfect or nearly perfect sequence repetition. DR act as recognition sequences for enzymes involved in excision of mobile genetic elements, thus contributing to the instability of a PAI flanked by DR. PAI often carry cryptic or even functional mobility genes such as integrases or transposases. Integrases, which may have been derived from lysogenic bacteriophages, mediate the integration of the phage genome into the genome of the host bacteria, as well as the excision needed to enter a lytic cycle. Such genes are still functional in certain PAIs, and the encoded proteins can mediate the excision of the PAI and its loss. Other PAIs contain genes that are similar to integrase and resolvase genes of transposons. PAI can also represent integrated plasmids, conjugative transposons, bacteriophages or parts of these elements (Hacker *et al.*, 1997).
- PAIs often are unstable and delete with distinct frequencies. Virulence functions encoded by certain PAIs are lost with a frequency that is higher than the normal rate of mutation. Several characteristic elements, such as integrases, transposases, and IS elements, have been identified that contribute to mobilization and as well as to PAIs instability.
- PAIs often represent mosaic-like structures rather than homogeneous segments of horizontally acquired DNA. Some PAIs represent an insertion of a single genetic element. Others show a more complex structure, since elements of different origin are present. During evolution, several genetic elements have been acquired independently at different time points and from different hosts. However, these DNA acquisitions integrated at the same position into the chromosome of the recipient bacterial cell. This will result in the accumulation of horizontally acquired elements at a certain location of the chromosome, and the same target structures (e.g. tRNA genes).

3. PAIs, as a particular case of “Genomic Islands” (GEIs)

Although the concept of PAIs was first established in pathogenic bacteria, the comparison of DNA sequences from different microorganisms, including the increasing number of complete bacterial genome sequences, has revealed that regions with features that are characteristic of PAIs can also be found in many non-pathogenic bacteria. Owing to the occurrence of PAIs in phylogenetically unrelated organisms and the different functions that they encode depending on the ecological context, it has become clear that these genetic structures are of more general relevance than was initially anticipated. Therefore, the designation “pathogenicity islands” has been extended to “genomic islands” (GEIs), which can encode a wide range of functions.

Depending on the functions they encode and the advantages they confer relative to the specific lifestyle of a bacterium, GEIs can be called pathogenicity, symbiosis, fitness, metabolic or resistance islands (Hacker and Carniel, 2001; Hentschel and Hacker, 2001). Furthermore, the presence of identical genes in pathogenic and non-pathogenic variants of one species — for example, in extraintestinal pathogenic and commensal *E. coli* — implies that some of these encoded functions contribute to general adaptability, fitness and competitiveness, rather than to particular virulence traits (Dobrindt *et al.*, 2003).

4. Evolution of genomic islands

According to modern evolutional theory, increased fitness results from progressive evolution. Bacterial fitness can be characterized as deriving from properties that enhance the survival and transmission of an organism in a specific niche (Preston *et al.*, 1998). Therefore, main evolutionary advantage of GEIs is that large numbers of genes (entire operons that confer new traits) can be horizontally transferred into the genome of the recipient, resulting in marked phenotypical changes of the recipient. GEIs might provide a selective advantage under specific growth conditions as they can enhance adaptability and competitiveness within a niche. The biggest evolutionary advantage of GEIs is probably the maintenance of genetic flexibility and the ability of GEIs to transfer large numbers of genes, which allows for more successful adaptation and increased fitness in a specific ecological niche.

The acquisition of foreign genetic elements is frequently counterbalanced by the loss of native genes. In some cases, this loss of function could be a selective advantage, for example, the complete genome sequences of obligate intracellular pathogens or symbionts show genome reduction (Moran, 2002). This emphasizes the similar mechanisms of genome optimization by gene loss and horizontal gene transfer in pathogens and symbionts, and highlights the fact that the optimization of ‘en bloc’ gene acquisition and gene loss shapes the architecture of the bacterial genome.

Little is known about the origin of GEIs, but it has been speculated that they might have been derived from integrating plasmids or phages that have lost the genes that are required for replication and self-transfer in exchange for a more stable association and inheritance with the host chromosome. Some GEIs exhibit features of integrative and conjugative elements (ICEs). These elements include conjugative transposons, integrative plasmids and other elements that are excised to form a circular molecule, which is then transferred by conjugation and integrated into

the host genome by site-specific recombination. GEIs might evolve from mobile genetic elements, such as bacteriophages or plasmids that can be transferred even between unrelated microorganisms (Burrus *et al.*, 2002). Following acquisition by horizontal gene transfer, chromosomal integration by site-specific recombination and positive selection, a mobile genetic element might develop into a GEI due to genetic rearrangements, or gene loss or acquisition. The inactivation or deletion of origins of plasmid replication, or genes that are involved in the mobilization and transfer of plasmids or bacteriophages leads to immobilization of GEIs. However, the presence of a functional integrase gene seems to be a typical characteristic of many islands, thereby allowing insertion and excision of this type of element (Schmidt and Hensel, 2004).

GEIs might evolve further by consecutive recombination events that result in gains or losses of genetic information. In this way, features of mobile genetic elements could also be regained, resulting in chromosomal excision of the island and enabling its transfer to another recipient. There is an increasing number of examples of genetic determinants from non-pathogenic and environmental bacteria that can be located on extrachromosomal replicons (plasmids or phages) or in the chromosome as part of GEIs. The presence of these determinants in such elements in closely related microorganisms reflects their mobility. This shows that extrachromosomal replicons are frequently able to integrate into and excise from chromosomes, thereby supporting the hypothesis of GEI evolution from mobile genetic elements that are able to integrate into chromosomes.

The self-transmissible megaplasmid pHG1 of *Ralstonia eutropha* H16 consists of clusters of functionally related genes that are flanked by complete or partial mobile genetic elements. These clusters contain genes that are required for lithoautotrophy, denitrification, mineralization of aromatic compounds and iron uptake, as well as for type IVand RP4-like sex pili. The large number of pHG1 genes that encode transposases and integrases/recombinases indicates the high recombinational activity of this plasmid, which is likely to have resulted in the accumulation of diverse traits, thereby broadening the metabolic capacity of the recipient (Schwartz *et al.*, 2003). The structure of pHG1 shows that the genetic information coding for several different traits, which might also be chromosomally encoded in other organisms, has been ‘collected’ and that different mobile and accessory genetic elements have been involved in the evolution of this megaplasmid. Insertion of this plasmid into a chromosome could easily lead to the evolution of a GEI. Site-specific recombination with a chromosomal tRNA gene is not only a typical feature of GEIs, but

also of many lysogenic bacteriophages and has been described for a large conjugative resistance plasmid of *Haemophilus influenzae* (Dimopoulou *et al.*, 1997).

The presence of conjugative plasmids in several thermophilic archaea and their ability to insert into the host genome using a plasmid-encoded integrase has been described (Stedman *et al.*, 2000). A similar plasmid has been inserted into the genome of the thermoacidophilic crenarchaeon *Sulfolobus tokodaii* strain 7, which has subsequently been assimilated by rearrangements and gene duplication so that the structural features of the ancestral plasmid have been lost (Kawarabayasi *et al.*, 2001). This shows that a mobile genetic element can become part of the chromosome and can develop into a GEI by consecutive genetic rearrangements, gene duplication and insertion of genes. If DNA regions, such as integrases or repeat structures that are required for mobility of the element are deleted or destroyed, the mobile element becomes stably inserted into the chromosome. The increasing use of various comparative genomic approaches and genome sequence data provides evidence that these types of mechanisms contribute to general genetic flexibility in bacterial pathogens, symbionts (Dobrindt *et al.*, 2002; Ochman and Moran, 2001) and environmental microorganisms.

5. GEIs strategies for lateral transfer

Horizontal gene transfer, the intraspecies and interspecies exchange of genetic information, plays an important role in the evolution of bacteria. Three major mechanisms, transformation, transduction, and conjugation, provide bacterial populations with access to a "horizontal gene pool," enabling them to rapidly respond to environmental challenges. Theoretically, genomic islands could utilize all mentioned above strategies for lateral transfer, but the exact way of transfer determined only for single representatives of this group of mobile elements.

Genomic islands with ICEs features excise by site-specific recombination and are transferred to the new host by conjugation (Burrus *et al.*, 2002). The best characterized members of this group are the SXT island of *Vibrio cholerae* (Beaber *et al.*, 2002) and the R391 island of *Providencia rettgeri* (Boltner *et al.*, 2002). Both code for a nearly identical phage-like integrase, which mediates site-specific integration into the 5’ end of the *prfC* gene of the *Escherichia coli* chromosome (Hochhut and Waldor, 1999). Comparative analysis of these elements revealed a conserved backbone that contains regions that are dedicated to the integration, transmission and regulation of these elements, and additional variable regions that are unique to specific elements.

A phage-mediated transfer of a GEI between bacterial isolates has only been reported for the Gram-negative bacterium *V. cholerae* and the Gram-positive bacterium *S. aureus* (O'Shea and Boyd, 2002; Ruzin *et al.*, 2001). In both cases the mechanism of transfer involved horizontal gene transfer and recombination mediated via a bacteriophage. In *S. aureus* the 15.2-kb SaPI1, which encodes the toxic shock syndrome toxin, requires a helper bacteriophage 80α to excise and replicate and is transduced to recipient strains at very high frequencies. However, it is uncertain whether the 15.2-kb SaPI1 entirely conforms to the definition of a PAI and may represent a defective phage. Indeed, it is possible that SaPI1 and the helper phage are genetically related and as suggested SaPI1 requires a helper phage similar to the P2/P4 interaction (Ruzin *et al.*, 2001). The *Vibrio* pathogenicity island (VPI) has been shown to be transferable between O:1 serogroup strains, the predominant cause of epidemic cholera, via a generalized transducing phage CP-T1 (O'Shea and Boyd, 2002).

In spite of increasing number of described GEIs, the mechanism of transfer of most of them remains unclear.

6. Structure and function of the HPI

The discovery of the *Yersinia* HPI traces back to reports on siderophore production and iron-regulated proteins (Irp) associated exclusively with mouse-lethal strains of human pathogenic *Yersinia* species (Carniel *et al.*, 1987; Heesemann, 1987). The first sequenced iron-regulated gene of *Y. enterocolitica* (irp2 gene) indicated that the predicted irp2-encoding protein might be involved in siderophore biosynthesis (de Almeida *et al.*, 1993; Guilvout *et al.*, 1993). The next characterized iron-regulated gene of *Y. enterocolitica* was the *fyuA* gene encoding for an outer membrane protein of 71.3 kDa (denoted FyuA for ferric-yersiniabactin [Fe-Ybt] uptake). The FyuA protein acts as a receptor for Ybt uptake and is involved in pesticin sensitivity and mouse virulence (Heesemann *et al.*, 1993; Rakin *et al.*, 1994).

By further sequence comparisons of the *fyuA* genes of yersiniae two distinct evolutionary lineages of the HPI could be established, namely the *Y. pestis*/*Y. pseudotuberculosis* (HPI_{Yps}) and the *Y. enterocolitica* 1B (HPI_{Yen}) lineage (Rakin *et al.*, 1995). The HPI of the two evolutionary lineages differ considerably in size, with 44.3 kb for the HPI_{Yen} and 36.5 kb for the HPI_{Yps} (Fig. 1). The size of the integrated HPI can be delineated by two short 18-bp direct repeats that represent the core part (‘‘O’’) of the hybrid attachment sites *attL* and *attR* formed as a result of the site-specific recombination of the HPI with the bacterial *attB* (*asn* tRNA gene) recognition site

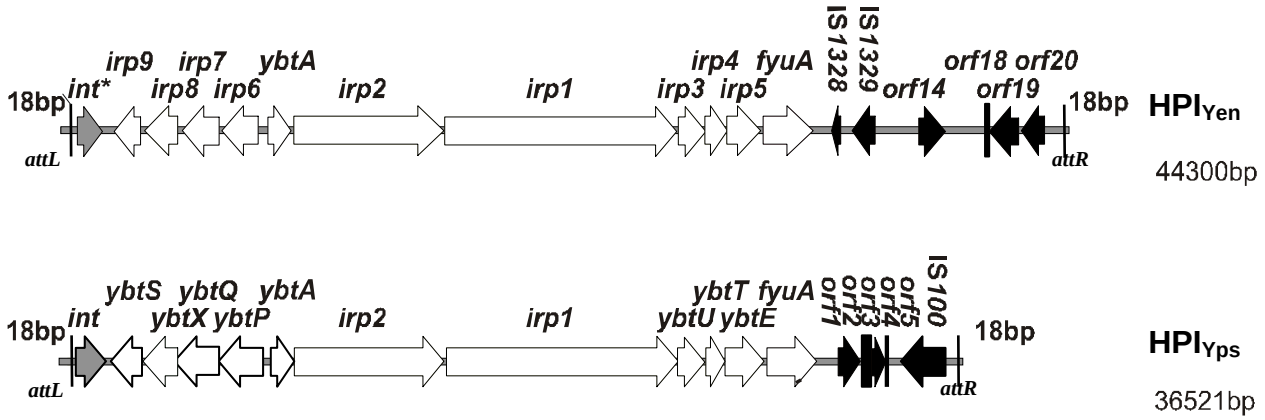


Fig. 1. Comparison of the high-pathogenicity islands of *Y. enterocolitica* and *Y. pseudotuberculosis* / *Y. pestis* evolutionary lineages

Yersiniabactin biosynthesis core genes are designated with thin arrows. The variable AT-rich part is designated with black arrows. Integrase genes (*int*) are shown with gray arrows.

(Rakin *et al.*, 2001). The difference in the size of the HPI resides in the presence of the variable AT-rich part (Fig. 1) that is completely different in both HPI lineages and greatly enlarges the HPI_{Yen} by acquisition of multiple IS elements (Bach *et al.*, 1999; Rakin *et al.*, 1999a).

Strikingly, the HPI_{Yen} is restricted to *Y. enterocolitica* biotype 1B strains (serotypes O:8, O:13, O:20 and O:21) which are typical North American isolates. It is worth mentioning that the HPI_{Yps} has also suffered a large deletion of the AT-rich part of the island in *Y. pseudotuberculosis* serotype O:3 strains (Buchrieser *et al.*, 1998a; Rakin *et al.*, 1995). The HPI_{Yps} of the *Y. pestis*/*Y. pseudotuberculosis* lineage might be accepted as an archetype HPI because it contains the complete set of genes necessary for production and transport of yersiniabactin (Ybt gene cluster or HPI core) and for mobility of the island (integration/excision module).

6.1 Yersiniabactin core

Yersiniae carrying the HPI secrete the low-molecular weight siderophore yersiniabactin (Ybt), which consists of catecholate, thiazoline, and thiazolidine residues and depicts high similarity to the siderophore pyochelin produced by *P. aeruginosa* (Cox *et al.*, 1981; Haag *et al.*, 1993). The genes encoding Ybt biosynthesis, transport and the transcriptional regulator are clustered within the functional core of the island (Carniel, 2001; Rakin *et al.*, 1999b). Six genes (designated *irp1 – irp5*, *irp9* in *Y. enterocolitica*, and *irp1-2*, *ybtU*, *ybtT*, *ybtE*, *ybtS* in *Y. pestis* and *Y. pseudotuberculosis*) are supposed to be involved in Ybt synthesis (Fig. 1). FyuA (Psn in *Y. pestis*) is the outer membrane receptor of the Fe-Ybt, and Irp6 and Irp7 (YbtQ and YbtP in *Y.*

pestis) are responsible for the inward Fe-Ybt transport across the cytoplasmic membrane. No periplasmic-binding protein has been defined for the Fe- Ybt import system so far. The expression of the biosynthetic and transport genes is repressed by iron and the ferric uptake regulator Fur, and is transcriptionally activated by the AraC-type transcriptional activator YbtA which also represses its own transcription (Anisimov *et al.*, 2005; Fetherston *et al.*, 1996). The function of the Irp8 protein (YbtX in *Y. pestis*) has not yet been established (Brem *et al.*, 2001). Ybt is synthesized by a mixed non-ribosomal peptide synthesis (NRPS)/polyketide (PK) strategy that follows modular assembly of the siderophore from salicylate, a residue from malonyl coenzyme A, three cysteine molecules and three methyl groups (Gehring *et al.*, 1998). Irp9/YbtS, the first gene in the Ybt biosynthesis gene cluster, directly converts chorismate into salicylate, the precursor of Ybt (Pelludat *et al.*, 2003). This contrasts to salicylate synthesis in *Pseudomonas*, where two enzymes, namely isochorismate synthase and isochorismate pyruvate-lyase, are involved and both are required to complement an *irp9* mutant in *Yersinia*. Irp5/YbtE salicyl-AMP ligase transfers the activated salicylate to HMWP2 (encoded by *irp2*). HMWP2 possesses six predicted NRPS domains involved in initial cyclization and condensation reactions. Irp3/ YbtU reduces the internal thiazoline ring to a thiozolidine structure while the first five domains of HMWP1 switch from NRPS-type assembly line molecules to a PK-strategy. Irp4/YbtT contains a thioesterase domain to remove aberrant structures from the enzymatic complex and, displays an editing function together with terminal HMWP1 domains. Generally, NRPS/PK synthetases are activated by phosphopantetheinylation mediated by P-pant transferase.

Obviously, the general P-pant transferase of the Ybt system (YbtD), is located outside the HPI (Bobrov *et al.*, 2002). Phosphopantetheinylation of a peptidyl carrier protein domain of HMWP1 was also demonstrated in vitro using a heterologous EntD from E. coli (Gehring *et al.*, 1998). Thus, synthesis of the Ybt by the HPI-encoded genes and Ybt-mediated iron acquisition is tightly linked to the biochemistry of the bacterial cell. It could also be demonstrated by GFP-reporter technology in a mouse model that Ybt production is high in Yersinia-infected spleen and Peyer's patches, but low in the lumen of small intestine of infected mice (Jacobi *et al.*, 2001).

6.2 Mobility of the *Yersinia* HPI

Most PAIs are only ‘‘ghosts’’ of their former selves still loaded with remnants of the delivery genes. At least in *Y. pseudotuberculosis* serotype O:1, the HPI_{YPS} can occupy any of the three *asn* tRNA genes suggesting its functional mobility (Buchrieser *et al.*, 1998). In contrast to *Y. pseudotuberculosis*, the HPI is ‘‘frozen’’ in a single *asn* tRNA locus in *Y. pestis* and *Y. enterocolitica* biotype 1B. Whole genome sequencing discovered a second, truncated copy of the HPI in *Y. pestis* (Deng *et al.*, 2002; Parkhill *et al.*, 2001). This HPI-2 is not co-linear with the HPI-1, it is not associated with any tRNA loci, and contains only some but not all genes necessary for Ybt production. The genes *ybtP*, *ybtQ*, *ybtX*, *ybtS* corresponding to genes *irp6-9*, *ybtU* (*irp3*) and a large portion of *irp2* with several internal stop codons followed by an IS100 element reside on HPI-2. Presence of IS100 favours its possible role in duplication of the HPI and secondary rearrangements in HPI-2. HPI-2 is absent from *Y. pseudotuberculosis* (Hinchliffe *et al.*, 2003). The functionality of the remaining genes of the HPI-2 remains to be proven.

The HPI-integrase, a unidirectional site-specific recombinase is the main part of the genetic dissemination machinery encoded by the island (Rakin *et al.*, 2001). Moreover, because the HPI lacks replication functions it has to rescue itself by integration into the genome of the host cell. The integration is mediated by the HPI integrase that interacts with two pairs of short DNA sequences on recombining DNA molecules, *attP* and *attB*. One, *attP* (designated *attP* (POP’) by analogy to well-studied phage attachment sites involved in site-specific recombination), resides on the island and another, *attB* (BOB’, chromosomal recognition site) (Fig. 2) is represented by several *asn* tRNA gene copies on the bacterial genome. As a result of recombination between *attP* and *attB* sequences, the HPI is physically integrated into the bacterial chromosome between two new hybrid sites, *attL* (BOP’) and *attR* (POB’), that are chimeras composed of two halves of the *attP* and *attB* sequences, respectively. The expression of the HPI recombinase is differently regulated depending on its free, circularized state or its integrated state (Rakin *et al.*, 2001). The promoter of the HPI-integrase, P_{int}, is located within the *attP* site. A bacterial *asn* tRNA promoter replaces P_{int} as a result of the HPI integration (promoter swapping) (Fig. 2). The island in its integrated form is inherited by the host as a part of its genome. As HPI-carrying pathogens benefit from Ybt production during host infection, there is a selection for genetic stabilization of the integrated form of HPI. This is not only achieved by replacement of the P_{int} promoter, but also by deletions of the *int* gene or the *attR* site (Karch *et al.*, 1999; Rakin *et al.*, 1999a; Schubert *et al.*, 1999). To be transferred to new hosts, the HPI must be properly excised from its integration site

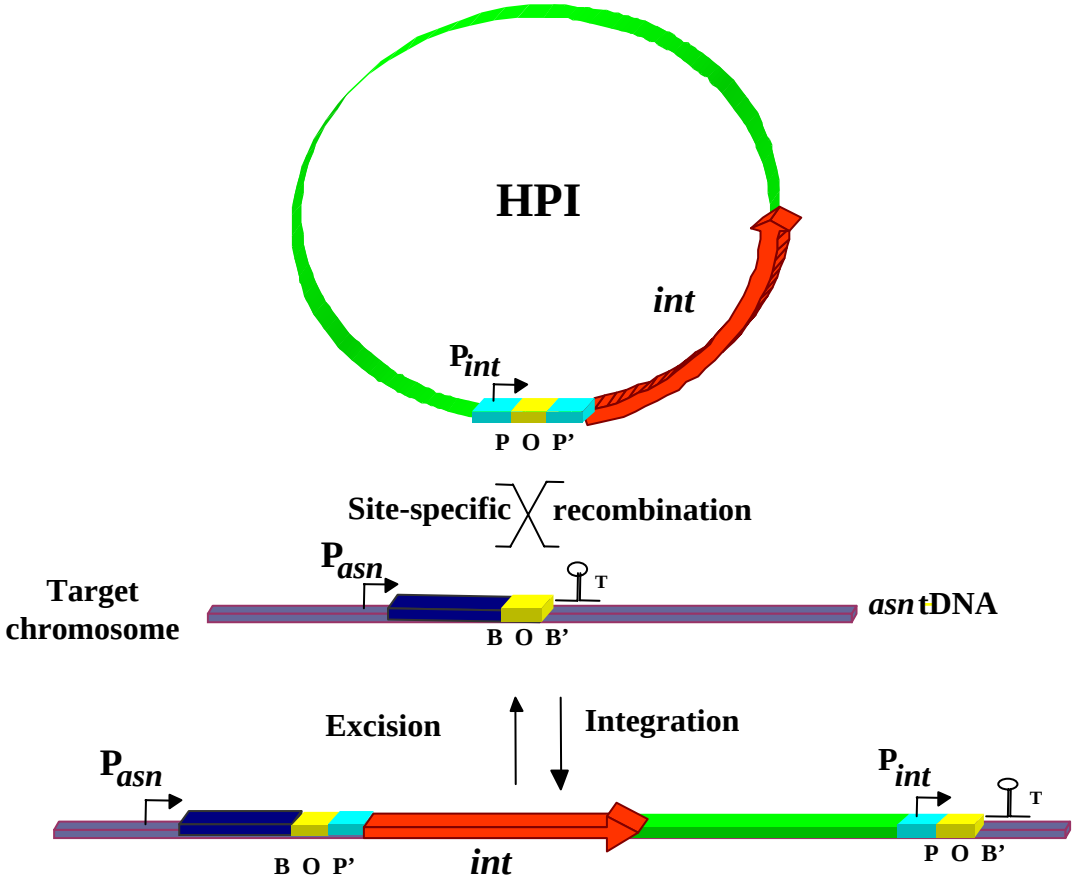


Fig. 2. Site-specific integration of the HPI into the asn-tDNA target on the chromosome.

Promoters of the *asn*-tRNA and *int* genes are shown as P_{asn} and P_{int}, respectively. "T" - rho-independent terminator structure of *asn*-tDNA. The 17 bp consensus core (yellow, "O") is bordered by arm-like sequences (P and P') in the *attP* site and also presented in the *attB* site (BOB'). Integrase gene (*int*) designated by red arrow.

on the bacterial chromosome being a reverse event to integration. However, integration and excision do not involve the same pair of reacting sequences and proteins. The directionality of site-specific recombination is controlled by the identity of the recombining sites and different proteins that mediate the two reactions. In case of prototype temperate bacteriophages, integration (*attB* x *attP*) requires the product of the gene *int*, the integrase, and the integration host factor IHF, while the excision (*attL* x *attR*) requires the product of an additional gene *xis*, an excisionase (also designated recombination directionality factor, RDF). Thus Int (and probably integration host factor IHF) is required for both reactions, while Xis (excisionase) plays an important role in controlling the direction of recombination. The HPI seems to utilize a selfish recombination module of the phage P4-like mobile group of elements for its dissemination. The HPI-integrase is a tyrosine recombinase homologous to the phage P4-like group of integrases. Also, site-specificity

of the integration mechanism of the HPI and implication of the tRNA loci as recognition sites lead to the conclusion that the HPI backbone "arrived in *Yersinia* via a bacteriophage" (Carniel, 1999). However, there is no evidence for this suggestion, e.g. there are no ''phage-associated'' genes besides those with similarity to recombination enzymes. It is of note that genes encoding phage-like recombination enzymes are present not only on temperate phages, but also on other mobile elements, e.g. conjugative transposons as well as ICEs (Burrus *et al.*, 2002). To become a phage-like mobile structure, the HPI sequence, which is highly degenerated if compared even to a satellite phage, must contain at least sequences necessary for its packaging to be recognized by a helper phage. Also, no specialized transducing phage has been assigned to the HPI. Generalized transduction cannot be ruled out, but the efficiency could not be expected to be high, due to the fact that the complete HPI including its *attL* and *attR* sites must be packaged and this structure must recircularize in the recipient cell to become recombinogenic. Parts of the island can be occasionally transferred by general transducing phages, but they have to recombine with complementary regions on the recipient chromosome. However, IS elements present on the island can supply regions of homology for such homologous recombination with the recipient genome. By definition, the HPIs of both evolutionary lineages in yersiniae are non-replicative, non-self-transmissible structural and genetic entities carrying fitness-associated genes and utilizing a site-specific mechanism of integration into conserved target sites (Rakin *et al.*, 1999b). Thus, the mechanism of HPI dissemination is completely obscure. An enlarged form of the *E. coli* HPI described recently in *E. coli* ECOR31 carries the complete HPI_{Yps} and a truncated 35 kb transfer region of a conjugative plasmid (Schubert *et al.*, 2004). The HPI_{ECOR31} can be considered as an integrative and conjugative element (ICE) (Burrus *et al.*, 2002). However, the HPI-ICE is restricted to a single *E. coli* strain and does not contain all the genes necessary for its self-transmission. Thus HPI_{ECOR31} could not be considered as a parental form of the HPI responsible for its wide dissemination.

On the other hand, certain transmissible plasmids (episomes) are able to integrate into the bacterial chromosome and "pick up" chromosomal sequences in course of incorrect excision (Jaoua *et al.*, 1990; Rigby and Fraser, 1989). Such "substituted" plasmids could carry small RNA genes that frequently serve as targets for integration of pathogenicity islands and other integrative elements (Williams, 2002). For example, pHCM2 plasmid from *Salmonella enterica* contains the putative *asn* tRNA gene (Kidgell *et al.*, 2002).

7. Aims of this research study

The aim of this research study was to characterize mechanisms and structures responsible for mobility and dissemination of *Yersinia pestis* High Pathogenicity Island.

The main goals of this project are:

- to discover the key elements, involved in mobility of *Y. pestis* HPI and responsible for its wide dissemination in *Enterobacteriaceae*;
- to define DNA/protein interactions of integrase, excisionase and attachment sites inside integrative complex;
- to develop a model of the HPI_{Yps} transmission, thus to explain how the island is transferred from one bacterial host to another and uncover the possible mechanisms involved in its transmission.

B. MATERIALS AND METHODS

1. Material

1.1 Equipment

Centrifuge	Sigma, Deisenhofen 1K1S, Table-centrifuge 3K30 with Rotor Nos. 12156 and 19776
Electrophoresis apparatus for SDS-PAGE	Bio-Rad, München Mini-Protean -II Cell and Western Blot Apparatus
Electroporation-apparatus	Bio-Rad, München, Gene Pulser, II Pulse Controller II
FACSscan (Flow Cytometer)	Becton Dickinson, Heidelberg
French Press	French Pressure Cell 40K SLM Aminco
HPLC system	SMART system, Pharmacia Biotech
Hybridization oven	Personal Hyb. Stratagene, Amsterdam
Incubator	Heraeus, Hanau Typ B20
Light Cycler	Light Cycler PCR and detection system, Roche Diagnostics
Luminometer	MicroLumat Plus LB96V Luminometer, Berthold Technologies
PCR-Cycler	PE Applied Biosystems, Weiterstadt Gene Amp 2400
pH Meter	Mettler, Toledo 320 pH Meter
Photometer	Pharmacia, Biotech Ultrospec 2000
Pipettes	Eppendorf, Hamburg Research P10-P1000
Phosphorimager	Pharmacia LKB ImageMaster DTS
Sequencer	PE Applied Biosystems, Weiterstadt ABI 377 DNA Sequencer
Shaking incubator	Braun, Melsungen Certomat BS-1
Sterile bank	Heraeus, Hanau Herasafe HS12
Transilluminator	Heralab, Wiesloch, UVT-20M/W
Video-equipment	Sigma, Deisenhofen, EASY (Enhanced Analysis System)

Vacuum blot	Pharmacia,-LKB, Uppsala, LKB 2016 Vacu Gene^{R} - Chamber
Scale	Sartorius, Göttingen Model R 160P and Pt 1200

1.2 Other materials

Plastic and related articles were purchased from the following firms: Nunc, Roskilde, DK; Sartorius, Göttingen; Falco/Becton Dickinson, Heidelberg; B. Braun, Melsungen; Eppendorf, Hamburg; Greiner, Nürtingen and Schleicher & Schüll, Dassel.Nylon membranes (Zeta Probe GT) were purchased from Biorad and Nitrocellulose membranes (Whatman-paper) from Schleicher & Schüll.

1.3 Chemicals and Enzymes

All chemicals and antibiotics were supplied by Merck (Darmstadt), Biochrom (Berlin), Roche (Mannheim) and Sigma (Deisenhofen). Media plates were supplied by Difco (Detroit, Michigan, USA) and enzymes were obtained from MBI Fermentas (St. Leon-Roth), Roche (Mannheim), and Gibco (Eggenstein).

2. Bacteria, Plasmids and Primers

2.1 Bacterial strains and plasmids (Table 1)

Strain / plasmid	Relevant Characteristics	References or source
Srains		
E. coli		
BL21 (DE3)	F^{-} *ompT hsdS*(r_B^- m_B^-) *gal* λ (DE3)	Stratagene
CC118λpir	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-I rpsE rpoB argE*(Am) *recA1*; lysogenized with λ*pir*	(Herrero *et al.*, 1990)
CFT073	Clinical isolate of uropathogenic *E. coli*	(Welch *et al.*, 2002)
DH5α	*end*A1 *hsd*R17(rk-mk+)*sup*E*44 thi*-1 *recA1 gyrA relA1* Δ(*lacZYA-argF*) U169 (ϕ80*lacZ*Δ*M15*)	(Hanahan, 1983)

JM109	{ *recA1 endA1 gyrA96 thi hsdR17*(r_k-, m_k+) *supE44 relA1* Δ(*lac-proAB*) [F' *traD36 proAB lacI*^{q}ZΔM15]}	(Yanisch-Perron *et al.*, 1985)
JM109 Nal^r	The spontaneous Nal^R mutant of *E. coli* strain JM109	MvP strain collection
HB101	*SupE*44 *hsdS*20(r_B^- m_B^-) *rec*A13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1	(Bolivar and Backman, 1979)
S17-1 λpir	*pir*$^+$ *tra*$^+$	(Simon *et al.*, 1983)
Y. enterocolitica		
WA-C	Plasmidless derivative of strain WA-314, serotype O:8	(Heesemann *et al.*, 1984)
WA-C *hsmYI*, *hsrYI*	*hsmYI*, *hsrYI*	this study
WA-CS irp1::Kan^r	*irp1*-mutant of WA-CS, Nal^r, Sm^r, Kan^r	(Pelludat *et al.*, 2002)
Y. pestis		
KUMA	BG M	R. R. Brubaker
Y. pseudotuberculosis		
YPS06	Clinical isolate	MvP strain collection
YPS06 *xis*	YPS06 with inactivated HPI excisionase gene	this study
Plasmids		
pCR2.1-TOPO	Topo cloning vector	Invitrogen
pCR2.1-TOPO/*yenI*	pCR2.1-TOPO with *yenI* ORF	this study
pCJ Luc	pCJFY-L derivate containing promoterless luciferase gene	(Jacobi *et al.*, 2001)
pCJ P_{intB}-Luc	pCJFY-L derivate containing intB promoter - luciferase fusion	this study
pCJ P_{orf1}-Luc	pCJFY-L derivate containing orf1 promoter - luciferase fusion	this study
pCJ P_{orf2}-Luc	pCJFY-L derivate containing putative orf2 promoter – luciferase fusion	this study
pCP20	Plasmid with thermo-inducible FLP recombinase	(Datsenko and Wanner, 2000)
pET-3C	Expression vector	Invitrogen
pET-3C *xis*	Excisionase expression vector	this study
pGEX-4T-3*int*	Integrase expression vector	this study

pQE-30*int*	Integrase expression vector	this study
pET-3C *int*	Integrase expression vector	this study
pET-3C *hsmYI*	Plasmid for expression of restriction-deficient Yen I protein	this study
pIE928	Plasmid with streptothricine resistance gene *sat3*	(Jelenska *et al.*, 2000)
pMOS*Blue*	Cloning vector Ap^r	Amersham
RP4	The conjugative plasmid	(Kim *et al.*, 1993)
RP4 *attB*	HPI-"trapping" construct	this study
pKD46	Plasmid carrying red recombinase system genes	(Datsenko and Wanner, 2000)
pKD3	Plasmid carrying Cm^r gene was used as a source of Cm^r cassete	(Datsenko and Wanner, 2000)
pKD4	Plasmid carrying Km^r gene was used as a source of Km^r cassete	(Datsenko and Wanner, 2000)
pKR528	*attP-int*B cloned in suicide vector pKAS32, Ap^r	(Rakin *et al.*, 2001)
pKR529	Suicide plasmid carrying the *attP* (POP´) part and the functional integrase of *Y. pestis* KUMA, Km^r, Ap^r	(Rakin *et al.*, 2001)
pKR529 *orf1-5r*	pKR529 with *orf1-5* of the variable part of the Yps-HPI	this study
pKR531	pKR528 with inactivated integrase gene	MvP collection
pKR600	Plasmid harbouring the *asn* tRNA gene	(Rakin *et al.*, 2001)
pGP1-2	vector with T7 RNA polymerase gene	(Tabor and Richardson, 1985)
pSAK2	Recombinant plasmid carrying a 5039 bp DNA fragment with *yenI* from *Y. enterocolitica* 8081 isolate	V.L. Miller

2.2 List of primers (Table 2)

Primer	5'-…..-3' sequence	Description
T7 (forward)	TAATACGACTCACTATAGGGA	amplifies the insert within the MCS of pMOS*Blue* vector

U19 (reverse)	GTTTTCCCAGTCACGACGT	amplifies the insert within the MCS of pMOS*Blue* vector
rms_for	ATGTTAGAAGAAGTTGATGAAATCCGAGTC	start of *yenI* gene
rms_rev	TTAGTTATGTGGACCTAAGAACCTGTCG	end of *yenI* gene
asnT167	CCCCAGAACTTTTTGCTCCT	forward primer for *asnT* tRNA gene
asnW761	GTCGGGTTGTAGTCGGTTATG	forward primer for *asnW* tRNA gene
asnU211	ACAACCTGGCGTAAAGCAGAG	forward primer for *asnU* tRNA gene
asnV251	AGTGCCGCCATTACTTACAAC	forward primer for *asnV* tRNA gene
c15-205	TACAGGCAGGTTCCCGATGAC	in int_{HPI} gene reverse
ORF2F Nde	CGCCATATGACATCTTATCAGTTACTAC	start of xis_{HPI} gene with *Nde*I site
ORF2R Bam	CGGGATCCCATCATCTTCTCCTCATTGCG	end of xis_{HPI} gene with *Bam*HI site
attB128	TTGGATCCGATGCGCCCCGTTCACAC	start of *attB*
attB320	AAAAGCTTGGAGAGGAAGGGTGCTGTTGA	end of *attB*
attP72	CGCAACTATTGGTGGTCATTA	start of HPI *attP*
attP72 Hind	TTCGGCCGCGCAACTATTGGTGGTCATTA	start of HPI *attP* with *Hind*III site
attP298 Bam	TTGGATCCGAACTAACCTGACCCCAGAT	in *attP,* reverse, with *Bam*HI site
attR241	AGCGGCCGCTTTGCGTCGGTAAGGGACATA	end of HPI *attP*, reverse
FyuAF	GACCGTTATCGCCATTCTG	*fyuA* promoter forward
FyuAR	CCGTGTCATTTTCATTGTTG	*fyuA* promoter reverse
exc1000R	AATAGACCGATAGTAGGATGTTGCCACTCAAGG	in excisionase reverse
exc914	CCGTTATAGTGACCCATGTTGAC	in excisionase reverse
exc941	TGCTTCCGTTATAGTGACCCA	in excisionase reverse
exc1010	TGGCAACATCCTACTATCGGTCTA	in excisionase forward
exc1025	ATCGGTCTATTTCCAGTCTCCT	in excisionase forward
exc1135R-P	Pho-CCATAAATAAAACCGTCCCTGT	in excisionase reverse
orf1P Hind	AAAAGCTTCCCCATAGGCCTGTACATGT	in *orf1* promoter, with *Hind*III site
orf1P Bam	TTGGATCCATCTCCTTCTACACAACAATTC	in *orf1* promoter, with *Bam*HI site
orf2P Hind	AAAAGCTTGCTGAAACACTGAAAAATGCG	in *orf2* promoter, with *Hind*III site
orf2P Bam	AACCATGGATCCTCCTGTGACTGAAATAA	in *orf2* promoter, with *Bam*HI site
HPI174	CAGGGCCTATTTTTATTGAAC	annealing in excisionase
HPI878	GGGGGCAAGAAAAACTAACC	annealing in *orf1*
Int_rev15	CGTGAGAATCGGAGACTTTAAAGG	forward for HPI *attP*
cftattp501	AACGAGTACAGATTGTAGATGTACG	reverse for Ecoc54N *attP*
cftattp601	GAACCATCACCATAATTTTTAGTGTC	reverse for Ecoc54N *attP*
Intcft698_rev	AAAGACATGTCTGTTCAGACGGGC	reverse for Ecoc54N integrase
Int_cft1087	GGTATCGTCCAGATCCGATTTCTGAA	reverse for Ecoc54N integrase

attR248	TTGGCTCCTCTGACTGG	amplifies within *attP*
CoreD	GATCCCAGTCAGAGGAGCCAA	amplifies within *attP*
attP135	TTAGCCAGTTGCTGGCAGAGGC	amplifies within *attP*
attP247	AAGAGTTTTCACACTAACCTGT	amplifies within *attP*
HPI 1220	TTTGTTTTATGGCTTTGGTAG	amplifies within HPI AT-rich region
fyu18	AGGCGACTGAACGGATGAACA	in *fyuA* gene, forward
aph_wild_for	GTGAACGATATTGATCGAGAAGAGC	for RP4 *aph* gene, forward
sat3_seq	AGATGACCAATTCACGCATTGA	for *sat3* gene, reverse
IS131	GCTACTCATTCCCTGCTTGTG	in IS131 of HPI, forvard
RP_mut_for	GCAGCCGCTGCCGTGCCCGAGAGCATGGCGGCT CACGTGATGGGATACGATGCGCCCCGTTCAC	for PCR-directed mutagenesis
RP_mut_rev	GCAGCTTGCGCCTATCCGGATCGGCAATGCCAT ATTGCGCAA CAAGCCACTCATTCATAACTCC	for PCR-directed mutagenesis
HPI_ins2	TTTGAGTAGTGTACCTGAGTGATATTTGTGTTAT GTATGCATTGATTGCAGTGTAGGCTGGAGCTGC TTC	for PCR-directed mutagenesis
HPI_ins2_rev	TCAACTCAAAACAATTTCGAAAACTCAAAGATT TCATCGGCAAAAACAGCATATGAATATCCTCCT TA	for PCR-directed mutagenesis

All the primers used in this work were supplied by Metabion (Martinsried). They were supplied in either a 100 pmol/µl solution or lyophilized. Lyophilized primers were dissolved in distilled, sterile water to a 100 pmol/µl end concentration. Table 2 gives a list of the primers used in this work.

3. Culture media, Antibiotics, Strain Cultivation and Storage

3.1 Culture media

Liquid media were sterilized by autoclaving (121 °C at 1 bar for 20 min). For solid agar, 15 g agar per liter of liquid media was used.

Luria-Bertani (LB) Medium	Trypton	10 g
	Yeast powder extract	5 g
	NaCl	5 g
	H_2O_{dest} to	1 L
	pH set to 7.4 - 7.6 with NaOH	
Minimal medium (M9)	Na_2HPO_4	6 g
	KH_2PO_4	3 g
	NaCl	0.5 g
	NH_4Cl	1 g
	Water to	1 L
	pH adjusted to 7.4, autoclaved and cooled	
Nutrient Broth (NB) Medium	Nutrient Broth	8 g
	NaCl	5 g
	Water to	1 L
NBD Medium	NB-Medium with 200 µM dipyridyl (in 70 % EtOH) (Dipyridyl functions as an intracellular complexer of Fe^{2+})	
CAS-Agar	Chromazurol S	60.5 mg
	1mM FeCl3 *6H2O	10 ml
	HDTMA	72,9 mg
	10X MM9 salts	100 ml
	Agar	15 g
	PIPES	30.4 g
	10X LB medium	30 ml
	20% Glucose	10 ml
	1M $MgSO_4$	2 ml
	1M Na_2SO_4	2 ml
	0,1M $CaCl_2$	1 ml

	Water to	1 L
Further media components	20% Glucose	
	1M $CaCl_2$	

3.2 Antibiotics

Name and concentration of antibiotics employed in this research are listed in table 3. Sterilization of all antibiotics was by filtration with 0.22 µm filters.

Table 3. List of Antibiotics

Antibiotic	Abbreviation	Dissolved in	End-concentration (µg/ml)
Ampicillin	Amp	H_2O_{dest}	100 (*E. coli*)
			400 (*Yersinia*)
Kanamycin	Km	H_2O_{dest}	50
Nalidixic acid	Nal	0.5 N NaOH	100
Chloramphenicol	Cm	70 % C_2H_5OH	30
Streptomycin	Sm	H_2O_{dest}	100
Streptothricine	St	H_2O_{dest}	100
Tetracycline	Tet	70 % C_2H_5OH	15

3.3 Cultivation and long term storage of bacteria

Bacteria were cultivated either on agar plates or in liquid medium by incubation on a shaker as follows:

- *Yersinia*: 200 rpm at 27°C
- *E. coli*: 200 rpm at 37°C. For long term storage, bacteria were suspended in LB-Medium fortified with 10% Glycerol and frozen at -80°C. Table 1 presents a summary of the strains and plasmids used in this study.

4. Molecular genetic methods

4.1 Isolation of chromosomal DNA with Qiagen Genomic-tip 100/G

The Qiagen Genomic-tip 100/G kit was routinely used for small scale isolation of chromosomal DNA (up to 100 µg). The isolation procedure was as recommended by the kit's manufacturer.

4.2 Isolation of plasmid DNA

4.2.1 Plasmid isolation with QIAprep Spin Miniprep kit (Qiagen)

The QIAprep Spin Miniprep kit was routinely used for small scale isolation of plasmid DNA (up to 20 µg). The principle behind it is based on alkaline lysis, coupled with anion-exchange chromatography. The isolation procedure was as recommended by the kit's manufacturer.

4.2.2 Plasmid isolation with Nucleobond AX100 Kit (Machery-Nagel)

The Nucleobond AX100 Kit was used for the isolation of high quality DNA in high concentration (up to 100 µg). The principle of DNA isolation is also based on alkaline lysis of cells, followed by purification of nucleic acids on the basis of anion-exchange chromatography. The isolation procedure was as recommended by the kit's manufacturer.

4.3 Purification DNA and determination of DNA concentration and purity

4.3.1 Phenol extraction and ethanol precipitation of DNA

Phenol extraction was carried out to remove contaminating proteins from a DNA preparation.

Procedure

- The DNA solution was mixed with an equal volume of TE - saturated phenol/chloroform/isoamyl alcohol (25:24:1) in a microcentrifuge tube and the mixture vortexed for 30 sec.
- The mixture was centrifuged at 14,000 rpm for 5 min at RT to separate the sample into phases.
- The upper aqueous layer was then removed into a clean tube, carefully avoiding denatured proteins found at the aqueous / phenol interface. This upper phase was then mixed with an equal volume of the phenol / chloroform / isoamyl alcohol solution mentioned above, the mixture

vortexed and centrifuged (14,000 rpm for 5 min). This step was repeated 2-3 times, and the DNA precipitated from the upper aqueous phase through ethanol precipitation.

Ethanol precipitation

This was carried out to remove contaminating salts from a DNA preparation or to concentrate a DNA preparation.

Procedure

• The DNA solution was mixed with 1/10 volume of 3 M sodium acetate and 3 volumes of ethanol.

• The mixture was incubated at -20°C for 30 min.

• The mixture was centrifuged at 14,000 rpm for 15 min at 4°C.

• The supernatant was removed and the DNA pellet was washed with 70% ethanol and centrifuged at 14,000 rpm for 5 min at 4°C.

• The pellet was air-dried and the DNA resuspended in water and stored at -20°C.

4.3.2 Determination of DNA concentration and purity

Nucleic acids have a maximum light absorption at 260nm wavelength. The isolated DNA was diluted with distilled water (1:100) and the absorbance at 260nm (A_{260}) against H_2O_{bidest} measured spectrophotometrically. The calculation of the DNA concentration was based on the following formula:

$$\mathbf{A_{260} = 1 \triangleq 50\ \mu g/ml}\ \text{for dsDNA}$$

$$\mathbf{A_{260} = 1 \triangleq 33\ \mu g/ml}\ \text{for ssDNA}$$

For determination of DNA purity, the A_{260}/A_{280} coefficient was photometrically determined (A_{280} - absorbance at 280nm). An $A_{260}/A_{280} < 1.8$ indicated contamination of the DNA preparation with protein or aromatic substances such as phenol, while an $A_{260}/A_{280} > 2.0$ indicated possible contamination with RNA (LAB FAQs, Roche).

4.4 Polymerase Chain Reaction (Saiki *et al.*, 1988)

The polymerase chain reaction (PCR) permits the selective *in vitro* amplification of a particular DNA region by mimicking the phenomenon of *in vivo* DNA replication. Typically, three steps are involved in a standard PCR reaction: denaturation, which achieves the dissociation of the template DNA molecules into single strands; annealing, which allows single stranded primers to bind to complementary sites on the template DNA; and lastly elongation which allows

for extension of the DNA strands, due to the effect of the thermostable DNA polymerase. As template DNA, either plasmid, cosmid or chromosomal DNA was utilized at a diluted concentration, or cooked cells were employed. Where cooked cells were used as source of template DNA, the procedure was as follows:

• A bacterial colony was isolated, suspended in 50 µl H_2O_{bidest}, boiled for 5 min and centrifuged (14,000 rpm for 1 min).

• The supernatant containing released DNA template was then utilized in the PCR reaction.

For a typical 50 µl reaction volume, the following components were pipetted into a PCR test-tube:

Reaction components	Template DNA	0,1-100 ng
	Primer 1 (100 pmol/µl)	0.2 µl
	Primer 2 (100 pmol/µl)	0.2 µl
	dATP, dCTP, dGTP, dTTP, 2mM	5 µl
	10 x Taq-Reaction buffer	5 µl
	Taq-polymerase (5 U/µl)	0.2 µl
	H_2O	ad to 50 µl
Cycling parameters	Denaturation 94°C	3 min
	Denaturation* 94°C	30 sec
	Annealing* x°C	30 sec
	Elongation* 72°C	y min
	Final extension 72°C	3 min

* 30 - 35 cycles
x: Annealing temperature dependent on the Tm (melting temperature) of primers
y: Elongation is typically 1 min pro kb of amplified DNA

A negative control with water as template DNA was always included in the reactions and 5 µl of the finished PCR product was checked on an agarose gel before purification with the Qiagen PCR purification kit.

4.4.1. Nested PCR screening for genomic islands excision

The same amount of genomic DNA (100ng) of *E. coli* CFT073 and *E. coli* JM109 (as a control) were amplified by first PCR using primer pair Int_cft1087/cftattp501. One microliter

from first-round reaction was used as template for second round PCR using primer pair Intcft698_rev/cftattp601 (Table 2).

4.4.2. Real Time PCR and quantification of *attP*-targets

The Light Cycler PCR and detection system (Roche Diagnostics, Mannheim, Germany) was used for amplification and online quantification. Int_rev15/attP72 and Intcft698_rev/cftattp601 primer pairs were used for amplification of the restored HPI-*attP* and Ecoc54N-*attP*, respectively (Table 2). For the amplification of chromosomal markers, primers coreD and Int_rev15 were used (Table 2). For amplification detection, the Light Cycler DNA FastStart Master Hybridisation Probes Kit (Roche Diagnostics) was used as described by the manufacturer. Following hybridization probes were used: attP_hyb1 (5'-CCC ATA TGT CCC TTA CCG ACG CAA A-Fluo-3') and attP_hyb2 (5'-LCRed-640-TCC GCA CCC TCA AGC CTT CTG ATA AA-Pho-3') for HPI-*attP*; attPcft_hyb1 (5'-CCA TAT GTC CCT TAA CGA CGC AAA-Fluo-3') and attPcft_hyb2 (5'-LCRed-640-TCC GTA GTC TCA AGC CCA CTG ATA AA-Pho-3') for Ecoc54N-*attP* (Metabion GmbH, Martinsried, Germany). Quantification was performed by online monitoring of the crossing points. The number of cycles of the log-linear phase was plotted against the logarithm of concentration of the template DNA. External standardization was performed using pKR528 plasmid, containing the HPI-*attP*-site. Statistical analysis and data processing was done using RelQuant 1.01 relative quantification software.

4.5 Agarose gel electrophoresis

The agarose gel was prepared by mixing an appropriate proportion of agarose (to a final concentration of 0.7 - 2% depending on the MW of the sample DNA) with 1 x TAE buffer, the mixture cooked and after cooling poured into precast agarose gel chambers. The DNA was then mixed with loading buffer, loaded onto spurs on the gel and electrophoretically separated by voltage application utilizing the 1 x TAE solution as the running buffer. Following the electrophoretic run, gels were stained in ethidium bromide solution and the DNA visualized under ultraviolet radiation.

Solutions:

1x TAE buffer	40mM Tris / HCl, pH = 8.2 20mM Acetic acid 2mM EDTA, pH 7.6
10 x Loading buffer for agarose gels	0.25% (w/v) Bromophenol blue 10% (v/v) Glycerol
Agarose gel	0.7% - 2% Agarose in 1 x TAE
Ethidium bromide staining solution:	1 µg Ethidium bromide pro ml H_2O

4.6 Enzymatic modification of DNA

4.6.1 Restriction digestion of DNA

Chromosomal or plasmid DNA samples were routinely subjected to restriction digestions. For a restriction endonuclease reaction, the following components were mixed together and incubated at the appropriate temperature (usually 37°C for most enzymes):

DNA	x µl
10 x Reaction buffer[x]	1 µl
Enzyme	2 - 3 units / µg DNA
H_2O	to 10 µl*

* For higher DNA concentrations, the reaction and volume were scaled up linearly.
x: Choice of reaction buffer depended on the type of enzyme employed.

Because all reaction enzymes are supplied in 50% glycerol, which can exert an inhibitory effect on digestion efficiency, care was taken that the glycerol concentration did not exceed 5% final digestion volume. Enzyme inactivation was either through heat treatment at 65°C for 20 min (Lab FAQs, Roche).

4.6.2 Dephosphorylation of DNA

This procedure removes the phosphate ends arising after digestion of a vector/plasmid DNA with restriction endonucleases, thus preventing dimerization or self-religation of vector or plasmid DNA. The vector DNA is then free to ligate with an insert DNA of choice. Shrimp alkaline phosphatase (SAP from Roche, Mannheim) was employed and the reaction proceeded at 37°C for 30 minutes, followed by heat inactivation at 70°C for 20 min.

4.6.3 Ligation of DNA molecules

Ligation of linear DNA molecules was with the enzyme T4 DNA ligase (Gibco, Eggenstein). Typically, a 1:5 vector to insert ratio was utilized for all ligations and the reaction proceeded at 16°C overnight.

4.7 DNA sequencing

DNA Sequencing was done by the dideoxy-chain terminating method on an automated ABI Prism DNA Sequencer. The ensuing chromatograms were processed with Chromas software and BLASTN and BLASTX programs provided by NCBI (National Center for Biotechnology Information) and TIGR (The Institute for Genomic Research), and also the *Y. pestis* and *Y. enterocolitica* gene banks from Sanger Center were employed for in-depth homology searches.

4.8 RNA analysis

4.8.1 RNA isolation

Precautionary steps

Due to high degradation potential of RNA, the following precautions were strictly followed: Special set of pipettes and tips (10, 100 and 1000 µl) exclusively set aside for RNA work; all solutions were prepared with water treated with diethylpyrocarbonate (DEPC), a strong RNAse inhibitor; RNA isolation procedures were rapidly carried out to prevent premature degradation of the RNA template. RNA samples were usually treated with DNase (see below) to remove contaminating DNA before use in reverse transcription assays.

Isolation of RNA

RNA isolation was carried out with the TRIZOL Reagent (a monophasic solution of phenol

and guanidine isothiocyanate) from Gibco as follows: Bacterial cells were pelleted and homogenized in 1 ml of TRIZOL reagent. The mixture was incubated at RT for 5 min to achieve complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added and the tubes were vigorously shaken by hand for 15 secs and incubated at RT for 3 min. Samples were centrifuged at 12,000 x g for 15 min at 4 °C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase, which is about 60 % of the volume of the TRIZOL reagent used for homogenization. The aqueous upper phase was then transferred to a fresh tube and the RNA precipitated by mixing with 0.5 ml isopropyl alcohol. The sample was incubated at RT for 10 min and centrifuged at 12,000 x g for 10 minutes at 4 °C. The RNA precipitate, often invisible before precipitation, forms a gel-like pellet. The supernatant was discarded and the RNA washed once with 1 ml 75 % EtOH and centrifuged at 7,500 x g for 5 min at 4 °C. The RNA was then air-dried and dissolved in RNase-free water.

4.8.2 DNase reaction

This was essential to remove DNA contaminants from the RNA preparation.

Procedure

Reaction components :

RNA (up to 1 µg)	x µl
DNase incubation buffer	1 µl
DNase	1 u
Incubation:	
RNase free water	ad 10 µl
	15 min at RT

Reaction stop:

25 mM EDTA (pH 8) 1 µl

10 min heat treatment at 65 °C

4.8.3 Reverse Transcription

Reverse transcription is an enzyme-catalyzed synthesis of cDNA from an RNA matrix in the presence of a gene specific primer and dNTPs. The Superscript™ II RNase H$^-$ .Reverse Transcriptase (Gibco, Eggenstein) was used in all reverse transcription analyses as described

below. To check whether *orf1* and *orf2* are located in the same operon, the generated cDNA was employed as a template for PCR using primers HPI878 (annealing in *orf1*) and HPI174 (annealing in *orf2*). As a negative control reaction, RNA sample without reverse transcriptase was also always included to exclude the possibility of false positive reactions due to DNA contamination.

4.8.4. Mapping the start of *orf1* transcription

The determination of the 5´terminus of *orf1* was performed by the RACE method (Tillett *et al.*, 2000). Total RNA of *E. coli* DH5α *asnT*::pKR529*orf1-2* was isolated using the "High pure isolation kit" from Roche Diagnostics GmbH. Phosphorylated primer exc1135R-P was used in RT-PCR that was accomplished with Superscript III RNase H^- Reverse Transcriptase from Invitrogen (Karlsruhe, Germany). The resulting cDNA was self-ligated with T4-RNA-ligase from Roche Diagnostics GmbH. For amplification of the transcript, 3 rounds of semi-nested PCRs were performed using exc1010/exc1003R primers for the first PCR, exc1025/exc941 primers for the second nested PCR and exc1025/exc914 primers for the third semi-nested PCR. The resulting PCR product was sequenced from both sites using the same primers as for the third PCR.

4.9 Bacterial transformation

Bacterial cells were made electrocompetent using standard procedures, and then transformed with plasmid/cosmid DNA as described below.

4.9.1 Production of electrocompetent cells

A modified protocol from Hanahan (Hanahan and Meselson, 1983) was employed. The procedure was carried out in the cold and under sterile conditions.

Procedure

50 ml LB medium was inoculated with an overnight culture of the bacterium (*E. coli* or *Yersinia*) and incubated with vigorous shaking at 27°C/37°C until an OD_{600} of 0.5 - 0.6 was achieved.

- The cells were chilled on ice for 10 - 15 min and transferred to 50-ml falcon tubes.
- Cells were then centrifuged at 4000 rpm for 25 min at 4°C.
- The supernatant was decanted and cells resuspended in 50 ml of sterile ice-cold water (sterile), mixed well and centrifuged under the same conditions as above.

• The above wash step was repeated, following which cells were washed with 50 ml ice-cold 10% glycerol (centrifuged in the cold at 4000 rpm for 25 min).
• The glycerol solution was decanted and the cell volume estimated and cells resuspended in an equal volume of ice-cold glycerol.
• Cells were then aliquoted in 50 µl volumes and stored at -80°C until required.

4.9.2 Transformation procedure

Electroporation with high voltage was achieved with the Gene Pulser II from Bio-Rad. The principle relies on the fact that short electrical impulses directed at bacterial cells generate pores in the cell membrane that facilitates entry of foreign or exogenous DNA into the cell (Dower *et al.*, 1988). The settings employed were 25 µF capacitance at 2.5 kV and 200 ohms. After electroporation transformed cells were mixed with 1 ml LB medium and incubated at 27°C/37°C with shaking for 50 min. Bacterial cells were then plated out in 100 - 200 µl aliquots on LB-agar plates containing the required antibiotics for selection of recombinants.

4.9.3 Preparation of X-gal/IPTG LB-agar plates for blue-white screening of recombinants

• For one plate 35 µl of 50 mg/ml X-gal and 20 µl of 100 mM IPTG were added to 30 ml LB-agar with an appropriate antibiotic.
• The medium was dropped on plates.
• The plates were left to soak for at least 30 min prior to plating.
• 10 - 50 µl of each transformant was then spread on the LB agar X-gal/IPTG plates.
Inverted plates were incubated overnight at 37°C.

4.10 Conjugation (Achtman *et al.*, 1978)

Bacterial conjugation is the transfer of genetic material between donor end recipient bacterial cells through cell-to-cell contact. For this purpose, 1 ml of the overnight culture of the recipient strain and 1 ml of the early-log phase culture of the donor strain were centrifuged, washed and resuspended in isotonic NaCl solution. The mating mixture was collected on a membrane filter (pore size, 0.22 µm). The membrane was transferred on a LB agar plate and incubated at 37°C for 5 h. Next, the cells were resuspended in 1 ml of isotonic NaCl solution and plated on selective plates.

5. Enzyme activity assays

5.1 Luciferase assay

The *intB* promoter and the putative promoters of *orf1* and *orf2* were amplified from *Y. pestis* KUMA chromosomal DNA by PCR using primers attP72 Hind and attP298 Bam for *intB*, orf1P Hind and orf1P Bam for *orf1* and orf2P Hind and orf2P Bam for *orf2*, introducing *Bam* HI and *Hin*d III restriction sites (Table 2). The PCR products were digested with *Bam* HI and *Hin*d III and ligated into vector pCJFY5Luc (Jacobi *et al.*, 2001) substituting *fyuA* promoter for *intB, orf1* or *orf2* promoters. To obtain a promoterless luciferase gene, the *fyuA*-promoter of pCJFY5Luc was eliminated by *Bam*HI/*Hin*d III digestion followed by filling-in the overhanging ends with Klenow enzyme and self-ligation of the construct. Electrocompetent *E. coli* DH5α was transformed by these plasmids.

The resulting transformants were grown at 37 °C in LB medium (supplemented with 20 μg/ml chloramphenicol) to an $A_{600\ nm}$ of 1.0. One milliliter of each cell culture was centrifuged (5 minutes, 2000 g) and the pellets were lysed with luciferase lysis buffer according to manufacturer's instructions (Roche, Germany). Luciferase activity was measured using the MicroLumat Plus LB96V Luminometer (Berthold Technologies, Germany) as previously described (Jacobi *et al.*, 2001).

5.2 Quantification of GFP fluorescence for GFP-reporter studies with iron-regulated promoters

A Becton Dickinson flow cytometer equipped with an argon 488-nm laser was used for determination of GFP fluorescence of single bacterial cells. In vitro iron-derepressed recombinant yersiniae (grown in NBD broth) were diluted as required, and the bacteria were detected by side scatter. The average intensity of fluorescence was determined. The scale was logarithmic, and fluorescence data and scatter data were collected for 10,000 and 50,000 events (Jacobi *et al.*, 2001).

6. *In vitro* DNA-binding assays

6.1 Electrophoretic Mobility Shift Assay (EMSA)

The ability of recombinant excisionase Xis_{HPI} or integrase Int_{HPI} to bind DNA was evaluated by electrophoretic mobility shift assays using ^{32}P-end labeled probes generated by PCR. The

213bp *attB* site was amplified from pKR600 (Rakin *et al.*, 2001) using attB128 and attB320 and 280bp HPI-*attP* site, from pKR528 using attP72 and attR241 primers. The fragments of HPI-*attP* was amplified as follows: Frag1 from pKR528 using attP72 and attR248 primers; Frag2 from pKR528 using CoreD and attR241 primers; Frag3 from pKR528 using attP135 and attR241 primers; Frag4 from pKR528 using attP72 and attP247 primers. The Ecoc54N-*attP* site was amplified using cftattp601 and attR241 primers. A non-specific competitive 176bp DNA probe was generated from genomic DNA of *Y. enterocolitica* O: 8 using primers FyuAF and FyuAR (Table 2). Approximately 50 fmol of the probe was incubated at 28°C with varying amounts of Xis-HPI, as appropriate, in 10 µl binding buffer containing 20 mM Hepes, pH 7,6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, Tween 20 0,2% (w/v), 30 mM KCl and 250 ng/µl Poly [d(I-C)], Poly [d(A-T)] each. After 1 h, the samples were applied to a 5% acrylamide gel (acrylamide/bis-acrylamide 29:1) gel in 0,25X TBE buffer and electrophoresed at 10 V/cm. Gels were dried and analysed by phosphorimaging.

6.2 DNase I footprinting assay

FAM-labeled *attP* DNA probe was generated by PCR using attP72 and FAM-attR241 primer pair. 0.1 pM of FAM-labeled *attP* DNA was added to different amounts of protein, diluted in reaction buffer (20 mM Hepes, pH 7.6, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, Tween 20 0.2% (w/v), 30 mM KCl and 250 ng/µl Poly [d(I-C)], Poly [d(A-T)]) and incubated for 30 minutes at room temperature in a reaction volume of 10 µl. Then, 10 mU of DNase I (Roche) was added and the reaction was stopped after 3 min by addition of an equal volume of phenol. Electrophoresis and gel scan was performed using the ABI 377 DNA sequencer (ABI Prism, Perkin-Elmer, Boston, U.S.A).

7. Protein biochemical studies

7.1 Sodium-dodecyl-sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Principle

In SDS polyacrylamide gel electrophoresis, proteins are separated as they migrate through a gel on the basis of their molecular weights. SDS is an anionic detergent that denatures proteins. The SDS also disrupts hydrogen bonds, blocks hydrophobic interactions, and substantially unfolds the protein molecules by eliminating the tertiary and secondary structures. Two types of buffer

systems are used in protein gel electrophoresis: continuous and discontinuous. In the discontinuous system employed in this work, a non-restrictive large-pore gel called a stacking gel is layered on top of a separating (resolving gel). The buffer composition for the two gel layers differs which in turn differs from the composition of the electrophoresis buffer. At the onset of an electrophoretic separation, the proteins migrate first through the stacking gel and then into the separating gel, where separation takes place. With the aid of a protein marker applied alongside the protein samples of interest, the MW of the proteins applied on the gel can be estimated. The following is the pipetting scheme applied for the preparation of two 11% acrylamide SDS-gels:

Separating gel	**Stacking gel**
2.15 ml H_2O	3.19 ml H_2O
3.75 ml 1 M Tris/HCl (pH 8.8)	0.83 ml 0.75 M Tris/HCl (pH 6.8)
3.7 ml Protogel	0.7 ml Protogel
0.2 ml SDS (10%)	0.1 ml SDS (10%)
40 µl APS (10%)	40 µl APS (10%)
200 µl TEMED (10%)	200 µl TEMED (10%)

10x Electrophoresis buffer

Tris	30.2 g
Glycine	142.6 g
H2O	ad 1 liter

4x SDS-loading buffer (pH 6.8)

Tris	0.4 g
SDS	1.2 g
Glycerol	7.5 ml
ß-Mercaptoethanol	2.5 ml
Bromophenol blue (2% solution)	0.5 ml
H_2O	ad 50 ml

Coomasie dye solution

0.125% Coomasie Brilliant blue (Serva)	250 mg

Destaining solution

Methanol	250 ml
Glacial acetic acid	350 ml
H_2O	ad 5 liter

The electrophoresis system from Bio-Rad was employed in this work and the assembly of glass plates and spacers for the production of the gels was according to manufacturer's instructions. For the SDS-gel run, the probes to be analyzed were mixed with the SDS-loading gel buffer and heated briefly at 95°C for 5 - 10 min and then applied on the gels. Electrophoresis proceeded at an applied voltage of 200 V (or at 20 mA) for 1 - 2 hr.

7.2 Cultivation and induction of bacteria

E. coli BL21(DE3) carrying the expression vector was cultivated at 37°C overnight in LB medium fortified with ampicillin. The culture was diluted 1:100 in LB-medium (containing ampicillin) and incubated at 37°C with shaking till an OD_{600} of 0.6 - 0.8 was achieved. The cells were then induced with 0.3 mM IPTG, incubated further at 16°C for 32 hr. The cells were then pelleted by centrifugation at 6000 rpm for 20 min at 4°C, and the pellet resuspended in PBS (containing 1 mM PMSF, 1 mM DTT for protein stabilization). For release of the soluble protein fractions from the cell, the bacterial suspension from above was subjected to French Press with the French Pressure Cell at 1000 psi (repeated four times).

7.3 Purification of the 6xHis fusion protein

The soluble fractions with the 6xHis fusion protein from bacterial lysates were rapidly purified with Ni-NTA Purification System (Invitrogen, USA). The principle is based on the strong affinity of the polyhistidine (6xHis) peptide to nickel-charged agarose resins which it binds specifically; allowing other proteins to flow though the column packed with the agarose beads. Through several wash steps, the unspecific bound proteins are washed through the column and the 6xHis-tagged protein is eluted under mild conditions with an elution buffer.

Procedure

Cell debris was dissolved in loading buffer (6 M Urea, 40 mM imidasole, 20 mM Tris - HCl, pH 8.0) and applied on a 3-ml Ni-NTA column (Bio-Rad) equilibrated with the same buffer. After loading, the column was washed with 10 bed volumes of loading buffer. 6xHis-fusion protein was produced by elution from the column under denaturating conditions (6 M Urea, 200 mM imidasole 20 mM Tris - HCl, pH 8.0). Eluted protein was collected in 0.4 ml portion and frozen at -30°C. Protein sample was analyzed by SDS-PAGE and Western-blot with AP Ni – NTA conjugate (Qiagen).

7.4 The Glutathione-S-transferase Gene Fusion System (Pharmacia Biotech)

The GST gene fusion system is an integrated system for the expression, purification and detection of fusion proteins produced in *E. coli*. The pGEX plasmids supplied with the system are designed for inducible, high-level intracellular expression of genes or gene fragments as fusions with *Schistosoma japonicum* GST. GST occurs naturally as a 26kDa protein that can be expressed in *E. coli* with full enzymatic activity. Fusion proteins are easily purified from bacterial lysates by affinity chromatography using Glutathine Sepharose 4B contained in the purification module. In this study, a GST-Int fusion was constructed (using the pGEX-4T-3 vector) to facilitate the purification of the Int protein.

7.4.1 Purification of the GST-fusion protein

The soluble fractions with the GST fusion protein from bacterial lysates were rapidly purified with Glutathone-Sepharose 4B (Pharmacia Biotech). The principle is based on the strong affinity of the GST protein for Glutathione to which it binds specifically, allowing other proteins to flow though the column packed with the sepharose beads. Through several wash steps with PBS, the unspecific bound proteins are washed through the column and the GST fusion protein eluted under mild conditions with an elution buffer containing reduced glutathione (0.5 M NaCl, 0.1 M Tris/HCl pH 8.0, and 0.02 M GSH -reduced glutathione).

8. Bioinformatics

Bioinformatic tools were powerfully utilized for sequence analysis, alignments and similarity searches. The two primary databanks that were extensively utilized were Genbank and EMBL (European Molecular Biology Laboratory).

Primary databanks

1. The Genbank in the USA is under the patronage of the National Center for Biotechnology (NCBI) and is an official Sequence data bank which contains more than 3 millions protein and nucleotide sequences. All sequences are identified or tagged with a unique accession number. A Genbank sequence is usually divided into two parts:

• the Annotation which contains a precise and detailed information about the sequence and

• the Sequence itself. The ENTREZ search machine is coupled with the Genbank and allows a specific search based on an accession number, organism, gene, protein or author.

2. The EMBL nucleotide sequence database is the European equivalent of the Genbank and utilizes the SRS (sequence retrieval system), a search machine similar to the ENTREZ for specialized searches of the database and many other databanks over the web interface.

3. The Islander Database of Genomic Islands (www.indiana.edu/~islander/) is a comprehensive online database containing genomic islands discovered in completely sequenced bacterial genomes.

BLAST

In addition to the text-based SRS and ENTREZ search engines described above, the BLAST search was also extensively utilized. The BLAST (basic local alignment search tool) search enables comparison of a particular sequence of interest with available databanks, leading to identification of similar sequences or relationships with previously described gene families. The following BLAST programs were employed in this work:

• BLASTN: compares a nucleic acid query sequence with nucleic acid databanks directly

• BLASTX: compares a translated nucleotide sequence with protein sequence databanks

• TBLASTX: compares a translated nucleotide sequence with a database of translated nucleotide sequences

• BLASTP: compares a protein query with a protein database.

The BLAST program provided by NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) and BLAST2 (http://www.ch.embnet.org/software/BottomBLAST.html?) maintained by the Swiss Institute of Bioinformatics were extensively used for sequence analysis.

FASTA: A very common format for sequence data is derived from conventions of FASTA, a program for FAST Alignment by W. R. Pearson. Many of the programs used in this work employed the FASTA format for reading sequences or for reporting results.

Sequence alignment: This is the assignment of residue-residue correspondences. Examples included:

- a Global match: all of one sequence was aligned with all of another
- a Local match: a region in one sequence was matched with a region of another
- a Multiple alignment: a mutual alignment of many sequences.

Neural Network Promoter Prediction version 2.2 (NNPP2.2): This is a promoter analysis tool, located at the Berkeley Drosophila Genome Project (BDGP), http://www.fruitfly.org/seq_tools/promoter.html.

C. RESULTS

1. Characterisation of the HPI integrase, as a DNA-binding trans-acting protein

1.2 Construction of recombinant integrase expression vectors

Native purified protein was required for investigation of the HPI integrase DNA-binding ability. Previous work of Wojciak *et al.* describes the importance of the N-terminal domain of the lambda integrase and loss of its activity in the case of N-terminal fusions. Therefore three different expression vectors for recombinant integrase purification were constructed. Two of them are N-terminal gene fusion vectors: pGEX-4T-3*int* and pQE-30*int*.

Like other GST gene fusion vectors, pGEX-4T-3 carries the gene for Glutathione S-transferase (GST) under control of the *tac* promoter for chemically inducible (with IPTG) high level expression. It also carries an internal *lac* I^q gene compatible for use in any *E. coli* strain. Utilizing the *Bam*HI and *Not*I recognition sites present on the MCS of pGEX-4T-3, the *int* gene was introduced into the vector generating plasmid pGEX-4T-3*int*) so that an N-terminal GST-Int fusion was achieved (Fig. 3).

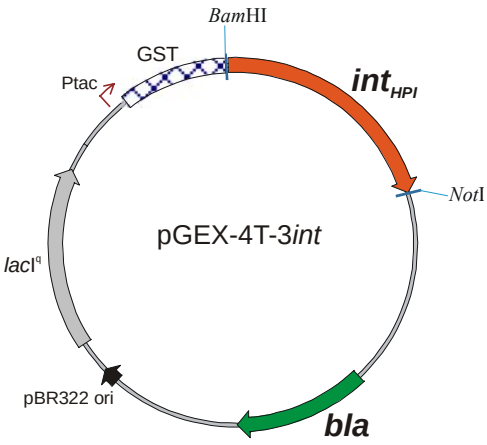


Fig. 3: Vector map of pGEX-4T-3*int*

The int_{HPI} gene is depicted by the orange arrow. Beta-lactamase gene is represented with a green arrow. pBR322 replication origin appears as black, and $lacI^q$ repressor is shown grey. The GST polypeptide is depicted as a crossed bar and P_{tac} promoter as a thin arrow. Cloning sites are marked on the map.

For construction of pQE-30*int* expression vector *int* gene was introduced into pQE-30 plasmid by *Bam*HI and *Hind*III recognition sites, thus providing an N-terminal 6xHis-fusion, which facilitates binding to Ni-NTA (Fig. 4). The tag-less expression plasmid pET-3c*int* was constructed by cloning of *int* gene in a common expression vector pET-3c by *Nde*I and *Bam*HI restriction sites (Fig. 5).

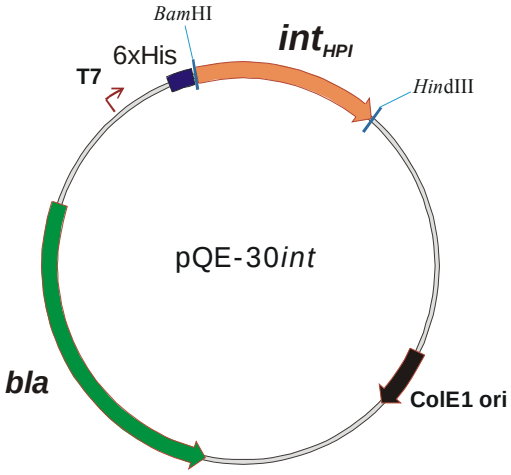


Fig. 4: Vector map of pQE-30*int*

The int_{HPI} gene is depicted by the orange arrow. Beta-lactamase gene is represented with a green arrow. Col E1 replication origin appears as black. The 6xHis polypeptide is depicted as a blue bar and T7 promoter as a thin arrow. Cloning sites are marked on the map.

All expression vectors were proven by sequencing and introduced into the *E. coli* expression strain BL21 (DE3). Expression was induced with IPTG. The induced cultures presented with a strong 48 kDa band in the case of pQE-30*int* and pET3c*int* expression vectors and 74 kDa band (comprising 26 kDa of GST and 48 kDa from the Int) in the case of pGEX-4T-3*int* vector.

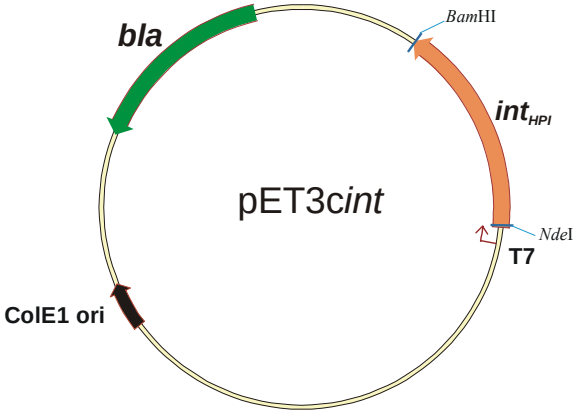


Fig. 5: Vector map of pET3c*int*

The int_{HPI} gene is depicted by the orange arrow. Beta-lactamase gene is represented with a green arrow. Col E1 replication origin appears as black. The T7 promoter as a thin arrow. Cloning sites are marked on the map.

1.3 Integrase activity assay

While only cis-activity of the HPI integrase protein was shown in the earlier study (Rakin *et al*, 2001), we here have developed an assay for trans-activity testing of different integrase derivatives.

The cells of each strain with different integrase expression vectors were grown in LB medium to an $A_{600\ nm}$ of 0.4, IPTG-induced and harvested at $A_{600\ nm}$ of 0.8. Such cells were subsequently used for the preparation of electrocompetent cells.

Each strain expressing integrase was electroporated with 100 ng of pKR531 suicide plasmid (Fig. 6), a pKR528 derivative (Rakin *et al*, 2001), containing reconstituted *attP* site of HPI, as well as inactivated gene of integrase. The described element, being assisted by trans-acting functional integrase protein, could only integrate into the BL21(DE3) chromosome. Otherwise pKR531 would be eliminated.

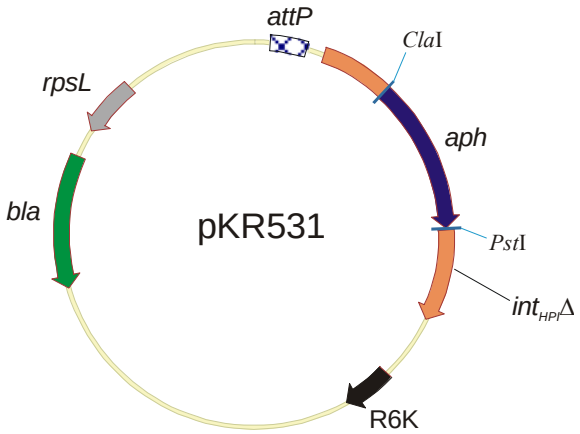


Fig. 6: Vector map of pKR531

The truncated *int*$_{HPI}$Δ gene is depicted by the interrupted orange arrow. Beta-lactamase gene is represented with a green arrow. R6K replication origin appears as black, and *rpsL* gene is shown grey. The *attP* site is depicted as a crossed bar and kanamycin resistance gene as a blue arrow. Cloning sites are marked on the map.

After electroporation BL21(DE3) cells were plated on LB agar supplemented with kanamycin and resistant clones were counted. Only one strain with pET-3c*int* construct showed stable efficiency of transformation by pKR531 plasmid (Table 4). This indicates, that even 6 additional N-terminal histidine residues in the case of 6xHis-Int fusion lead to the crucial loss of integrase activity.

Table 4: Transformation efficiency of different integrase-producing strains by pKR531 construct

Strain	PFU
E. coli BL21(DE3)[pGEX-4T-3*int*]	0
E. coli BL21(DE3)[pQE-30*int*]	0
E. coli BL21(DE3)[pET-3c*int*]	$2.3X10^2$

The specificity of *int*-mediated integration of pKR531 plasmid in *E. coli* BL21(DE3) chromosome was proven by PCR. To detect integrants and determine the exact chromosomal location of the integrative module, four direct *asn* primers (asnT167, asnW761, asnU211 and asnV251, Table 2, Fig. 5) were used in the PCR, together with the reverse C15-205 primer

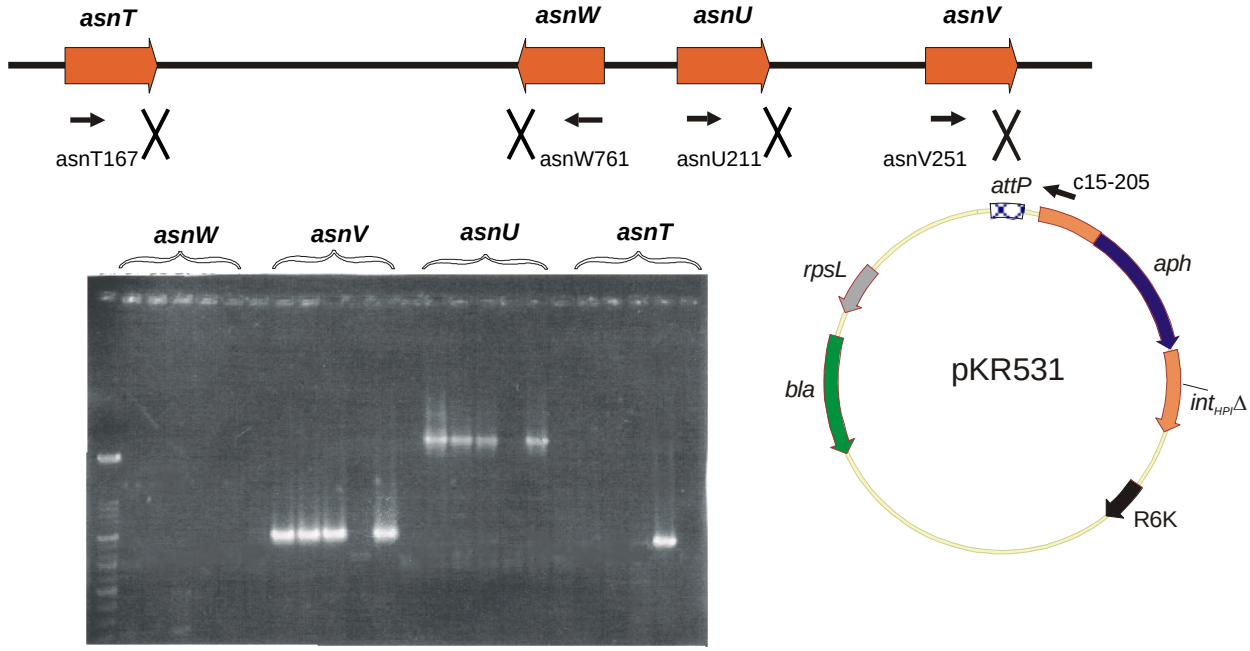


Fig. 7: *E. coli* cluster of *asn* genes and PCR-analysis of integration sites

The black arrows depict the position of the primers. The solid black line with orange arrows denotes the fragment of *E. coli* chromosome with *asn* tRNA genes. Criss-crossed lines with vector map denotes one of the four possible pKR531 integration sites. DNA-bands on the agarose gel are *asn* tRNA/pKR531 integrant-specific PCR-fragments.

(located in the first half of the inactivated integrase gene). All tested clones showed site-specific chromosomal integration of the pKR531 construct (Fig. 7), it means that *E. coli* BL21(DE3)[pET-3c*int*] strain produces active HPI integrase, and can be used for isolation of recombinant protein protein.

1.4 Purification of recombinant Int_{HPI}

The BL21(DE3)[pET-3c*int*] strain was used for purification of integrase protein. Cells were grown in LB medium with glucose (supplemented with carbenicillin) at 37 °C to logarithmic phase and induced with IPTG for 5 h. Cells were lysed by several French press passages and clear cell lysate containing Int_{HPI} was applied onto heparin-sepharose affinity column (with high affinity to DNA-binding proteins) and eluted with with a gradient to 1 M KCl (Fig.8).

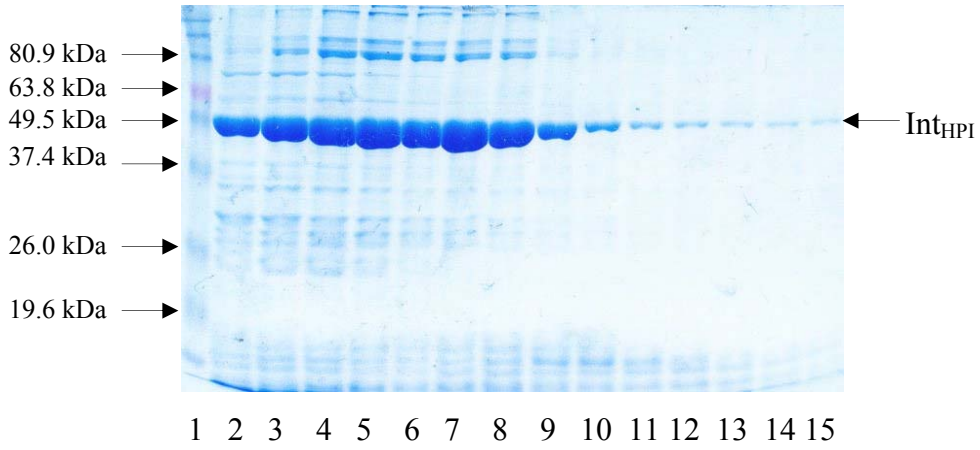


Fig. 8: Coomassie-stained SDS-PAGE of protein fractions after elution by salt gradient from heparin-sepharose affinity column

Lane 1: protein molecular weight marker. Lanes 2-15: protein fractions.
Relevant protein marker sizes are indicated on the left with arrows. The stained Int$_{HPI}$ protein band is indicated on the right with arrow.

After the first step, fractions 2-8 were mixed and applied onto size-exclusion chromatography column. The most pure fraction (Fig. 9, lane 3) was used for the next experiments. Protein purity was evaluated visually and comprised 99% (SDS-gel estimated).

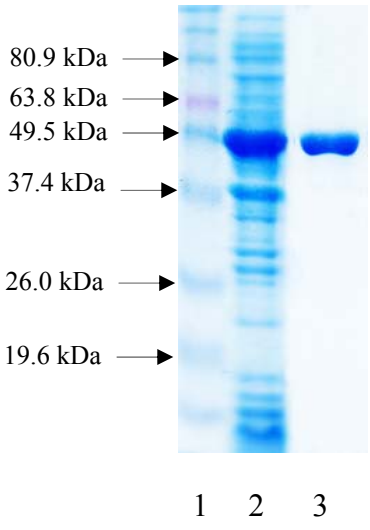


Fig. 9: SDS-PAGE of purified Int$_{HPI}$ protein.

Lane 1: molecular weight marker; lane 2: *E. coli* BL21(DE3)[pET-3c*int*] cell lysate;
lane 3: purified Int$_{HPI}$ protein.
Relevant protein marker sizes are indicated on the left with arrows.

1.5 Integrase/*attP* electrophoretic mobility shift assay

To promote its integrase activity, Int_{HPI} must possess DNA-binding ability, i.e. it must bind and interact with *attP* attachment site, involved in site-specific recombination of the HPI. The ability of recombinant Int_{HPI} to bind DNA was evaluated by electrophoretic mobility shift assay using ^{32}P-end labelled DNA probes generated by PCR. The 358bp fragment containing HPI *attP* was efficiently bound by the Int_{HPI} protein (Fig. 10). In the case of higher protein concentration we were able to observe two mobility shift bands per lane. It suggests HPI integrase binding to the *attP* site as a dimer or that *attP* contains two Int_{HPI} -binding sites.

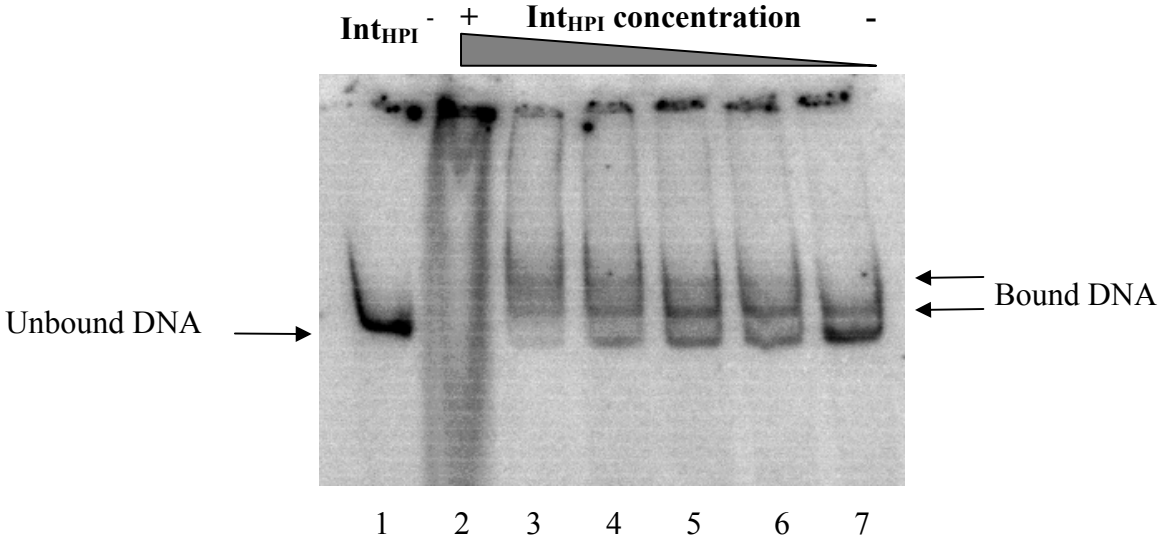


Fig. 10: Mobility of Int_{HPI} /*attP* complexes in native 5% PAGE

Lane 1: labelled DNA without Int_{HPI} protein; lanes 2-7: 30, 10, 5, 2, 1 and 0,5 pMol Int_{HPI} protein respectively.

1.6 IHF/*attP* electrophoretic mobility shift assay

Sequence analysis of HPI *attP* showed presence of a DNA sequence with high similarity to integration host factor (IHF) binding site (Rakin *et al*, 2001). Possible influence of IHF could be uncovered using IHF/*attP* electrophoretic mobility shift assay. Pure *E. coli* IHF protein was supplied by Steven Goodman (University of Southern California, Los Angeles, USA) and prepared by the protocol of Nash (Nash and Robertson, 1981). As expected, IHF efficiently binds *attP* site of HPI. It also bind cooperatively, as we could observe an additional band with lower mobility at higher protein concentration (Fig. 11). It seems like IHF plays role in recombination of HPI. It has been shown previously that IHF creates bends in lambda *attP* DNA to help *attP* condense into a compact structure that is activated for recombination (Robertson and Nash, 1988).

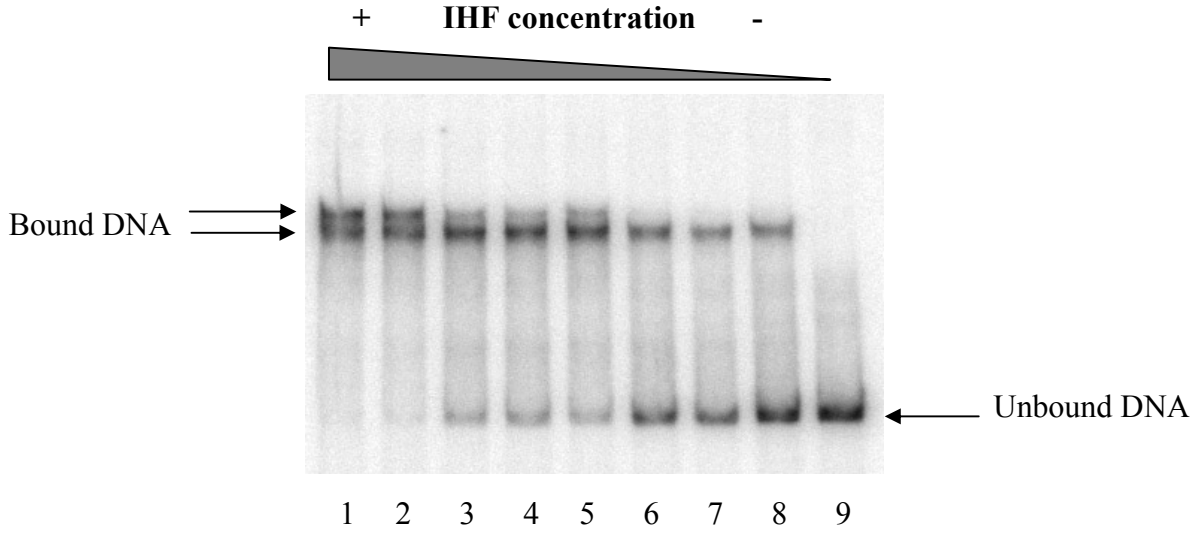


Fig. 11: Mobility of IHF/*attP* complexes in native 5% PAGE

Lanes 1-8: 30, 15, 10, 5, 3, 1, 0.5, and 0.1 pMol IHF respectively; lane 9: labelled DNA without IHF protein.

2. Recombination Directionality Factor of the HPI_{Yps}

2.1. Bioinformatic analysis of AT-rich region of HPI, defining of the putative excisionase of the HPI_{Yps}

The HPI has a mosaic structure and consists of two defined parts, the yersiniabactin "core" and the variable AT-rich part that differs in two evolutionary lineages, HPI_{Yen} and HPI_{Yps}. It is significant, that the recombinase and AT-rich ORFs sequences showed markedly different codon biases. The recombinase gene has an average G+C content (52% G+C overall and 56% G+C at 3rd base positions), while the AT-rich ORFs have a low G+C content (43% overall and 42% at 3rd base positions). By comparing the two AT-rich variable parts of the high-pathogenicity island of *Y. pestis* / *Y. pseudotuberculosis* with that of *Y. enterocolitica* two HPI_{Yps} ORFs with potential HTH DNA binding motifs, *orf2* and *orf5*, were selected as possible candidates for the role of an excisionase (Fig. 12). Both ORFs were aligned with a group of recombination directionality factors (RDFs) of Gram-negative bacteria (Fig. 13). It turned out that *orf2* is 50% similar and 35.1% identical to the phage P4 Vis excisionase (Y9K_BPP4, Fig. 13) belonging to the L5-pSAM2-SLP1 family of putative RDFs (Lewis and Hatfull, 2001). In contrast, another ORF with a putative DNA-binding domain, *orf5*, had no obvious similarity to RDFs but contains a Zink fingers domain that is more typical for transcriptional regulators. The putative excisionase Orf2 consists of 61 aa, has a molecular mass of 7200 and is predicted to have a basic pI of 9.69.

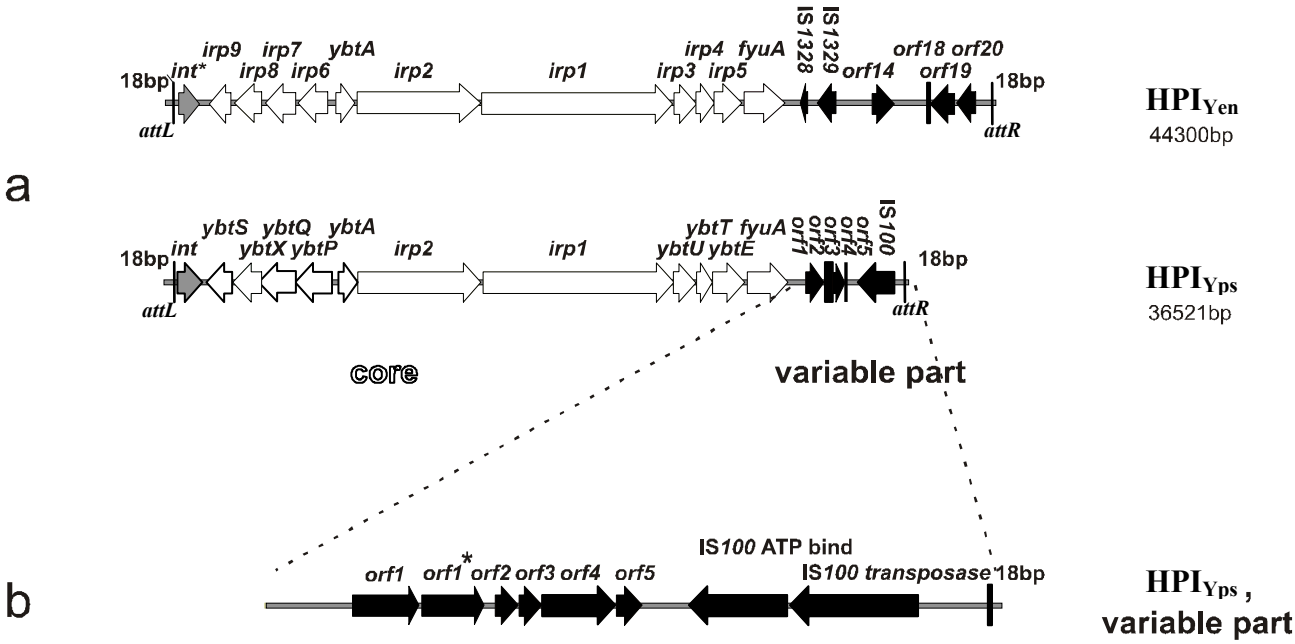


Fig. 12: Physical map of HPI_{Yen} and HPI_{Yps} evolutionary lineages (a); AT-rich part of HPI_{Yps} (b).

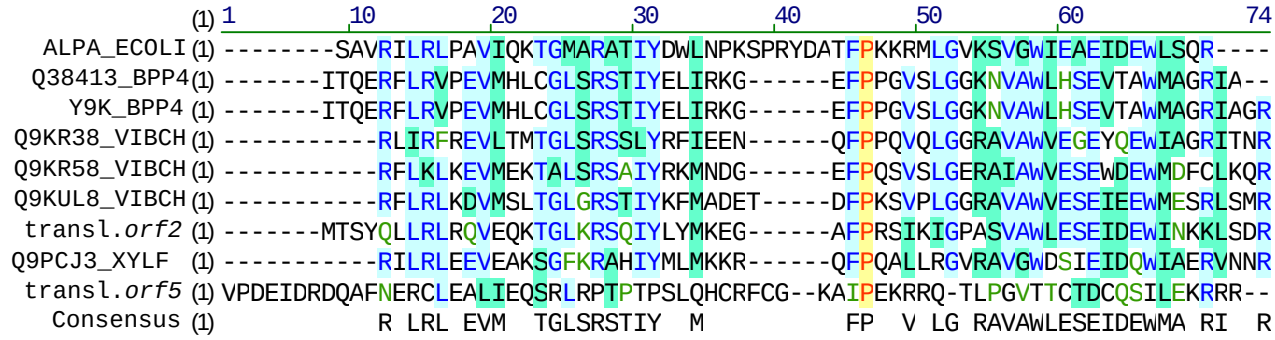


Fig. 13: Comparison of *orf2* and *orf5* with other potential excisionases of *E. coli* (ALPA_ECOLI), bacteriophage P4 (Q38413_BPP4, Y9K_BPP4), *Vibrio cholera* (Q9KR38_VIBCH, Q9KR58_VIBCH, Q9KUL8_VIBCH), *Xylella fastidiosa* (Q9PCJ3_XYLFA).

2.2 Promoters of *orf1* and *orf2*

Orf2 located 139 bp downstream of the *orf1* stop codon. We were not able to identify the position of the *orf2* promoter using available computational programs aimed to localize prokaryotic promoters (Neural Network Promoter Prediction 2.2). Also our attempts to localize the *orf2* promoter using RACE and primer extension methods failed. The reason for this might be a low activity of the possible *orf2* promoter, if at all present. To prove this we fused the regions located 141-bp upstream of the *orf1* translation start and the region between the end of *orf1* and the start of *orf2* to a luciferase gene reporter. The fusions showed different activities, with *orf1* promoter having at least 2,75x higher activity than the putative *orf2* promoter. In contrast, the int_{HPI} promoter of the recombinase gene has a much higher activity (77 times higher than the *orf2* promoter). For comparison, *asn*T tRNA gene promoter showed nearly the same activity with *orf1* promoter (only 1,1x times higher).

By RT-PCR with primers HPI 878 (annealing in *orf1*) and HPI 174 (annealing in *orf2*) we proved that *orf1* and *orf2* are transcribed as one mRNA and the *orf1* promoter might also control the activity of the *orf2* gene. Thus we mapped the promoter of *orf1* in HPI_{Yps}. The transcriptional start of the *orf1* promoter mapped by RACE resides 79 bp upstream of the translational start of *orf1* (Fig. 14).

A

AATAGTCACCCCATAGGCCTGTACATGTTCACTCAG**AAATAT**ACATCCTTTTCTCTG

T**CATAAA**CCCTCT**G**ATTAATCATAAATAAATACTTGTGACACCAATCTTTTTCCTT

AACGGAACGAATTGTTGTGTAG**AAGGAG**ATAATATT**ATG**...

B

...CCAGCTTTCTCTCAGGCTTCCACTGTGTTTTTTTATTCTCCGGCCACCGTTTATTTC

AGTCAC**AGGAG**GATAT**ATG**

Fig. 14: Predicted promoter structure (-35, -10 elements and +1 transcriptional start) and ribosome binding sites (SD) of *orf1* (a) and *orf2* (xis_{HPI}) (b).

2.3 Construction of *orf*2 mutant

We proposed that *orf2* gene might encode a putative excisionase protein essential for efficient HPI excision. To prove this we inactivated this gene by the method of one-step inactivation of chromosomal genes was applied (Datsenko and Wanner, 2000). A plasmid pKD3 was used as a template for generation of PCR fragment containing chloramphenicol resistance cassette flanked by FRT-sites and 50-nt homology arms for *orf2* gene (Fig. 15). For this primer pair Exc_rev/ Exc_for were employed (Table 2). *Y. pseudotuberculosis* YPS06 cells harboring pKD46 plasmid were grown in the presence of arabinose to induce Red recombinase. Such competent cells were transformed by the purified PCR product. Recombinant clones were selected on LB agar plates containing chloramphenicol. Replacement of *orf2* by chloromphenicol resistance cassette was confirmed by PCR and sequencing. The resistance cassette was removed using thermoinducible FLP recombinase on pCP20 plasmid. The resulting strain was designated YPS06 *xis*.

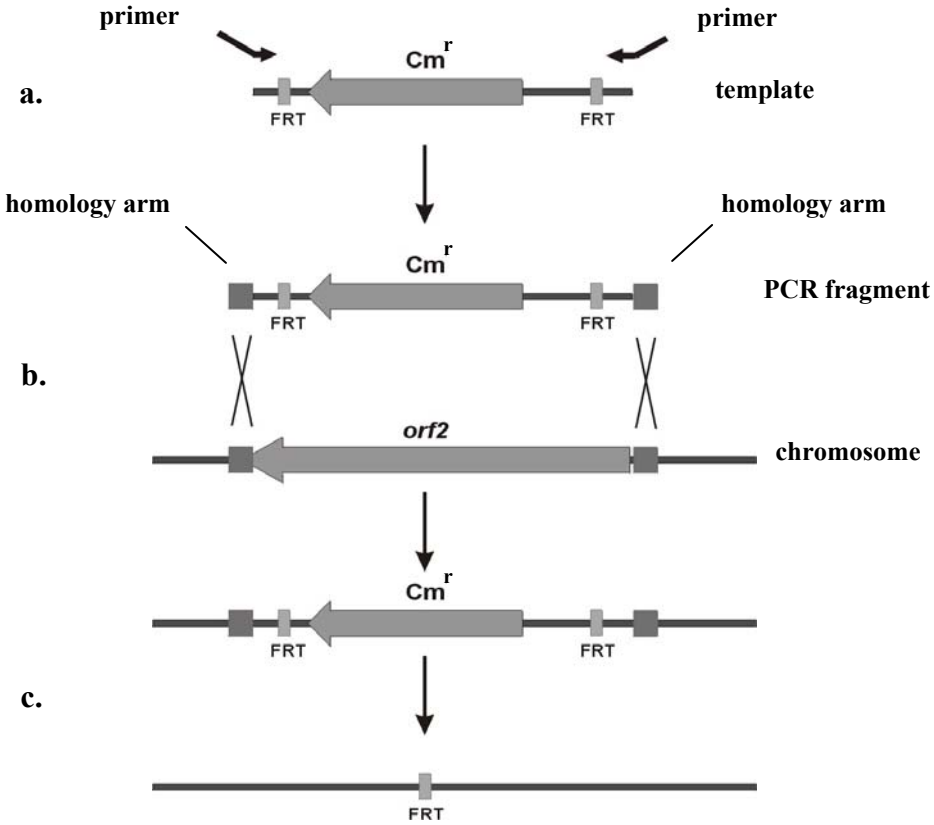


Fig. 15: Construction of the *orf2*-deficient mutant by one-step gene inactivation.

a. Preparation of PCR product containing chloramphenicol resistance gene (Cm^r), FRT recombination sites and 50 bp homology regions for homological recombination.
b. Red recombinase-mediated homological recombination.
c. Removing of resistance cassette by FRT-recombination sites mediated by FLP recombinase.

2.4 Effect of *orf2* on excision of HPI in *Y. pseudotuberculosis* YPS06 and YPS06 *xis* strains

To prove that *orf2* has an excisionase activity, we compared HPI excision rates in *Y. pseudotuberculosis* YPS06 wild type and YPS06 *xis* strains. To determine the efficiency of excision we quantified *attP*-targets in both strains by Real Time PCR. For quantification of the *attP* DNA, pKR528 plasmid (Table 1) was serially diluted (ranging from $2x10^9$ to $2x10^0$ copies) and amplified by the LightCycler (Fig. 16).

Total DNA isolated from YPS06 and YPS06 *xis* was analysed in several dilutions. The concentration of *attP*, or, correspondingly, the circular form of the excised island, was approximately eightfold higher in the wild type strain, than in its isogenic xis_{HPI} mutant (Fig. 17). This implies a direct role of *orf2* in excisive recombination of the HPI. Orf2 was therefore denoted as Xis_{HPI} for HPI excisionase. 1µg of *Y. pseudotuberculosis* YPS06 wild type genomic DNA contains 150-200 copies of the excised island. The same amount of chromosomal DNA of YPS06 contains $1,4x10^8$ copies of a single-copy chromosomal marker *attL*. That corresponds to $1,4x10^8$ cells excising 150-200 HPI copies. The frequency of excision of the HPI can be estimated 10^{-6}. Inactivation of the xis_{HPI} gene reduced this frequency approximately eightfold, to 10^{-7}.

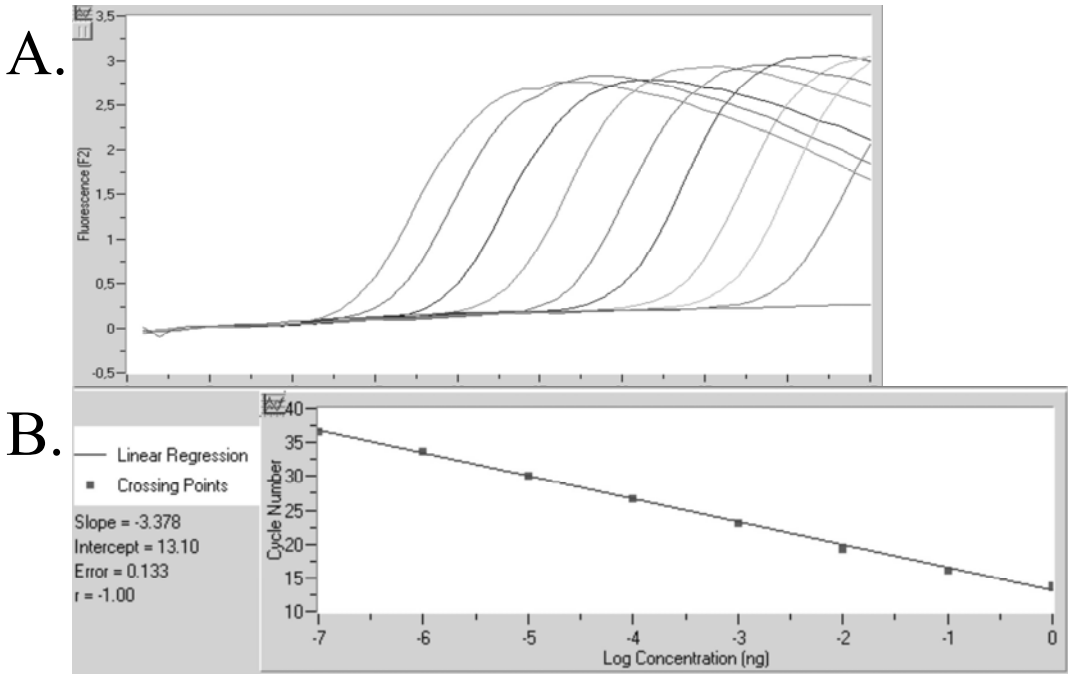


Fig. 16: Amplification of the serially diluted plasmid pKR528 for external standardization (a) and Linear regression of the LightCycler assay (b).

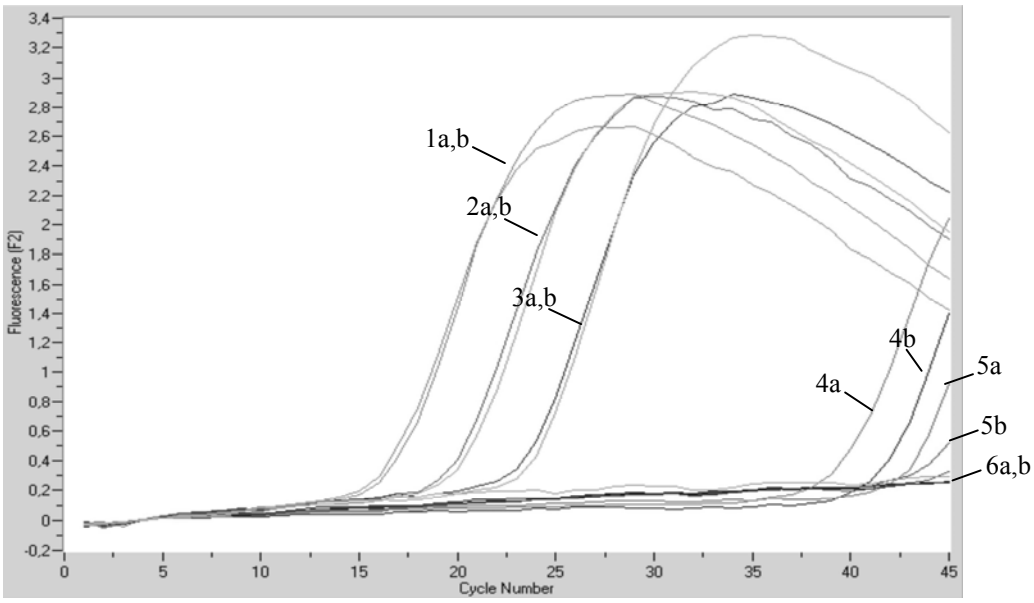


Fig. 17: Real Time PCR quantification of the circular form of the HPI.

1-3: control reaction for the chromosomal marker *attL* - 1µg, 100ng and 10ng of the chromosomal DNA, respectively.
4-6: specific reactions for the *attP* site - 1µg, 100ng and 10ng of chromosomal DNA, respectively.
a- YPS06 wild type, b- YPS06 *xis*.

2.5 Construction of recombinant excisionase expression vector.

The excisionase gene *xis*$_{HPI}$, was amplified from *Y. pestis* KUMA chromosomal DNA by PCR using primers ORF2F Nde and ORF2R Bam (Table 2), introducing *Nde*I and *Bam*HI restriction sites. The PCR-product was cloned into vector pMos*Blue* using the pMos*Blue* blunt ended cloning kit (Amersham International, Little Chalfont, U.K.). The resulting plasmid was

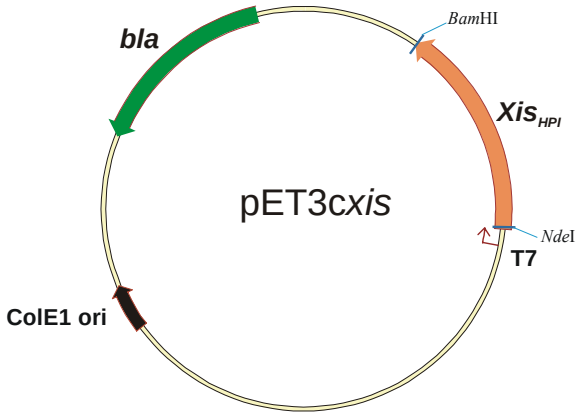


Fig. 18: Vector map of pET3c*xis*

The *xis*$_{HPI}$ gene is depicted by the orange arrow. Beta-lactamase gene is represented with a green arrow. Col E1 replication origin appears as black. The T7 promoter as a thin arrow. Cloning sites are marked on the map.

digested with *Nde*I and *Bam*HI and the insert was cloned into expression vector pET-3c to generate plasmid pET-3c*xis* (Fig. 18). Expression vector was proved by sequencing and introduced into the *E. coli* expression strain BL21 (DE3).

2.6 Excisionase protein expression and purification

The *orf2* gene encoding excisionase was overexpressed in *E.coli* BL21 (DE3) containing pET-3c*xis*. Cells were grown in LB medium with glucose (supplemented with carbenicillin) at 37 °C to logarithmic phase and induced with IPTG for 5 h. Cells were lysed by several French press passages. For isolation of excisionase standard scheme for DNA-binding

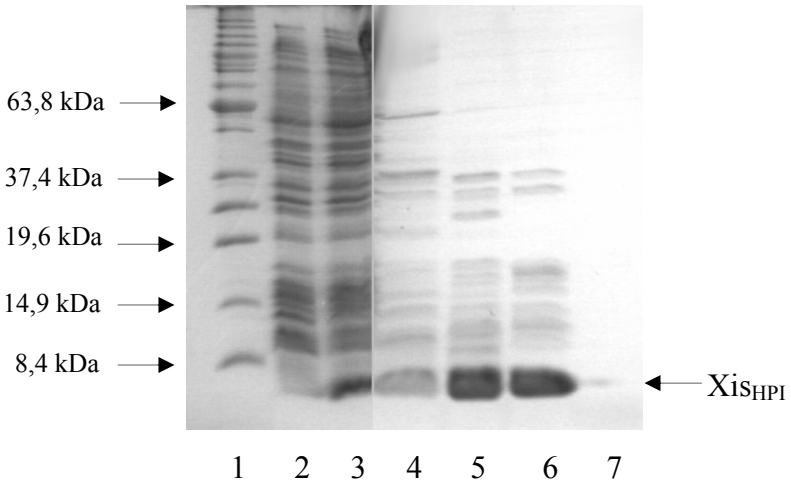


Fig. 19: Purification of excisionase. Protein fractions after elution by salt gradient from heparin-sepharose affinity column

Lane 1: Molecular weight marker; lane 2: uninduced cells; lane 3: induced cells; lanes 4-7: protein fractions. Relevant protein marker sizes are indicated on the left with arrows. The stained Xis$_{HPI}$ protein band indicated on the right with arrow.

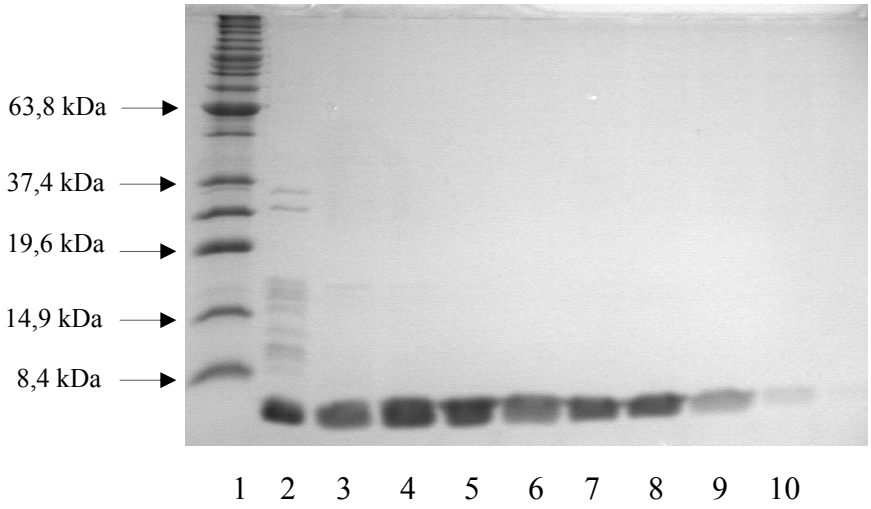


Fig. 20: Purification of excisionase. Protein fractions after size-exclusion chromatography

Lane 1: Molecular weight marker; lane 2: protein sample before gel-filtration; lanes 3-10: different through-fractions of the sample. Relevant protein marker sizes are indicated on the left with arrows.

proteins purification was applied. Cleared lysate was loaded onto a heparin column using a SMART system (Pharmacia Biotech) and proteins were eluted with a salt gradient. Fractions containing the excisionase protein (Fig. 19) (elution at approximately 0.7 M NaCl) were pooled and concentrated. Concentrated protein sample after heparin-sepharose column was gel-filtrated on a Superdex 75 HR 10/30 column and the purity of collected fractions were analyzed by SDS-PAGE (Fig. 20). The most pure fractions (Fig. 20, lanes 5-8) were used for next experiments. Protein purity was estimated by SDS-gel visual overview and comprised 99%.

2.7 Excisionase-DNA binding experiments

To promote its excisionase activity Xis_{HPI} should possess DNA binding ability, that is to bind and interact with the attachment sites involved in site-specific recombination of the HPI. Indeed, the Xis_{HPI} amino acid sequence contains predicted H-T-H DNA binding motive. To test its DNA binding ability, we performed an electrophoretic mobility shift assay using a purified recombinant excisionase and DNA fragments containing *attP* and *attB* regions, labelled with ^{32}P. The fragment containing *attP* was efficiently bound by the Xis_{HPI} protein. In contrast, Xis_{HPI} did not specifically change the mobility of the *attB*-carrying fragment (Fig. 19).

To prove specificity of protein-DNA binding, the same EMSA experiments were conducted, but with 100-fold excess of the nonspecific competitor DNA. For this purpose a non-specific competitive 176 bp DNA probe was generated from genomic DNA of *Y. enterocolitica* O: 8 using primers FyuAF and FyuAR (Table 2). Addition of the nonspecific competitor DNA (up to 1:100 excess) did not alter the excisionase-*attP* specific binding (Fig. 20).

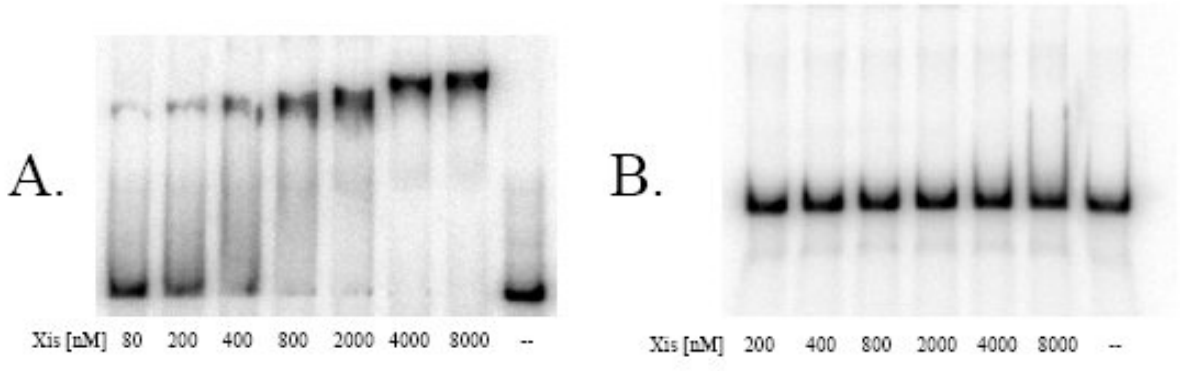


Fig. 21: Comparative electrophoretic mobility shift assay (EMSA) of the excisionase with (A) *attP* and (B) *attB* DNA fragments.

Digits below the gels represent the quantity of the applied Xis protein; (--), probe without addition of protein.

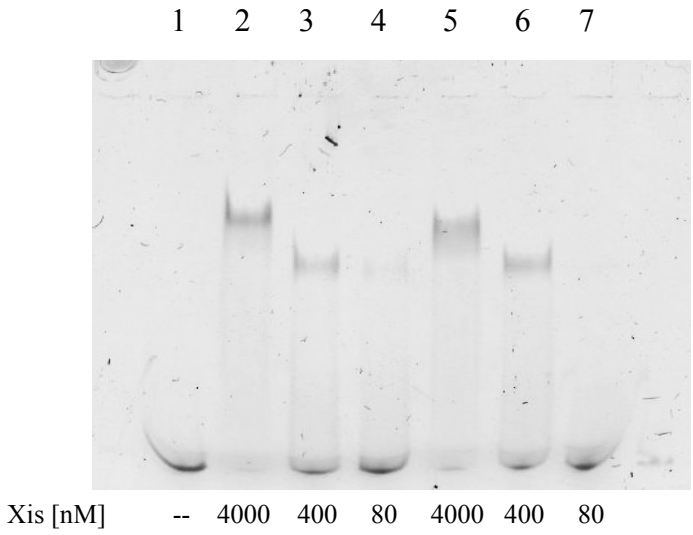


Fig. 22: EMSA assay of the excisionase with *attP* DNA fragment without (lanes 2-4) and with (lanes 5-7) addition of 100X-excess of non-specific competitor.

Digits below the gel represent the quantity of the applied Xis protein; (--), probe without addition of protein.

To localize the binding regions, we performed EMSA of Xis_{HPI} with overlapping fragments of *attP* site, labeled with fluorescent dye FAM. The purified excisionase demonstrated efficient binding with all fragments besides one, representing the left part of *attP* (Fig. 23).

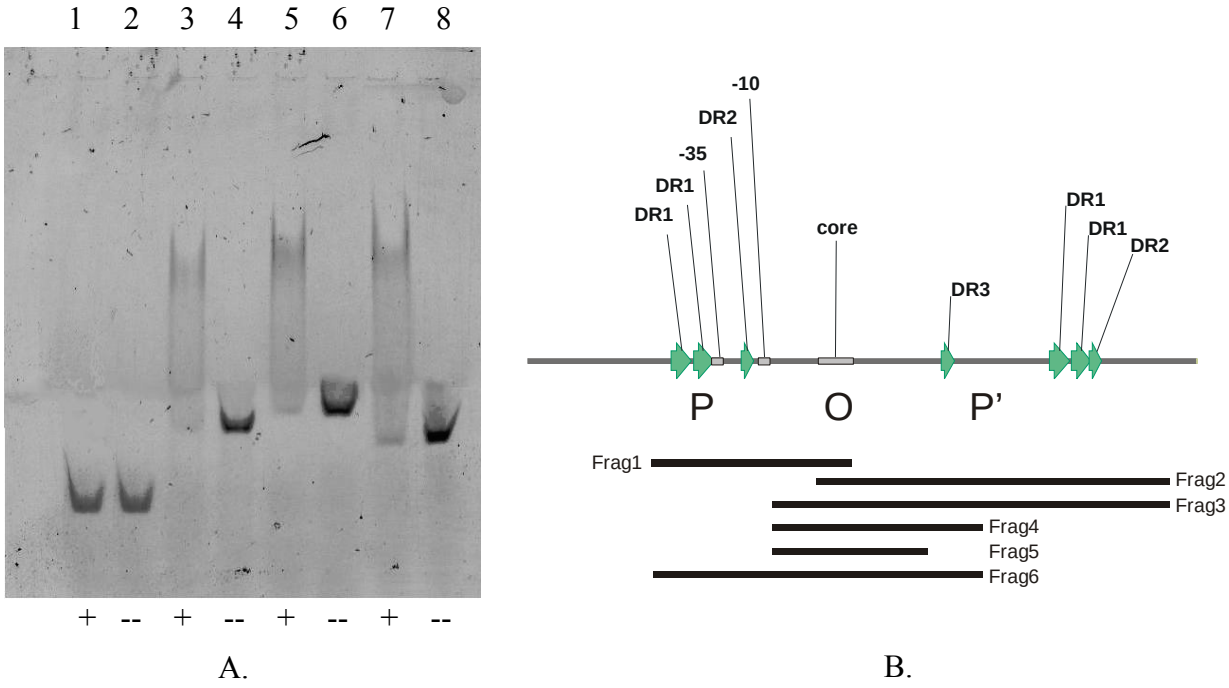


Fig. 23: a. EMSA of the excisionase protein (8 µM) with different fragments of HPI-*attP*; b. A diagram of HPI-*attP* site (POP')

Lanes 1,2: Frag1; lanes 3,4: Frag. 2; lanes 5,6: Frag. 3; lanes 7,8: Frag. 4.
(+) - probe with addition of protein, (--) - probe without addition of protein.
DRs – direct repeats, -35; -10 – promoter regions of int_{HPI}.

To delimit Xis_{HPI} binding sites, we performed DNase I protection experiments with FAM-labeled *attP* DNA fragment of HPI_{Yps} and recombinant Xis_{HPI} protein (see Materials and Methods, p. 32). Footprinting analysis proved the presence of one protected region extending between the core and the DR3 direct repeat in the right part of the *attP* (Fig. 24). Thus, Xis_{HPI} recognizes the right moiety of the *attP* site that is presented in the recombinant *attL* site in the integrated form of the HPI and serves as a recognition site for the excisive recombination.

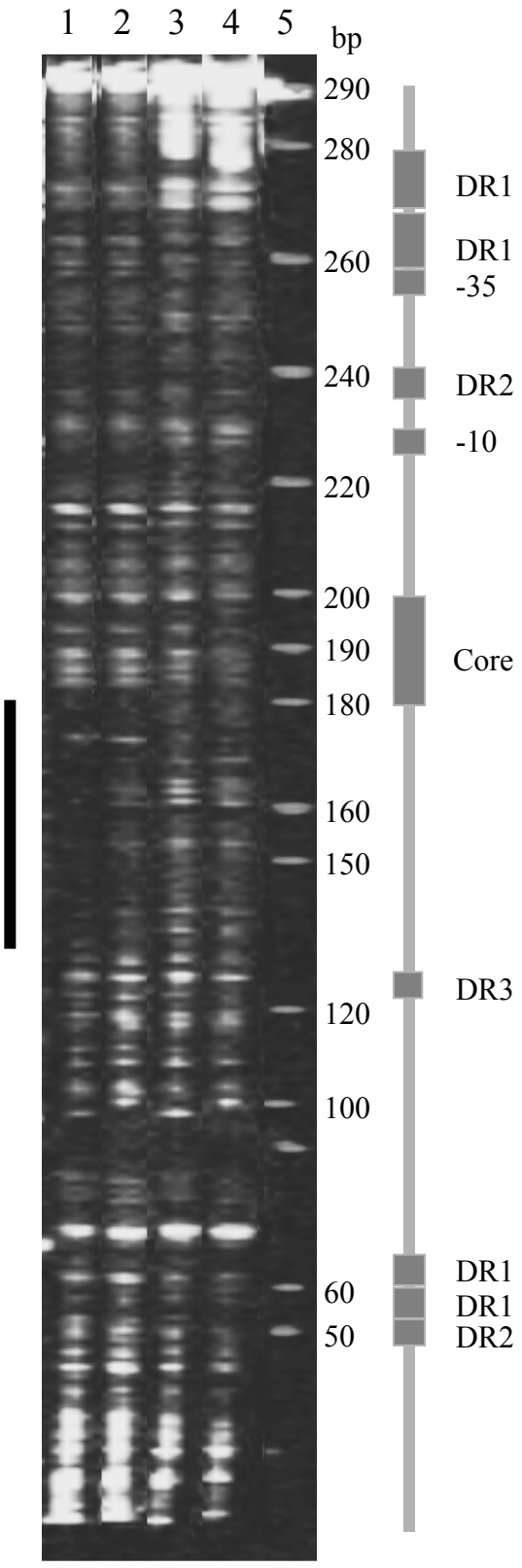


Fig. 24: DNase I footprinting of *attP*-Xis$_{HPI}$ complexes and schematic structure of the *attP*-site

Lanes 1-4: 8, 0.8, 0.08, and 0 µM excisionase protein, respectively; lane 5: DNA molecular weight marker. Protected region is shown with vertical black line on the left.

3. Evolution of recombination apparatus of GEIs integrated in *asn* tRNA genes

3.1 Comparison of HPI with *E. coli* Ecoc54N GEI

The HPI is known to integrate into *asn* tRNA genes by means of a site-specific recombinase or integrase, Int_{HPI} (Rakin *et al.*, 2001). The search for other genomic islands that utilize the same recombination target within the Genomic Islands Database (www.indiana.edu/~islander/) revealed a new uncharacterised genomic island Ecoc54N in the genome sequence of the uropathogenic *E. coli* CFT073 (Welch *et al.*, 2002). It seems like that Ecoc54N also recognizes *asn* tRNA gene for integration (*asnW* tDNA). The comparison of Ecoc54N and HPI_{Yps} uncovered surprising homology of their recombination apparatus (Fig. 25). The recombinase genes have 80% homology at nucleotide level and 83% homology in amino acids. Also sequences of the attachment sites show 90% similarity (Fig. 26). Besides recombinase gene and attachment sites, Ecoc54N carries a cluster of putative polyketide synthesis genes with no similarity to the HPI core part. Thus the homology between these two islands is restricted only to sequences involved in recombination. Although some of the HPI "core" genes also encode polyketide synthases, they demonstrate no obvious similarity to the sequences found on Ecoc54N. Also no genes potentially involved in replication or transfer could be recognized on Ecoc54N.

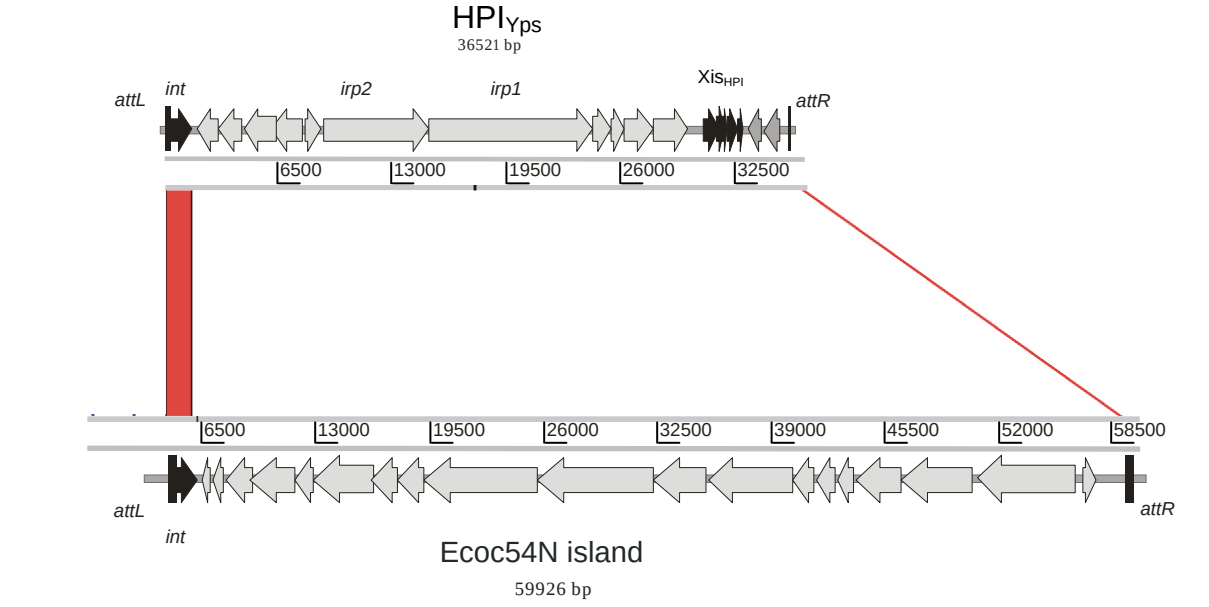


Fig. 25: Comparison of two *asn* tRNA gene-recognizing genomic islands: HPI_{Yps} and Ecoc54N

Open reading frames are depicted by the grey arrows. Homology regions are shown with the red lines. The depiction of both islands is proportional to genetic distance indicated by scale (in bp).

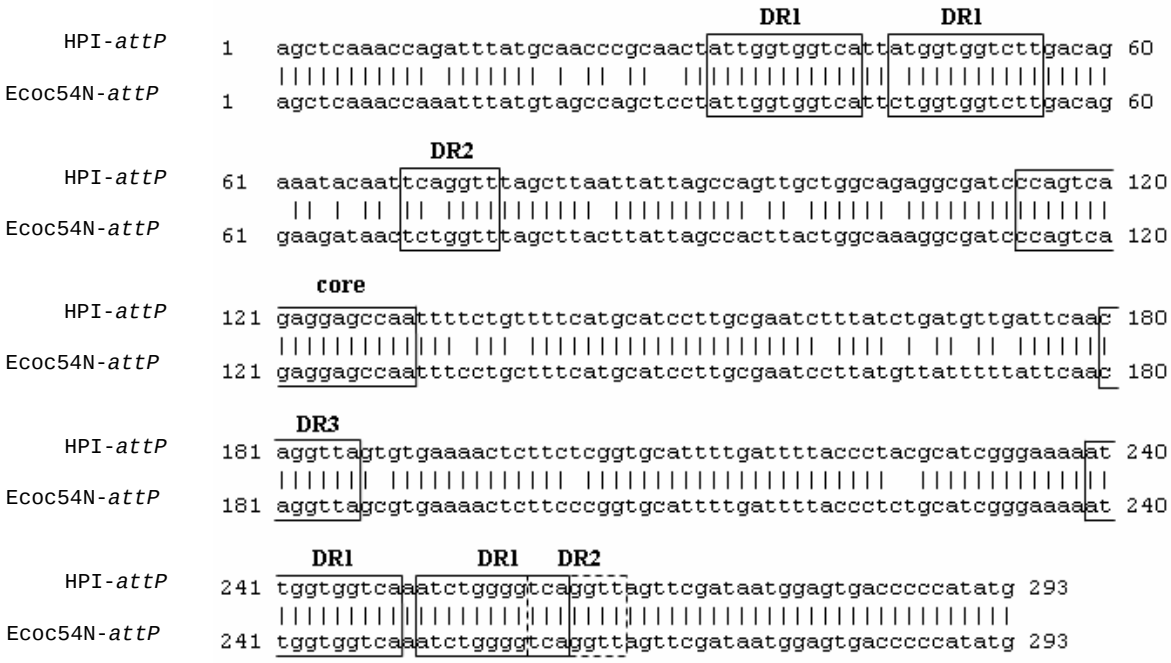
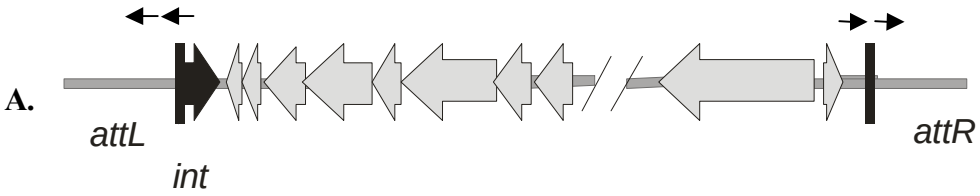


Fig. 26: Alignment of HPI and Ecoc54N attachment sites

Structural elements of attachment sites depicted with squares.

3.2 Recombinase of Ecoc54N island is active and able to promote excision

We have tested Ecoc54N island for excision with the same approach applied for HPI. To detect this we tested the total DNA from *E. coli* CFT073 for the presence of the circular form of the island by nested PCR with primers Int_cft1087/ cftattp501 (1st PCR) and Intcft698_rev/cftattp601 (2nd PCR) (Table 2, Fig. 27a). Indeed the 890 bp PCR product (Fig. 27b) contained the restored *attP* site of the Ecoc54N island that was proved by sequencing. PCR for the restored *attB* site yielded the product of the expected size. These data favor the presence of a functional recombinase in Ecoc54N island and demonstrate that Ecoc54N also appeared to be a mobile genomic island. However, no potential excisionase gene can be detected in Ecoc54N.



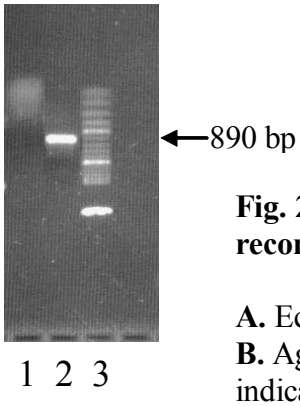


Fig. 27: Nested-PCR amplification of Ecoc54N *attP* recombination site and *attP*-integrase containing fragment

A. Ecoc54N island and positions of primers for nested PCR.
B. Agarose gel electrophoresis of nested-PCR products, arrow indicates the length of amplified 2nd PCR product. Lane 1: 1st PCR of Ecoc54N *attP* fragment; lane 2: 2nd PCR of Ecoc54N *attP* fragment; lane 3: DNA molecular weight marker.

3.3 Xis_{HPI} does not assist the Ecoc54N excision

To test whether Xis_{HPI} excisionase can assist excision of *asn*-tRNA-related island, Ecoc54N, we cotransformed *E. coli* CFT073 strain with pET-3C *xis* and pGP1-2 plasmids (Table 1). After induction of the excisionase, we purified the total DNA from CFT073 and CFT073 [pET-3C *xis* /pGP1-2] strains. Quantification of *attP*-targets in wild type CFT073 compared to CFT073 [pET-3C *xis* /pGP1-2] was performed by Real Time PCR. Total DNA was analysed in several dilutions. However, we detected no differences in *attP* concentration or, correspondingly, in the excised circular form of the Ecoc54N island in both strains (Fig. 28). Thus, Xis_{HPI} did not effect the excisive recombination of the Ecoc54N island.

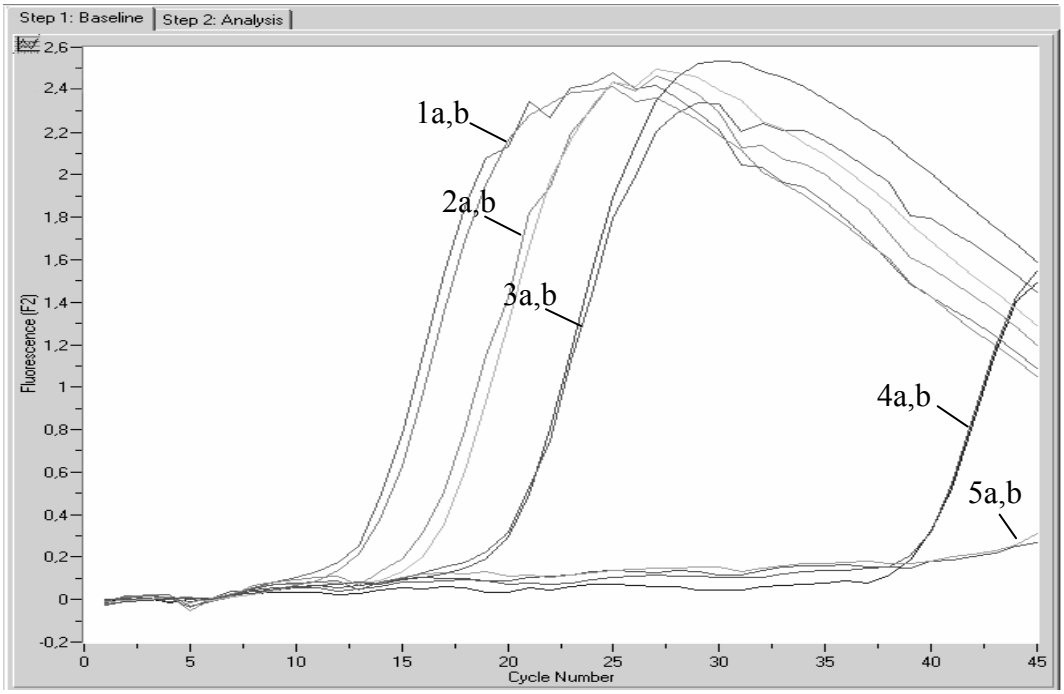


Fig. 28: Real Time PCR quantification of the circular form of the Ecoc54N

1-3: control reaction for the chromosomal marker *attL* - 1μg, 100ng and 10ng of the chromosomal DNA, respectively.
4-5: specific reactions for the *attP* site - 1μg and 100ng of chromosomal DNA, respectively.
a- CFT073 wild type, b- CFT073 [pET-3C *xis* /pGP1-2].

3.4 Xis_{HPI} did not bind to Ecoc54N *attP* recombination site

The reconstructed Ecoc54N *attP* site showed 90% similarity with HPI *attP*. To examine the possibility of the recombinant Xis_{HPI} excisionase to bind to Ecoc54N *attP* site, we carried out electrophoretic mobility shift assay with FAM-labelled Ecoc54N *attP*-bearing PCR fragment. However, even with high concentration of the excisionase (4μM) we detected no visible

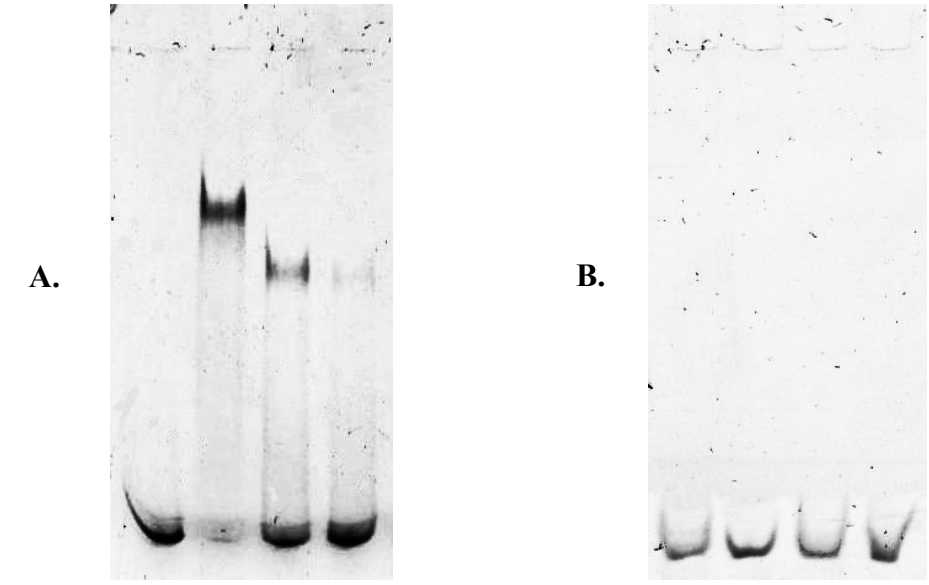


Fig. 29: EMSA of the Xis_{HPI} excisionase with (A) HPI-*attP* and (B) Ecoc54N-*attP* DNA fragments

Digits below the gels represent the quantity (nM) of the applied Xis_{HPI} protein;
(--) - probe without addition of protein (control).

fragment mobility shift on the gel (Fig. 27). Failure of Xis_{HPI} to bind to Ecoc54N *attP* site is possibly due to nucleotide substitutions in *attP* site (Fig. 24) that might explain Xis_{HPI} inability to assist excisive recombination of Ecoc54N.

4. Mechanisms of GEIs dissemination

We propose a mechanism of lateral transfer of GEIs, incapable for self-dissemination (like HPI_{Yps}) based on site-specific recombination of the excised island with the *attB*-presenting conjugative shuttle plasmid. The resulting cointegrate can be transferred by conjugation to a new host. This hypothesis was proven using RP4 - a wide host range IncP1 conjugative plasmid

4.1 Construction of the shuttle plasmid

The RP4 plasmid presenting *attB* attachment site (*asn* tRNA gene) suitable for HPI "trapping" was constructed in two steps (Fig. 30). Firstly, the *asn* tRNA gene was combined with the *sat3* streptothricine resistance marker to facilitate insertion of *asn* tDNA into RP4 plasmid. To do this, plasmids pKR600 and pIE928 were digested with *Bam*HI and equimolar amounts of linearized plasmids were ligated. The resulting product of the ligation reaction served as template for PCR amplification with primers RP_mut_for and RP_mut_rev (Table 2). The resulting PCR product contains the *sat3* gene from pIE928 plasmid, the *asn* tRNA gene from pKR600 plasmid and 50bp-homology arms to the *aph* gene. A promiscuous plasmid RP4

(IncP1) with a wide host range was selected as a trapping vehicle for *Yersinia* HPI. The kanamycin resistance gene of RP4 was substituted by this *sat3-asn* tRNA gene cassette by Red recombination. Recombinant clones were selected on LB agar containing streptothricine (50 μg/ml). Insertion of the *asnT* RNA gene was confirmed by PCR and sequencing. The resulting plasmid was designated as RP4' *asn.* We suppose that such transmissive *attB*-presenting plasmid (a "shuttle") can be used for "trapping" and subsequent transfer of any functional integrative genetic element that recognizes *asn* tRNA gene as an attachment site.

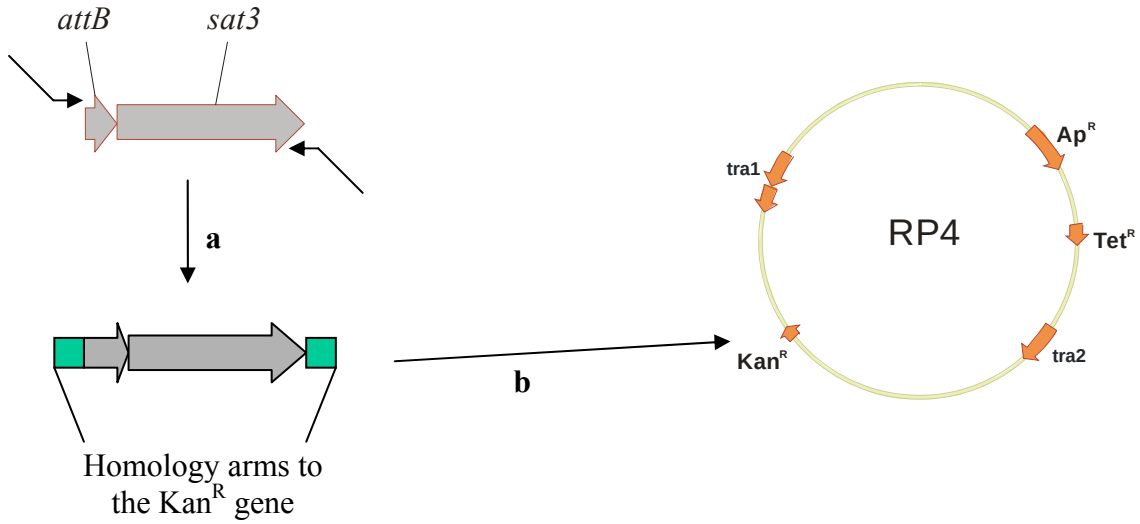


Fig. 30: Constructing of the RP4' *asn* "trapping" plasmid.

a. Amplification of *attB*/*sat3* PCR product with addition of Kan^R gene homology arms.
b Introduction of *attB*/*sat3*gene fusion in RP4 plasmid with the help of lambda Red recombinase system

4.2 Trapping of the "mini-island"

To prove the ability of the RP4'*asn* trapping plasmid to capture integrative elements, we tested RP4'*asn* for its ability to recombine with pKR549 *orf1-5r* plasmid. The latter suicidal construct contains the integrative module of the high-pathogenicity island (Rakin *et al.*, 2001) with addition of the entire gene cluster from the AT-rich region of the HPI carrying the excisionase (Lesic *et al.*, 2004) (Fig. 31). Thus, this suicide plasmid might be considered as a free circular (or excised) form of the HPI lacking its core part that is responsible for the yersiniabactin biosynthesis. *E. coli* JM109 strain was transformed with pKR549 *orf1-5r* plasmid and clones with plasmid integrated into the *asnT* tRNA gene were selected. The RP4'*asn* construct was subsequently transferred into JM109 *asnT*::pKR549 *orf1-5r* integrant by conjugation. To estimate the efficiency of RP4'*asn*-mediated mobilization of the "mini"-HPI,

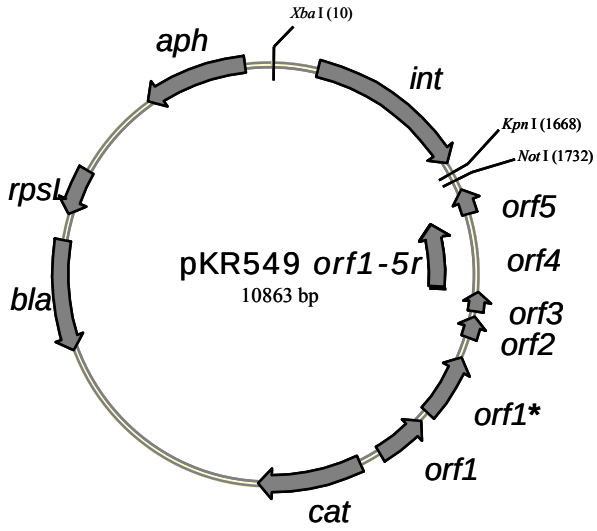


Fig. 31: Plasmid map of the suicide "mini-island" construct pKR549 *orf1-5r*.

we carried out conjugation between RP4'*asn*-bearing JM109 *asnT*::pKR549 *orf1-5r* strain as a donor and HB101 as a recipient. Transconjugants were selected on two different selective media. The first one was supplemented with streptomycin, tetracycline and chloramphenicol, to select for mobilization of the pKR549 *orf1-5r* by RP4'*asn*, while the second one lacks chloramphenicol to estimate the transfer rate of the shuttle plasmid alone. We also transferred original RP4 (without *asn* tRNA gene) plasmid into JM109 *asnT*::pKR549 *orf1-5r* integrant. RP4-bearing JM109 *asnT*::pKR549 *orf1-5r* strain was used as a negative control in "mini-island" trapping experiments. RP4'*asn* was efficiently transferred with $3.2X10^{-1}$ per donor CFU frequency whereas the integrated pKR549 *orf1-5r* was mobilized by RP4'*asn* with a frequency of $2.6X10^{-7}$ to $5.8X10^{-8}$ per donor CFU. In contrast, in negative control experiments efficiency of RP4 transfer was the same as for RP4'*asn*, but RP4 failed to mobilize the integrated pKR549 *orf1-5r* plasmid (no Cm^r clones). Transconjugant clones resistant to Sm, Tc and Cm were examined for the presence of the cointegrate of RP4'*asn* with pRK549 *orf1-5r* by PCR with aph_wild_for and c15-205 primers (Table 2). All 20 tested clones were positive and thus contain the recombinant plasmid.

In order to investigate whether the growth conditions of the donor affect "mini-HPI" excision, we also performed conjugation with donor obtained from different growth phases (log- and stationary phases), but detected no effect on mobilization efficiency. Reducing the temperature to 26°C also did not increase, or even slightly decreased the frequency of cointegrate transfer.

4.3 Horizontal transfer of the whole HPI_{Yps}

In previous experiments we demonstrated relatively high level of excision of the HPI in *Y. pseudotuberculosis* YPS06 strain. This strain carrying the HPI in *asn3* tRNA gene (for better comparison we use designations of the *asn* tRNA genes proposed by B. Lesic *et al.* (Lesic *et al.*, 2004)) was selected for HPI "trapping" experiments.

4.3.1 Introduction of resistance marker in to HPI_{Yps}

To facilitate selection of the mobilized integrative element, the HPI was labeled with a selective marker. For introduction of a Cm^r cassette a non-coding promotorless region of the HPI between *fyuA* and *orf1* in YPS06 was selected (Fig. 32). The modified method of one-step inactivation of chromosomal genes was applied (Datsenko and Wanner, 2000). A plasmid pKD3 was used as a template for generation of PCR fragment containing chloramphenicol resistance cassette flanked by FRT-sites and 50-nt homology arms for non-coding region of the HPI between *fyuA* and *orf1*. For this primer pair HPI_ins2 and HPI_ins2_rev were employed (Table 2). *Y. pseudotuberculosis* YPS06 cells harboring pKD46 plasmid were grown in the presence of arabinose to induce Red recombinase. These competent cells were transformed by the purified PCR product carrying the Cm^r cassette.

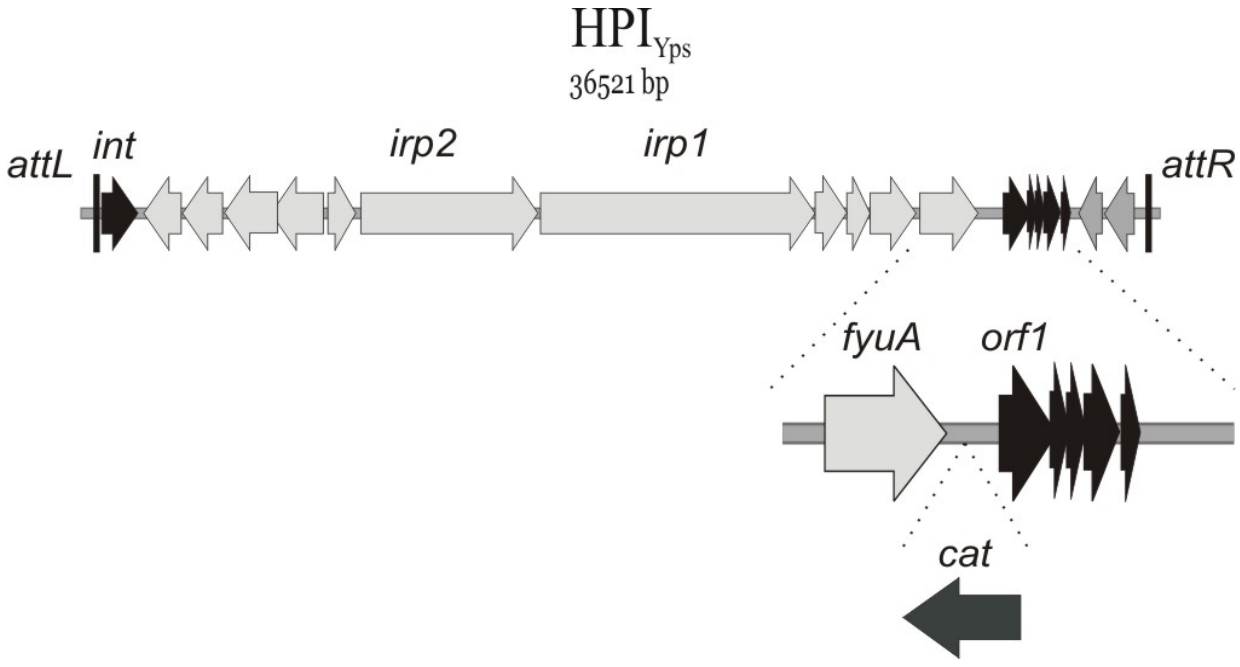


Fig. 32: Labelling of Yps HPI by a resistance marker

Recombinant clones were selected on LB agar containing chloramphenicol. PCR with primers HPI 1220 and fyu18 (Table 2) confirmed insertion of the chloramphenicol resistance cassette. The resulting strain was designated YPS06 HPI Cm^r.

4.3.2 Conjugative transfer of the HPI_{Yps}

The RP4'*asn* plasmid was subsequently transferred into YPS06 HPI Cm^r strain by conjugation and YPS06 HPI Cm^r (RP4'*asn*) strain was used as donor for further conjugation with HB101 recipient. The frequency of HPI Cm^r marker transfer was 1.8×10^{-8} per donor CFU. The size of the plasmid DNA isolated from Cm^r transconjugants was analyzed by the standard method of Kado and Liu (Kado and Liu, 1981). The plasmid DNA isolated from Sm/Tc/Cm-resistant clones was significantly larger than the original RP4'*asn* plasmid (Fig. 33). Hence one can assume that this larger plasmid represents a cointegrate of RP4'*asn* with the mobilized HPI Cm^r pathogenicity island. The accuracy of the HPI Cm^r integration into *asn* tRNA gene of RP4'*asn* and preservation of the recombination attachment sites was proven by PCR with aph_wild_for/c15-205 and sat3_seq/IS131 primer pairs (Table 2) and subsequent sequencing of the PCR products (Fig. 34). Presence of the intact *attL* and *attR* recombination sites confirmed the precise character of the site-specific recombinbation.

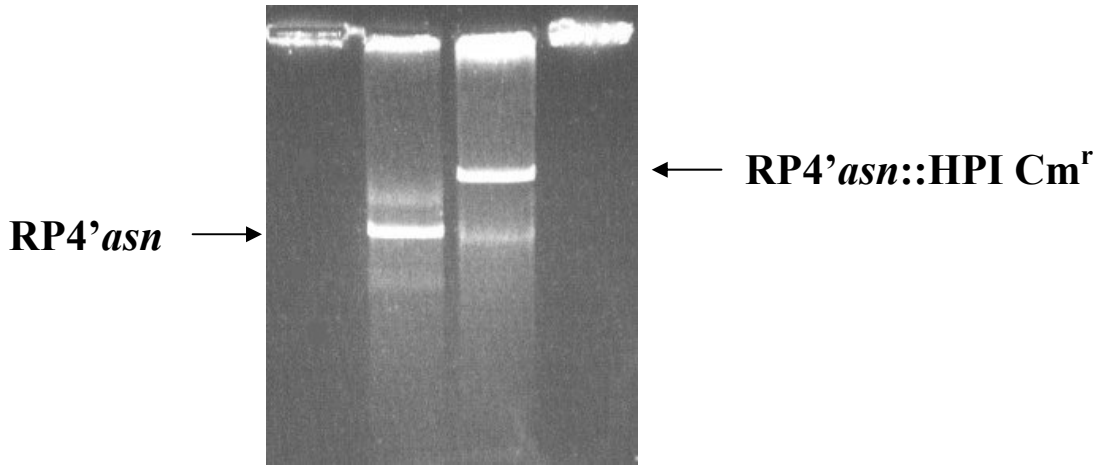


Fig. 33: Comparison of electrophoretic mobility in agarose gel of RP4'*asn* trapping plasmid with RP4'*asn*::HPI Cm^r cointegrate

A.

```
  1 GTGCGGATTT TTGCGTCGGT AAGGGACATA TGGGGGTCAC TCCATTATCG
 51 AACTAACCTG ACCCCAGATT TGACCACCAA TTTTTCCCGA TGCGTAGGGT
101 AAAATCAAAA TGCACCGAGA AGAGTTTTCA CACTAACCTG TTGAATCAAC
151 ATCAGATAAA GATTCGCAAG GATGCATGAA AACAGAAAAT TGGCTCCTCT
201 GACTGGACTC GAACCAGTGA CATACGGATT AACAGTCCGC CGTTCTACCG
251 ACTGAACTAC AGAGGAATCG CGTGAACGGG GCGCATCGTA TCCCATCACG
301 TGAGCCGCC
```

B.

```
  1 ACAGACCTTT ATTATAAATA ATATAATAAC TTTCTTTATT TTCAATAAGT
 51 TTTAAAAAAT AAATCATAGC AATGCCATGA AAAATACCAT GCTCAGAAAA
101 GGCTTAACAA TATTTTGAAA AATTGCCTAC TGAGCGCTGC CGCACAGCTC
151 CATAGGCCGC TTTCCTGGCT TTGCTTCCAG ATGTATGCTC TTCTGCTCCT
201 GCAGGCATGC AAGCTTGGAG AGGAAGGGTG CTGTTGAGCC GCTGAGACTA
251 GACGTAGATA AGCGAGGAGA GTAACTCAGT GATAGAAAAG CAAAAATCCC
301 GCTTAGTTTC CTAAACGGGA TTTTCTAAAT TTGGCTCCTC TGACTGGGAT
351 CGCCTTTGCC AGCAACTGGC TAATAATTAA GCTAAACCTG AATTGTATTT
401 CTGTCAAGAC CACCATAATG ACCACCAATA GTTGCGGGTT GCATAAATCT
```

Fig. 34: DNA sequences of the *attL* (A.) and *attR* (B.) attachments sites of the RP4'*asn*::HPI Cm^r cointegrate plasmid

The intact core-sites are highlighted in bold italics.

4.4 Reconstruction of CAS-phenotype in *Y. enterocolitica* WA-TH⁻ strain

To prove that the mobilized HPI retains all the core genes responsible for yersiniabactin production, we transferred the RP4'*asn*::HPI Cm^r cointegrate into the HPI-cured *Y. enterocolitica* WA-TH⁻ strain (Pelludat *et al.*, 2002). Transconjugants of WA-TH⁻ obtained the ability to synthesize the yersiniabactin (CAS-positive phenotype, Fig. 35a) after acquisition of RP4'*asn*::HPI Cm^r cointegrate. To prove the identity of yersiniabactin production by transconjugants, we tested their ability to feed *Y. enterocolitica irp1, fyuA-gfp* tester strain unable to synthesize yersiniabactin that contains a GFP reporter fused to the yersiniabactin receptor FyuA (Brem *et al.*, 2001). The supernatants of both *E. coli* HB101 RP4'*asn*::HPI and *Y. enterocolitica* WA-TH⁻ RP4'*asn*::HPI transconjugants contained the siderophore utilized by the FyuA receptor (Fig. 35b). Thus the complete functional HPI could be trapped by RP4 *attB*-presenting shuttle plasmid.

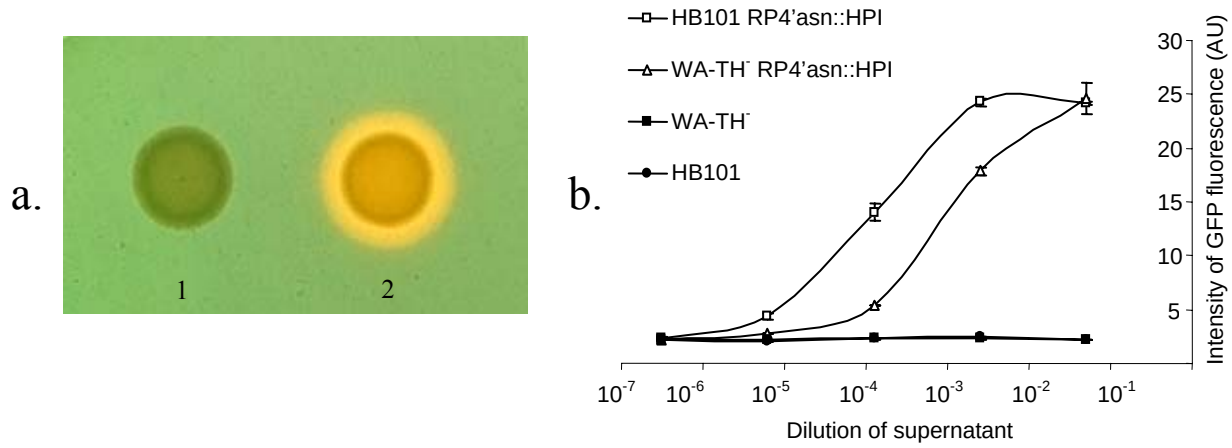


Fig. 35: Detection of yersiniabactin production by CAS-agar assay (A) and Fe-Ybt/*fyuA-gfp* reporter assay (B)

A culture spot of *Y. enterocolitica* WATH$^-$ (1) and its RP4'*asn*::HPI Cm^r -bearing derivative (2).

GFP fluorescence (arbitrary units) of *Y. enterocolitica* WA-CS irp1::Kan^r (pCJFY5G3) reporter strain grown in NBD medium supplemented with culture supernatants from tested strains.

4.5 Cointegrate instability and HPI reintegration

RP4'*asn*::HPI Cm^r turned out to be relatively stable. However, the cointegrate might dissociate into RP4'*asn* plasmid and non-replicating HPI Cm^r that has to rescue itself by reintegration into any non-occupied *asn* tRNA target gene. For example, the transposition of the HPI into the chromosomal *asnW* RNA gene (detected by PCR with c15-205/W761 primers) occurred by propagation of HB101 (RP4'*asn*::HPI Cm^r) under standard conditions (LB, 37°C, 18 hours). To isolate individual integrants with the HPI Cm^r inserted into chromosomal *asn* tRNA targets, we performed enrichment of chloramphenicol resistant and tetracycline sensitive clones by cultivation of the HB101 (RP4'*asn*::HPI Cm^r) with the plasmid-curing agent plumbagin (Bharathi and Polasa, 1991). Selected clones were analyzed by PCR with c15-205 primer for the HPI integrase gene and primers T167, U211, V251 and W761 (Table 2, Fig. 5) for *asnT*, *asnU*, *asnV* and *asnW* tRNA genes, respectively, to prove chromosomal localization of the HPI. By this approach we were able to select 24 clones with HPI integrated in *asnW*, 11 clones - with HPI in *asnU* and 9 clones - with HPI in *asnV* (out of 44 analyzed). We were unable to select individual clones with HPI insertions in *asnT* locus, although PCR with the total cell lysate was positive with c15-205/T167 primer pair (Table 2) after plasmid curing.

4.6 Efficiency of cointegrate transfer in *Y. enterecolitica* WA-C wild strain

The RP4'*asn*::HPI Cm^r cointegrate were tested for the efficiency of conjugative transfer to the *Y. enterocolitica* O:8, strain WA-C. Conjugation was carried out with *E. coli* HB101 (RP4'*asn*::HPI Cm^r) and two different recipient strains: *Y. enterocolitica* O:8 WA-C and *E. coli* JM109. The transconjugants were selected on nalidixic acid (marker for the both *Y. enterocolitica* and *E. coli* JM109 strains), tetracycline and chloramphenicol (cointegrate

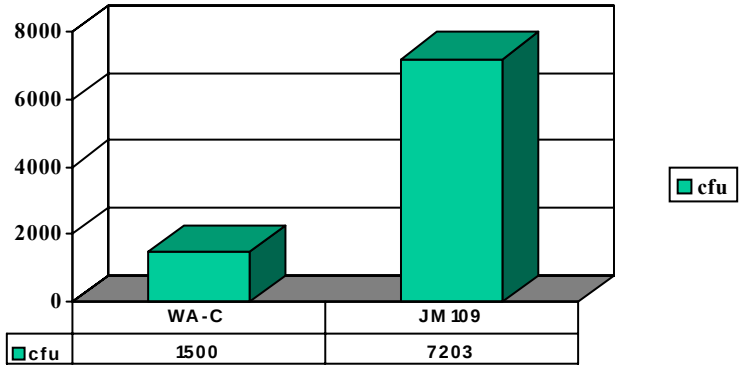


Fig. 36: Efficiency of RP4'*asn*::HPI Cm^r cointegrate transfer to the *Y. enterocolitica* O:8 WA-C (left bar) and *E. coli* JM109 (right bar).

selective markers). The frequency of RP4'*asn*::HPI Cm^r transfer was calculated as a qantity of resistant colonies (Nal^R, Cm^R, Tc^R) per donor CFU. The *E. coli* JM109 strain was shown to accept the cointegrate more efficiently than *Y. enterocolitica* WA-C strain (Fig. 36). According to our data the *Y. enterocolitica* WA-C strain may posess a powerfull restriction system.

5. Factors reducing the freqency of the lateral gene transfer

5.1 Determination of nucleotide sequence of the new restriction-modification (RM) system *Yen*I

The frequency of conjugative transfer of RP4'*asn*::HPI Cm^r cointegrate to *Y. enterocolitica* WA-C strain was significantly reduced. This character can be ascribed to restriction-modification (RM) system in *Y. enterocolitica* WA-C. For sequencing of the latter the pSAK2 recombinant plasmid was applied (a kind gift of Virginia L. Miller), which carried 5039-bp DNA fragment with an *yenI* locus from *Y. enterocolitica* 8081 isolate. The sequence was deposited at the GenBank under the accession number AJ414030. The partial sequencing of the homologous ORFs from *Y. enterocolitica* WA-C showed complete sequence identity. Figure 37 represents the organization of the *yenI* sequence and harbouring regions. DNA sequence analysis identified a single 2481 bp open reading frame that encodes 826 aa large polypeptide.

We analysed sequences neighbouring the *yenI* gene and found a copy of IS*1222* insertion sequence 667 bp upstream of the start codon of *yenI*. Multiple copies of IS*1222* are present in the genome of *Y. enterocolitica* 1B. Also a 60-bp sequence with extremely high similarity (91-88%) to phage PhiR73 and P4 phage sequences is located 562-bp upstream of the ATG start codon of *yenI*, between *yenI* and IS*1222*. Moreover, the G+C content of the *yenI* gene turned out to be atypically low for *Y. enterocolitica*, 37,8 G+C% in contrast to 46 G+C% of the yersiniae house-keeping genes.

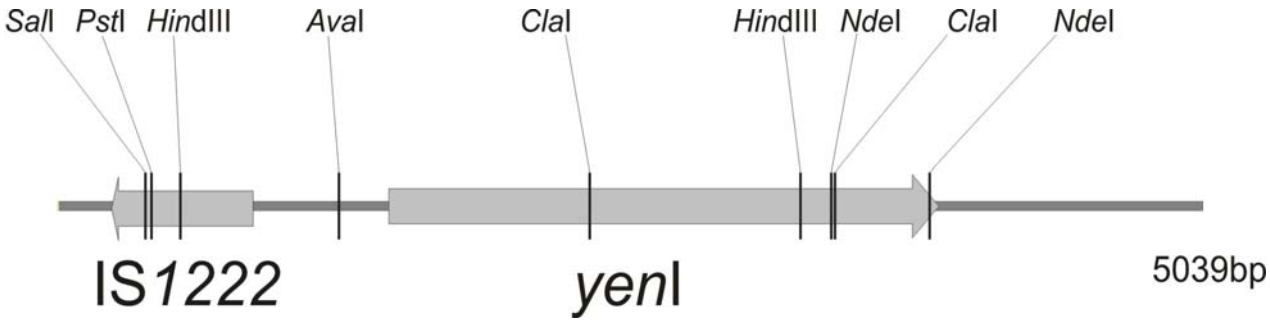


Fig. 37: Organization of the *Yen*I restriction-modification system.

5.2 Bioinformatic analysis of the *yen*I locus and comparison with other known restriction-modification systems

A resulted sequence was subjected to homology search in the NCBI databank. Analysis revealed two highly similar RM systems from *Pst*I-isoschizomeric group, namely *Pst*I and *Bsu*I. The first one, *Pst*I, is composed of two convergently transcribed genes, *hsmPI* and *hsrPI*, encoding *Pst*I methyltransferase (MTase) and endonuclease (ENase), while *Bsu*I has an operon of two genes, *hsmBI* and *hsrBI*, transcribed in tandem. In contrast, *Yen*I RM system has one large ORF showing homology both to MTase and ENase of *Bsu*I and *Pst*I in its N- and C-termini, respectively (Fig. 38). Thus, the YenI polypeptide shares two alternative functions, restriciton and modification. The N-terminal part of the YenI has 45% and 40% identity (61% and 58% positives) to PstI MTase and BsuI MTase, respectively, while the C-terminal part depicts 55% and 45% identity (76% and 59% postitives) to ENases of both isoschizogenic enzymes. Restriction endonucleases usually do not have extensive homology at amino acid sequence level, even when they recognize the same DNA sequence. *Pst*I/*Bsu*I /*Yen*I isospecific group is one of the rare exceptions showing a high degree of homology.

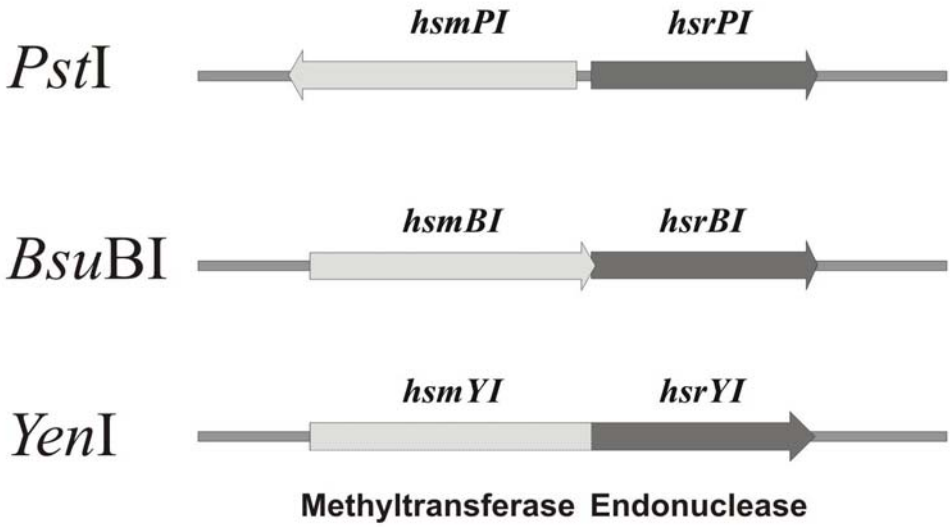


Fig. 38: Comparison of the three *Pst*I isoschizomeric restriction-modification systems
hsm - methylase (Mtase) and *hsr* - endonuclease (ENase) encoding genes, respectively.

5.3 Construction of YenI expression plasmid

Y. enterocolitica 0:8, strain WA-C was used for the isolation of the functional *yenI* gene. The restriction-modification gene was amplified from chromosomal DNA of WA-C by PCR using primers RMS Nde and RMS Bam (Table 2), introducing *Nde*I and *Bam*HI restriction sites

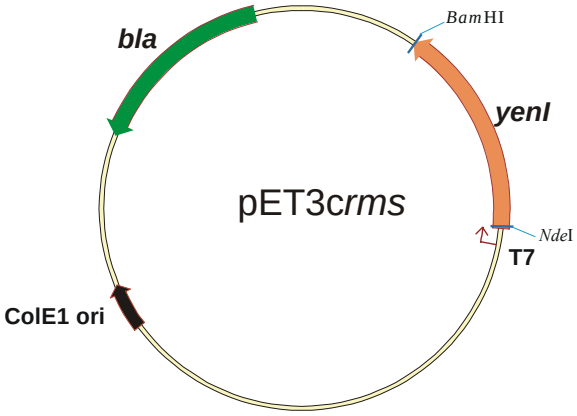


Fig. 39: Vector map of pET3c*rms*

The *yenI* gene is depicted by the orange arrow. Beta-lactamase gene is represented with a green arrow. Col E1 replication origin appears as black. The T7 promoter as a thin arrow. Cloning sites are marked on the map.

at the ends of the product. After partial hydrolysis of the PCR product by NdeI endonuclease, the full-length (2481 bp) DNA fragment was isolated from the gele and ligated into *Nde* I - *Bam* HI linearized expression vector pET-3c. The resulted plasmid was named pET-3c*rms* (Fig. 39). Expression vector was proved by sequencing and introduced into the *E. coli* expression strain BL21 (DE3).

5.4 Construction of endonuclease-deficient *yenI* ORF

To create a restriction-deficient *yenI* mutant we deleted a *Nde*I - *Nde*I fragment inside *yenI* ORF, encoding the endonuclease activity of YenI (Fig. 40). For that, a PCR product containing full-length *yenI* ORF was digested by *Nde* I endonuclease, the resulted fragments mixture were

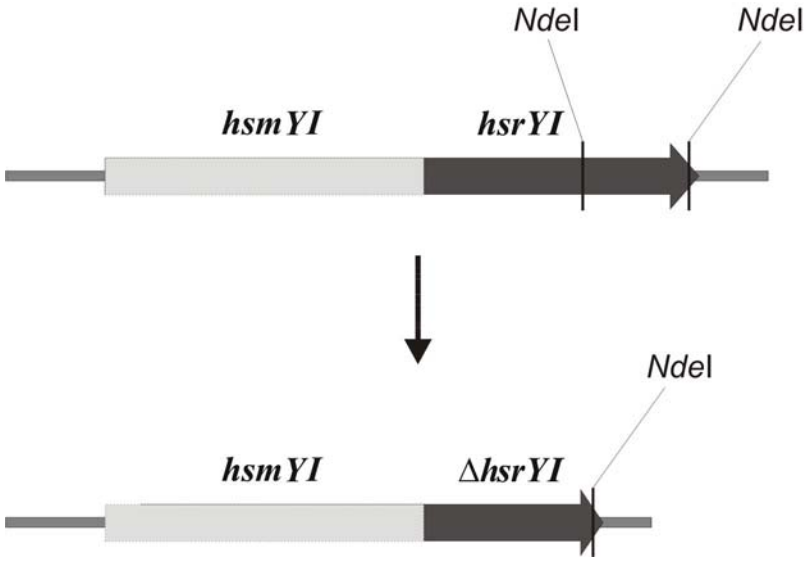


Fig. 40: Construction of *hsrYI* deletion mutant of *Yen*I restriction-modification system

The hsm and hsr parts of the *yenI* ORF are designated with gray and black colors, respectively.

ligated and used as a template for PCR amplification with RMS Nde and RMS Bam primers (Table 2). The products of the amplification were separated by agarose gel-electrophoresis and a product with approximate molecular mass of 2kb was isolated from the gel and cloned into pET3c plasmid. Sequencing proved the deletion of a *Nde*I - *Nde*I fragment inside *yenI* ORF. The resulted ORF was named *yenIΔhsrYI* and plasmid was named pET-3c*rms*Δ. Expression vector was introduced into the *E. coli* expression strain BL21 (DE3).

5.5 Expression of *yenI* and *yenIΔhsrY*

The *yenI* and *yenIΔhsrYI* genes were overexpressed in *E.coli* BL21 (DE3) containing pET-3c*rms*.and pET-3c*rms*Δ, respectively. Cells were grown in LB medium with glucose (supplemented with carbenicillin) at 37 °C to early logarithmic phase and induced with IPTG for 5 h. A band with the molecular mass of 93 kDa corresponding a full-lenght Yen I protein appeared on SDS-PAGE, as well as a band with the predicted reduced molecular mass of 78 kDa corresponding to a truncated YenI recombinant protein (Fig. 41).

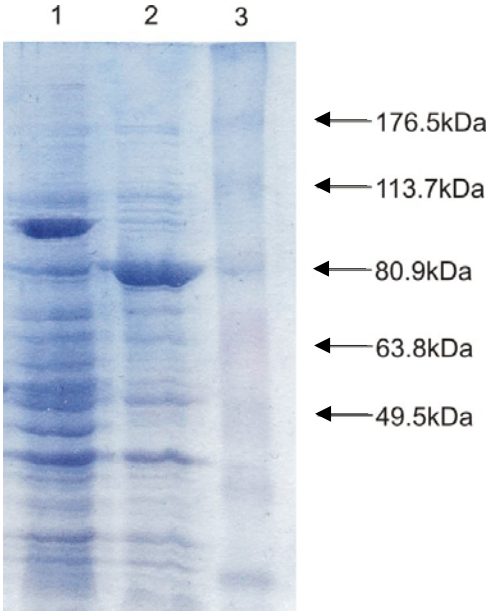


Fig. 41: Recombinant YenI protein (lane 1) and endonuclease-deficient derivative YenI^x (lane 2) after SDS-PAGE separation of *E. coli* producer strains cell lysate. Lane 3: protein molecular weight marker. Relevant protein marker sizes are indicated on the left with arrows.

5.6 Construction of Yen I endonuclease-deficient mutant

The modified method of one-step inactivation of chromosomal genes was applied (Fig. 42). The *yenI* ORF was cloned in pCR2.1-TOPO vector resulting pCR2.1-TOPO/*yenI* construct. Subsequently, pCR2.1-TOPO/*yenI* plasmid was digested by *Cla*I endonuclease and blunt ended. The linear vector was ligated with Kan^r cassette-bearing PCR fragment amplified from pKD4

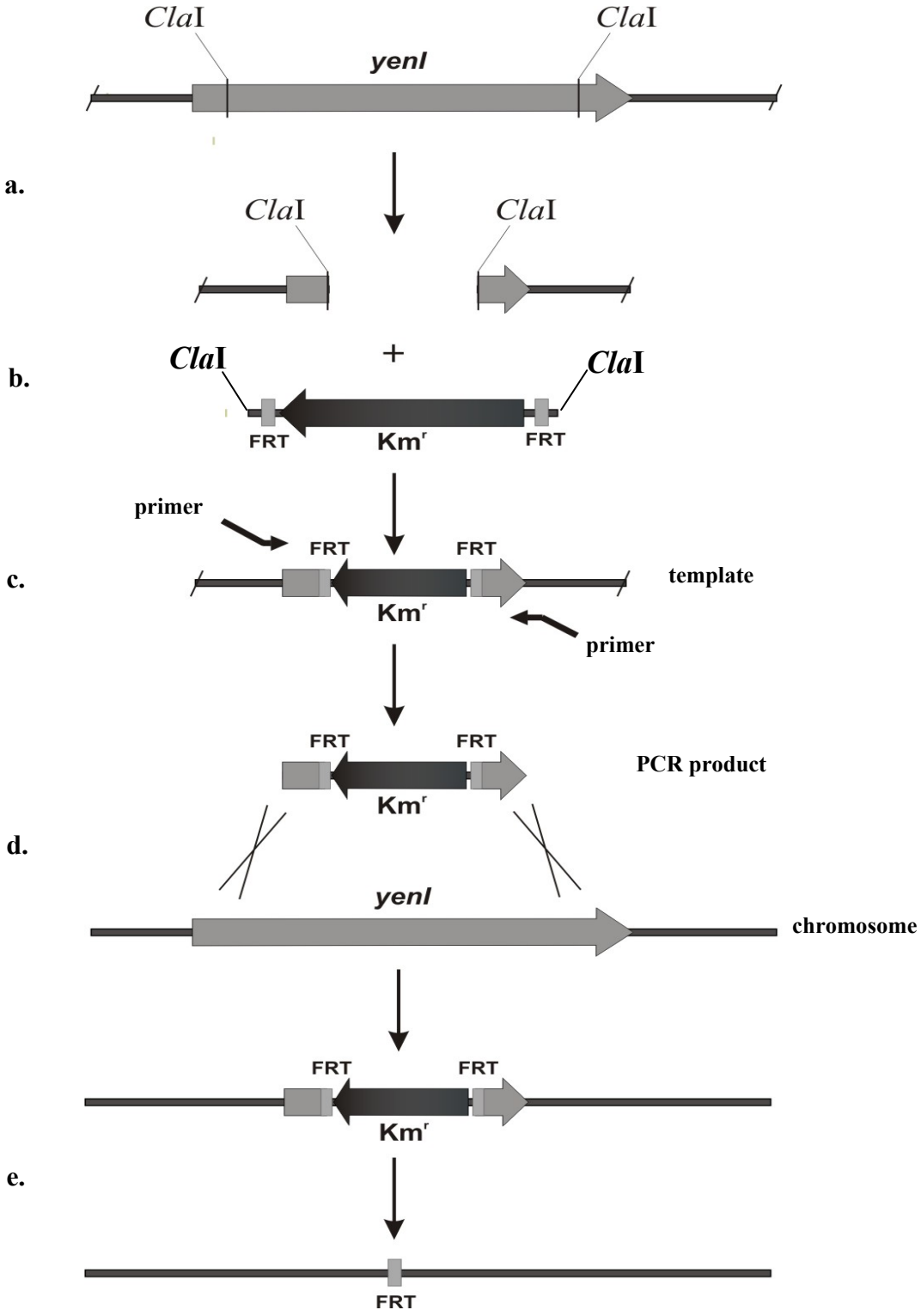


Fig. 42: Schematic presentation of construction of the *yenI*-deficient mutant by one-step gene inactivation

a. Digestion of pCR2.1-TOPO/*yenI* plasmid by *Cla*I cutting sites.
b. Insertion of the Kan^r cassette from pKD4 plasmid.
c. Preparation of PCR product with particular homology arms.
d. Red recombinase-mediated homologous recombination.
e. Removing of resistance cassette by FRT-recombination sites.

plasmid using standard primers (Datsenko and Wanner, 2000). Resulting plasmid was used for PCR amplification of a fragment, carrying Kan^r cassette and flanked by *yenI* homology arms with rms_for and rms_rev primers (Table 2). The purified PCR product was transformed into *Y. enterocolitica* WA-C cells harbouring pKD46 and grown in the presence of arabinose to induce the Red recombinase. Recombinant clones were selected by plating on LB-agar containing kanamycin (25 μg ml^{-1}) and confirmed by PCR. The resistance cassette was removed using thermoinducible FLP recombinase on pCP20 plasmid. The resulting strain harbouring a deletion in *yen*I gene was proved by PCR and sequencing and named WA-C *hsmYI, hsrYI*.

5.7 Methylation activity of YenIΔhsrYI protein

An *E. coli* strain BL21 (DE3) containing pET-3c*rms*Δ and producing truncated restriction-deficient YenI protein was tested for its ability to methylate the single *Pst*I site in the pET3c plasmid. To determine the methylation efficiency, methylated and non-methylated pET3c

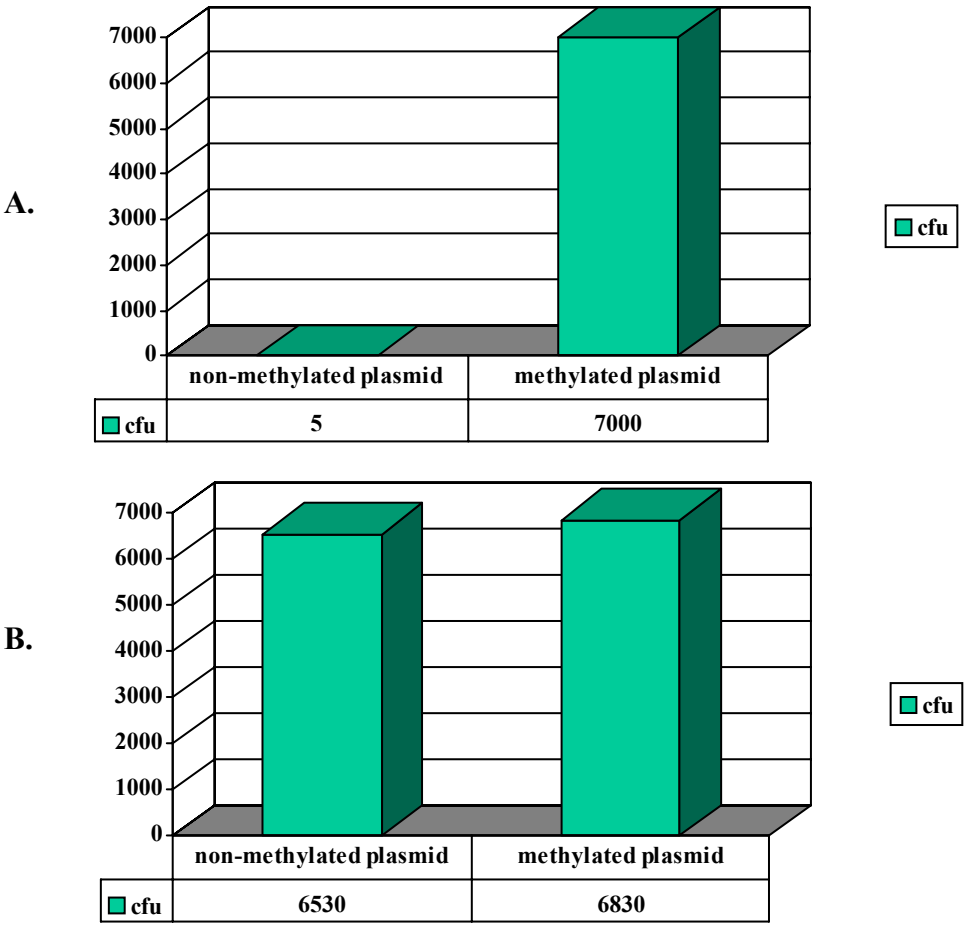


Fig. 43: Efficiency of electroporation (cfu/μg DNA) of non- M*Yen*I methylated and M*Yen*I methylated plasmid DNA

Y. enterocolitica WA-C wild type strain (**A**) and WA-C *hsrYI, hsmYI* mutant (**B**).

plasmids were electroporated in to *Y. enterocolitica* O:8 WAC wild type and its restriction deficient derivative WA-C *hsmYI, hsrYI*, in which the complete *yen*I ORF was deleted, as described above.

Electroporation of non-MYenI methylated plasmid DNA into wild type strain showed a dramatic reduction of transformants (5 cfu/μg) in comparison with MYenI methylated plasmid DNA ($7x10^3$ cfu/μg). Similarly, the elimination of *Yen*I restriction activity increases electroporation efficiency by at least 1000-fold (Fig. 43). Thus, both approaches namely, inactivation of endonuclease activity in recipient and *Yen*I methylation in a donor strain might be applied to increase the efficiency of genetic transfer to *Y. enterocolitica* biotype 1B.

5.8 Efficiency of RP4'*asn*::HPI Cm^r cointegrate transfer in *Y. enterecolitica* WA-C and WA-C *hsrYI, hsmYI* mutant

To prove that *Yen*I restriction-modification system was the factor reducing RP4'*asn*::HPI Cm^r conjugative transfer to *Y. enterecolitica* WA-C, we carried out conjugation experiments with *E. coli* HB 101 (RP4'*asn*::HPI Cm^r) as a donor strain and two different *Y. enterocolitica* O:8 recipients: WA-C and WA-C *hsrYI, hsmYI*. The transconjugants were selected on nalidixic acid (marker for the both recipient strains), tetracycline and chloramphenicol (cointegrate selective markers). The frequency of RP4'*asn*::HPI Cm^r transfer was calculated as a proportion of resistant colonies (Nal^R, Cm^R, Tc^R) per donor CFU. As expected, the *yenI* mutant strain acquired the cointegrate more efficiently ($6.4x10^3$ cfu) than *Y. enterocolitica* WA-C strain ($1.5x10^3$ cfu) (Fig. 44). Thus, we can conclude that RM systems play reductive role in dissemination of genomic islands.

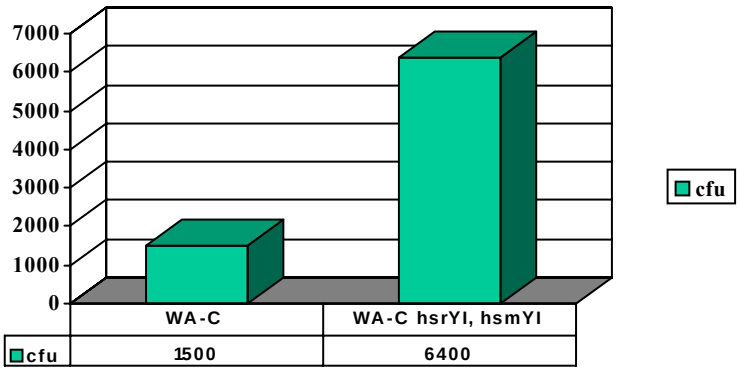


Fig. 44: Efficiency of RP4'*asn*::HPI Cm^r cointegrate transfer to the *Y. enterocolitica* O:8 WA-C (left bar) and *Y. enterocolitica* WA-C *hsmYI, hsrYI* (right bar)

D. DISCUSSION

1. Key elements, involved in mobility of *Y. pestis* HPI

Genomic islands (and pathogenicity islands, PAIs, as part of this group of mobile elements) are, in contrast to temperate phages and plasmids, non-self replicating, non-transmissive genetic elements that utilize element-encoded highly efficient site-specific mechanism of recombination to rescue themselves. PAIs carry a recombinogenic module consisting of recombinase genes (encoding integrase and excisionase) and are flanked by two recombination sites, *attL* and *attR*. In addition to recombinase module, PAIs contain an insert of different size encoding various virulence-associated traits.

The high-pathogenicity island (HPI) of *Yersinia* contains two main parts, the "core" and the variable AT-rich part. The "core" contains a functional cluster of genes coding for biosynthesis, transport and regulation of the siderophore yersiniabactin, and a recombinogenic module consisting of the recombinase gene (or integrase int_{HPI}) and two hybrid recombination *attL* and *attR* sites recognized in excisive recombination. HPI integrase plays the main role in both integration and excision of the island. To promote its function Int_{HPI} binds as a dimer to *attP* site, like in the case of lambda integrase (Wojciak *et al*., 2002). Another factor - IHF also efficiently binds *attP* site of HPI and might play role in recombination of HPI. It has been shown previously that IHF creates bends in lambda *attP* DNA so as to help *attP* condense into a compact structure that is activated for recombination (Robertson and Nash, 1988). To assist excision the HPI contains an excisionase that plays an architectural role in the reversion of integrative site-specific recombination. Such a recombination directionality factor is represented by Xis_{HPI} (corresponding to Orf43 of the *Y. pestis* KIM *pgm*-locus and YPO1904 of *Y. pestis* CO92 complete sequence; (Deng *et al.*, 2002; Parkhill *et al.*, 2001) and Hex in *Y. pseudotuberculosis*; (Lesic *et al.*, 2004) which is encoded in the variable AT-rich part of the island distinct from the location of the HPI-integrase (Fig. 12). In contrast to the clustering of integrase and excisionase genes in λ-like mobile elements, the xis_{HPI} gene is separated from its corresponding P4 phage-like integrase gene by a large insert of the yersiniabactin gene cluster.

Interestingly, the variable AT-rich part of the HPI differs markedly in two evolutionary lineages, *Y. enterocolitica* (HPI_{Yen}) and *Y. pestis* / *Y. pseudotuberculosis* (HPI_{Yps}). And only the AT-rich part of the HPI_{Yps} group carries the excisionase gene. Also only the HPI_{Yps} is widely distributed among *Enterobacteriaceae*, especially in extraintestinal pathogenic isolates of *E. coli* (ExPEC) (Schubert *et al.*, 2000; Karch *et al.*, 1999). However, the AT-rich variable part is deleted in most *E. coli* apart from E. coli ECOR31 that contains the ICE form of the HPI

(Schubert *et al.*, 2004a). This might indicate the loss of the AT-rich part in *E. coli*, but also the possibility that this part of the island has not yet been acquired by the HPI_{Yps} in *E. coli*. Absence of the right recombination site, *attR*, in *E. coli* makes it difficult to speculate further on any of these possibilities. Thus the excision of the *E. coli* HPI integrated into the *asnT* site might be abolished by deletion of the sequences encoding the excisionase as well as *attR* recombination site. In contrast, in *Y. enterocolitica* the HPI_{Yen} was rendered incapable of excision by another mechanism, namely by inactivation of the integrase gene, although this mechanism was also described for certain *E. coli* strains (Karch *et al.*, 1999). The previous proposal that the HPI_{Yen} is immobile in *Y. enterocolitica* 8081 (Carniel *et al.*, 1996) due to deletion of the recombination site seems to be wrong, because the 18-bp "core" fragment as well as the complete *attR* site is revealed in the genome sequence of *Y. enterocolitica* strain 8081 (genome sequence on the Sanger site http://www.sanger.ac.uk).

We were not able to detect a functional promoter in the intergenic region between *orf1* and xis_{HPI}. However, the *orf1* promoter seems also to be a promoter for xis_{HPI} because both genes are transcribed as one mRNA molecule. On the other hand, *orf1* promoter has also a much lower activity when compared to the integrase promoter. This fits well with the proposal on cooperative activities of both enzymes, integrase and excisionase, necessary for excisive recombination. If the excisionase gene has a low activity promoter, then an excess of the integrase will be obtained and the integrative recombination will exceed the excisive one. Decreased expression of the integrase (that might be effect of stress or other environmental factors) results in an increased rate of excision and correspondingly survival of the egoistic element, the HPI. However by an extremely low activity of the integrase, the excision is blocked due to the fact that integrase as a recombinase plays the main role in both processes.

We have reconstructed a minimal recombinogenic part of the HPI to follow the recombination activity of the complete island using the integrative module (consisting of int_{HPI} and reconstituted *attP* site) by addition of the xis_{HPI} gene coding for the excisionase. Such a minimal recombinogenic module of the island was able to integrate into any unoccupied *asn* tRNA gene on the chromosome of *E. coli* although with a diminished frequency when compared to the integrative module lacking the *xis* gene (Antonenka *et al.*, 2006). This might be explained as a result of Xis_{HPI} activity that reduced the integration rate of the recombinogenic module by increasing its excision or by direct competition with the integrase activity. Another ORF inside the HPI with DNA-binding ability, ORF5, did not influence excisive but perhaps might have effected the integrative recombination.

In this study we have demonstrated the high affinity of binding of the recombinant Xis_{HPI} to the *attP*, but not to the *attB* recombination site (Fig. 21). This is in good agreement with the architectural role of the excisionase – to recognize the recombinant *att* sites in order to bring them together to the access of the recombination enzyme – the integrase. As it was shown in DNAseI footprinting experiments, Xis_{HPI} recognized the part of an *attP* site between the core and the DR3 repeat (Fig. 24). Particularly this region becomes a part of the recombinant *attL* site as a result of HPI integration in the host chromosome. This implies that the HPI excisionase acts asymmetrically by binding efficiently to *attL* recombination site to facilitate excisive recombination promoted by the Int_{HPI}. A similar manner of preferential binding of the excisionase to the *attL* was also demonstrated for phage λ (Cho *et al.*, 2002), which is in good agreement with our results.

The second *asn* tRNA gene-targeted genomic island, Ecoc54N, was defined by the bioinformatic approach (www.indiana.edu/~islander/). It is supposed to carry the genes involved in polyketide synthase synthesis. According to our data Ecoc54N also contains a functional recombinase with high homology to Int_{HPI} that recognizes *asnW* tRNA gene as its corresponding target and carries complete *attL* and *attR* attachment sequences (Antonenka *et al.*, 2006). This enables us to take an insight into the evolution of the recombination systems having the same recognition pattern. However, no open reading frame with similarity to known excisionases could be defined within the island. Thus, in contrast to HPI_{Yps}, Ecoc54N does not contain the directionality factor that might assist the recombinase to accomplish the excisive recombination necessary for further dissemination of the island. This supplies us with the opportunity to access the effect of the HPI excisionase in another closely related recombination system. Nevertheless, the Xis_{HPI} was not promiscuous enough to support the excisive recombination by the “non-cognate” Ecoc54N recombinase (Fig. 28).

Moreover, we could show the effect of Xis_{HPI} on excision of the HPI to be insignificant in *Y. pseudotuberculosis*. Only an eightfold increase was detected by Real Time PCR (Fig. 17) and 5 to 6-fold increase was documented by mobilization of the integrated mini-derivatives of the HPI carrying or lacking the excisionase by the RP4 *attB* trapping plasmid (Antonenka *et al.*, 2006). Analysis of the AT content and codon usage pointed out at possible different origin and independent acquisition of the recombinase and excisionase genes of the mosaic HPI. Thus one can speculate that the AT-rich part of the HPI with the excisionase has been acquired by the HPI by horizontal gene transfer at a different time and source than the recombinase. Perhaps the fine-tuning of the acquired excisionase to its “cognate” recombinase and recognition site could explain its minute effect on the excisive recombination.

Nevertheless, the ability of both islands to excise with low but detectable rates even in the absence of the excisionase emphasizes the supplementary role of the promiscuous directionality factor in excision of the island. The main role in this process plays the recombinase responsible for both types of recombination, integrative and excisive. However, acquisition of an additional factor that could increase the excision rate might be advantageous for the successful dissemination of the genomic island.

2. The model of the HPI_{Yps} dissemination

The fate of the excised non-replicating HPI depends on its ability to recombine with the available free recognition site in the genome of the bacterial cell. If such *attB* recombination site is present on a conjugative plasmid the HPI becomes a part of the transmissive cointegrate and could be passively mobilized to a new recipient within the host range of the shuttle plasmid. However, the ongoing activity of the element-encoded recombinase may result in resolution of the cointegrate with liberation of a free form of the HPI able to re-occupy any available attachment site in the genome of the new host. The transconjugant, in its turn, might serve as a secondary donor and accordingly maintain the circulation of the non-transmissive island.

According to our hypothesis there are three main requirements for the “shuttle”-mediated mobilization of the integrative elements (IEs): presence of a functional recombinase on the integrative element (integrase and directionality factor that increases the excision rate), non-interrupted sequences involved in site-specific recombination of the IE (element-encoded *attP* and bacterial *attB*), and acquisition of a shuttle *attB*-presenting conjugative episome (Antonenka *et al*., 2005). Most site-specific systems are rather independent from host cell enzymatic machinery and thus element-encoded functions are mainly responsible for the fate of the integrated element.

However, the activity of the HPI recombinase and consequently transposition of the HPI to new locations in the host chromosome is controlled by the mechanism of “promoter swapping”. In the integrated state the HPI recombinase gene is subjected to regulation of the *asn* tRNA gene promoter that is substituted by the reconstituted promoter in the *attP* site in a free from of the island (Rakin *et al.*, 2001). Thus, the activity of the HPI recombinase in the cointegrate is regulated by a weak *asn* tRNA gene promoter controlled by the bacterial cell. This low activity of the recombinase protects the cointegrate from dissociation. After resolution of the cointegrate the recombination *attP* site is reconstituted and the recombinase gene becomes subjected to a much stronger promoter (p.46). Such swapping upregulates the activity of the recombinase and

increases probability to rescue the non-replicating island in any free recognition site in the chromosome of the host. Thus the bacterial cell may influence acquisition / loss of the HPI by mechanism of the promoter swapping.

We were not able to collect individual clones with the HPI integrated in *asnT* tRNA gene although such insertions were detected in total cell lysates by PCR. This might be explained by possible disadvantages of such integration. The HPI, except for the ICE HPI_{ECOR31}, is integrated into the *asnT* tRNA gene in *E. coli*. However, the recombination apparatus of the HPI is inactivated in these integrants either by deletions in the recombinase gene or attachment sites (Karch *et al.*, 1999; Schubert *et al.*, 1999) making the further transposition of the HPI impossible. The recombinase gene is also inactive in all *Y. enterocolitica* 1B isolates (Rakin *et al.*, 1999). However the integrative module of the HPI demonstrated no preference for integration in all four available attachment sites in *E. coli* (Rakin *et al.*, 2001). Thus the HPI could integrate in any free recognition site, but its association with the *asnT* tRNA gene seems to be somehow unstable. Inactivation of the recombinase or attachments sites could stabilize insertion of the HPI.

Plasmids, able to recombine with the chromosome (episomes) can also mobilize the neighboring DNA sequences as a result of incorrect excision (Fig. 45). Such substituted episomes carrying parts of the host bacterial chromosome are described in literature (also those isolated from pathogenic bacteria) (Holloway and Low, 1987). These episomes „loaded“ with the

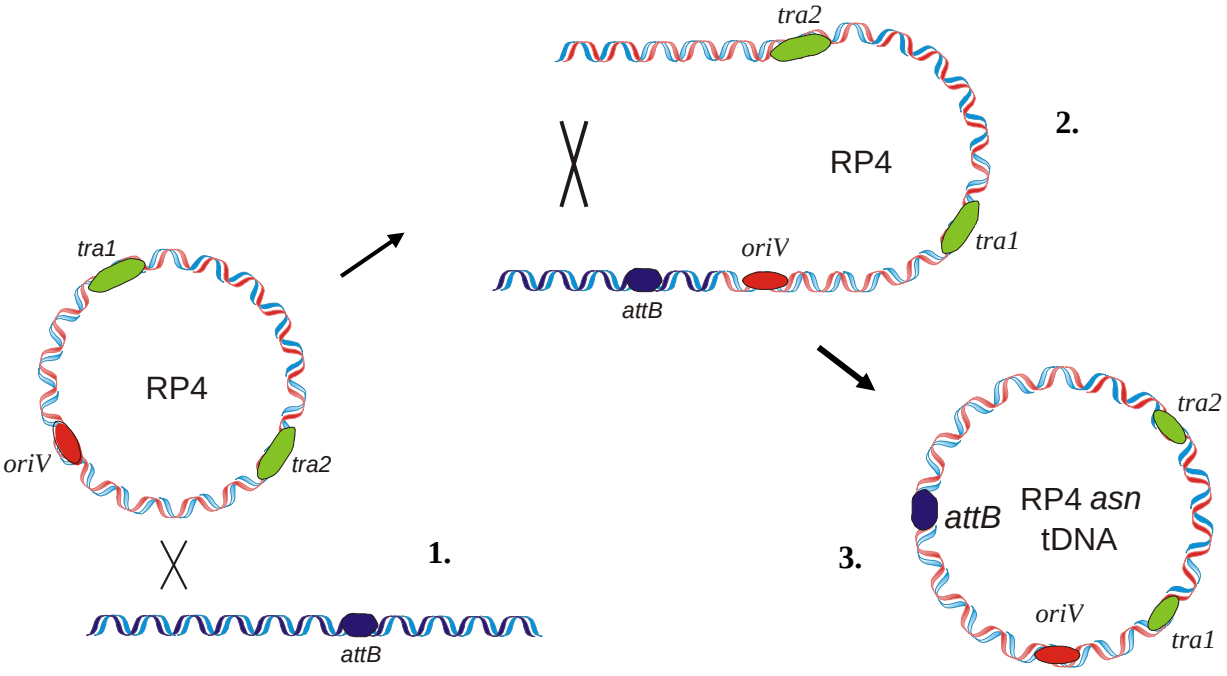


Fig. 45 Possible mechanism of “shuttle” plasmid formation by incorrect excision from the host chromosome.

1. Recombination of the transmissive plasmid with host chromosome. 2. Incorrect excision. 3. Possible „trapping“ shuttle plasmid

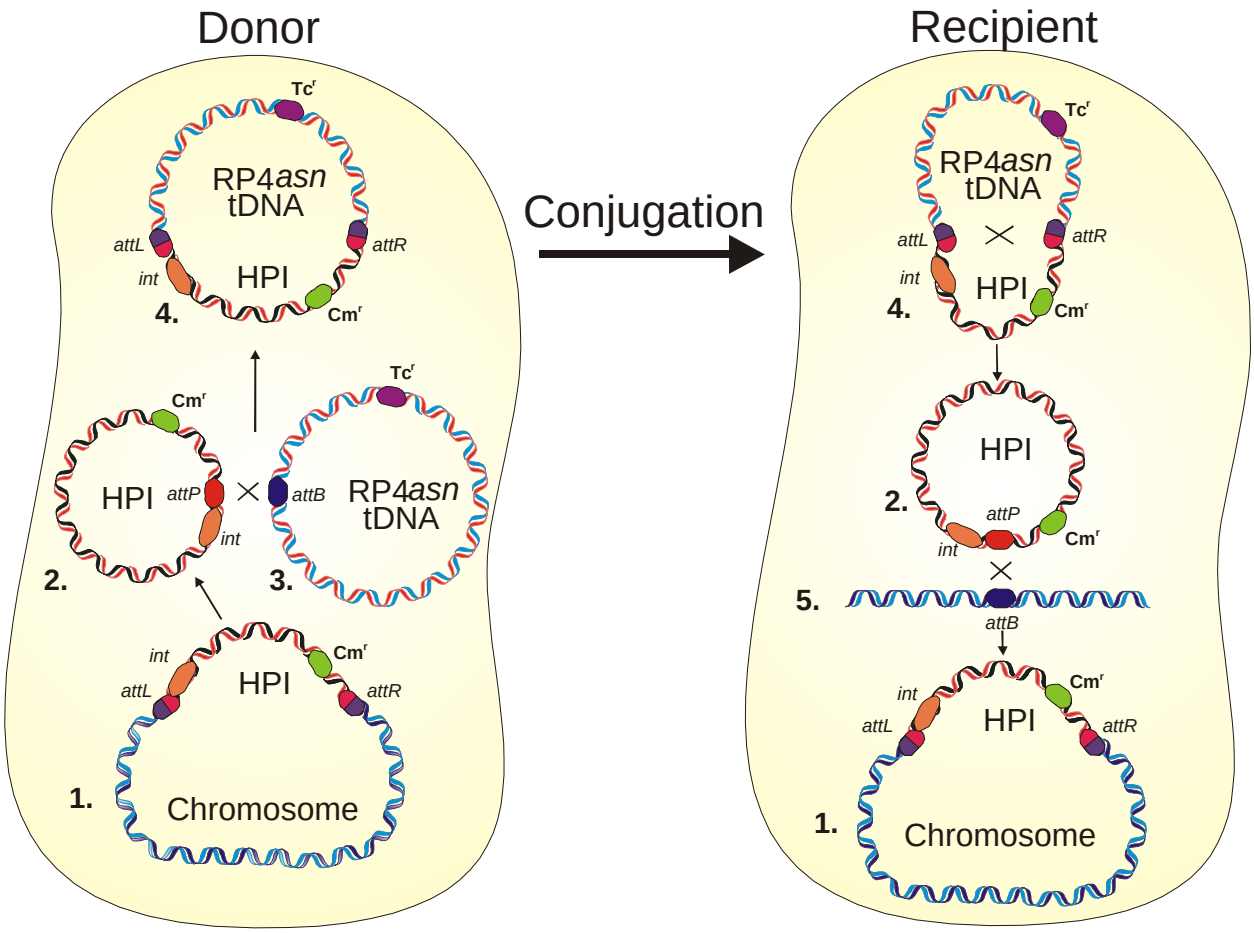


Fig. 46. Mechanism of the RP4'*asn*-mediated horizontal transfer of the HPI.

(1) Bacterial chromosome with the integrated HPI, delineated by *attL* and *attR* recombination sites. (2) Excised free form of the HPI with exposed *attP* recombination site. (3) RP4'*asn* trapping plasmid exposing *attB* recombination site. (4) RP4'*asn*::HPI cointegrate. (5) Bacterial chromosome with exposed *attB* recognition site.

recognition site for the integrative elements might serve as potential carriers of the integrated elements including genomic or pathogenicity islands. RP4 is a wide host range IncP1 plasmid that is able to integrate into the chromosome of the host and form RP4-primed derivatives carrying various chromosomal markers including the targets for the site-specific recombination of the IEs. We have remodeled this process of incorrect excision of the RP4 plasmid by introducing *attB* site for *Yersinia* HPI (the *asn* tRNA gene) in vitro and produced a conjugative vehicle as shuttle for targeting the HPI (Fig. 46). Conjugative plasmids of other incompatibility groups able to integrate in the chromosome can also be good candidates as "shuttles" for IEs.

The HPI belongs to most active integrative elements known today. It is widely disseminated in *Enterobacteriaceae*. However, the mechanism of its circulation is completely obscure. A phage-based mode of the HPI distribution might be proposed due to the presence of the phage-like sequences in the HPI (P4 phage-like recombinase, directionality factor and *att*-

sites with certain similarity to P4 phage attachment sites) (Buchrieser *et al.*, 1998; Lesic *et al.*, 2004). However, such resemblance of the HPI genes to phage P4 ones might reflect the evolutionary linkage of all recombination structures involved in site-specific recombination, rather than their particular phage origin. Large size of most islands excludes efficient transduction by most phages especially by a satellite P4 phage. Thus, a shuttle plasmid-mediated mechanism of the HPI dissemination seems more probable although does not rule out other possibilities. Existence of the HPI with the remnants of a conjugative plasmid, ICE HPI_{ECOR31}, also speaks in favor of this mechanism (Schubert *et al.*, 2004b). However, in contrast to the predominant non-conjugative form of the HPI, HPI_{ECOR31}, is restricted to a single *E. coli* isolate (Schubert *et al.*, 2004a).

Taken together, the *attB*-presenting conjugative plasmids can serve as shuttle vectors for integrative elements including the pathogenicity islands. Such plasmids loaded with the recombination target for PAIs can be readily generated in vivo as a result of incorrect excision. Minimal dependence of site-specific recombination on the host cell greatly facilitates plasmid-mediated trapping of non-replicating group of the integrative elements.

3. Restriction–modification systems as lateral gene transfer reducing factors.

Unlike eukaryotes, which evolve principally through the modification of existing genetic information, bacteria have obtained a significant proportion of their genetic diversity through the acquisition of sequences from distantly related organisms. Horizontal gene transfer produces extremely dynamic genomes in which substantial amounts of DNA are introduced into and deleted from the chromosome. Genomic islands are important but not the only contributors to this process. The main barrier for a wide dissemination of GEIs and other mobile genetic elements are host restriction–modification systems. RM systems are composed of genes that encode a restriction enzyme and a modification methylase. They are often linked with mobile genetic elements such as plasmids, viruses, transposons and integrons. The comparison of closely related bacterial genomes also suggests that, at times, RM genes themselves behave as mobile elements and cause genome rearrangements (Kobayashi, 2001; Naderer *et al.*, 2002). The newly characterized *Yen*I RM system in *Y. enterocolitica* O:8, biotype 1B is not exception of the rule. We analysed sequences neighbouring the *yenI* gene and found a copy of IS*1222* insertion sequence 667 bp upstream of the start codon of *yenI* (Fig. 37). Multiple copies of IS*1222* are present in the genome of *Y. enterocolitica* 1B, one of them a part of the high pathogenicity island of *Yersinia.* Also a 60-bp sequence with extremely high similarity (91-88%) to phage PhiR73 and P4 phage sequences is located 562-bp upstream of the ATG start codon of *yenI*, between *yenI*

and IS*1222*. Moreover, the G+C content of the *yenI* gene turned out to be atypically low for *Y. enterocolitica*, 37,8 G+C% in contrast to 46 G+C% of the yersiniae house-keeping genes. The presence of mobile genetic element in the vicinity of the *yenI* gene and atypical G+C content speak in favour of a horizontal acquisition of the *yen*I gene by biotype 1B *Yersinia enterocolitica*.

The organisation of the *Yen*I shown to be atypical in other means. It contrasts to the other two characterized RM systems of the *Pst*I group of the isoschyzomers, *Pst*I and *Bsu*I. Both of them consists of two ORFs, encoding MTase and Enase (Smith *et al.*, 1976; Xu *et al.*, 1992). In contrast, *Yen*I RM system has one ORF showing homology both to MTase and Enase (Fig. 38). Thus, the *Yen*I polypeptide shares two different functions, restriciton and modification. The N-terminal part of the *Yen*I ORF has 45% and 40% identity (61% and 58% positives) to *Pst*I MTase and *Bsu*I MTase, respectively, while the C-terminal part depicts 55% and 45% identity (76% and 59% postitives) to ENases of both isoschyzogenic enzymes. A specific recognition sequence, typical to the type II RM systems and single peptide organization, typical to type IV RM systems, make *Yen*I unique among known restriction-modification systems (Antonenko *et al*., 2003).

Inactivation of *Yen*I restriction-modification system resulted *Y. enterocolitica* WA-C *hsrYI, hsmYI* strain with high potential of foreign DNA acquisition. Our data shows increasing of transformation efficiency in such strain by at least 1000-fold in comparison with wild strain. Similarly, in vivo MYenI-methylated plasmid transforms *Y. enterocolitica* WA-C with high rates (Fig. 43). Also the frequency of conjugative transfer of HPI-loaded trapping plasmid in *Y. enterocolitica* WA-C *hsrYI, hsmYI* was as high as that in restriction-deficient *E. coli* laboratory strains (Fig. 36, 44). Thus, both approaches namely, inactivation of endonuclease activity in recipient and *Yen*I methylation in a donor strain might be applied to increase the efficiency of genetic transfer to *Y. enterocolitica* WA-C.

E. SUMMARY

The high-pathogenicity island encodes a highly efficient yersiniabactin system of iron acquisition responsible for mouse lethality in *Yersinia*. Although the HPI is widely disseminated among *Enterobacteriaceae* it lacks functions necessary for its replication and transmission. Therefore the mechanism of its horizontal transfer and circulation is completely obscure. On the other hand, the HPI is a genetically active island in the bacterial cell. It encodes a functional recombinase and is able to transpose to new targets on the chromosome. Here we report on a possible mechanism of the HPI dissemination based on site-specific recombination of the excised HPI with the *attB*-presenting (*asn* tRNA gene) RP4 promiscuous conjugative shuttle plasmid. The resulting cointegrate can be transferred by conjugation to a new host, where it dissociates, and the released HPI integrates into any unoccupied *asn* tRNA gene target in the genome. This mechanism has been proven with complete HPI labeled with an antibiotic resistance marker. After acquisition of the mobilized complete form of the HPI, the ability of the HPI-cured *Y. enterocolitica* WATH$^-$ strain to produce yersiniabactin has been restored. Such „trapping“ of pathogenicity islands and subsequent shuffling to new hosts by a conjugative replicon carrying a suitable *attB* site could be applied to other functional integrative elements and explain wide dissemination of PAIs.

Another genomic island Ecoc54N targets the same *asn* tRNA genes to integrate into the bacterial chromosome. Ecoc54N island encodes a polyketide synthase with an unknown function in the uropathogenic *E. coli* CFT073 strain. A recombinase orthologue with high similarity to int_{HPI} that promotes site-specific recombination (both integrative and excisive) with its corresponding attachment targets is also present in Ecoc54N. In addition, the HPI_{Yps} of the *Y. pestis* / *Y. pseudotuberculosis* evolutionary lineage encodes the excisionase (recombination directionality factor, Xis_{HPI}) that facilitates excision of the island. However, no sequence resembling excisionase gene could be found in Ecoc54N. The rate of the HPI_{Yps} excision estimated by Real Time PCR was 10^{-6} in *Y. pseudotuberculosis*. The presence of the excisionase increased the efficiency of the excisive recombination only 8-fold. However, the introduction of the xis_{HPI} in *E. coli* CFT073 did not influence the excision of Ecoc54N. The Xis_{HPI} is encoded by the variable AT-rich part of the HPI_{Yps} and substantially differs from its cognate recombinase in A+T content and codon usage. Also the Xis_{HPI} – protected region, defined in HPI attachment site, has suffered several nucleotide substitutions in Ecoc54N that could influence interaction with the excisionase. We propose that the pathogenicity islands targeting *asn* tRNA genes ($PAIs_{asn\ tRNA}$) might have acquired recombinase and excisionase (HPI) genes independently and sequentially.

Genetic manipulations with enteropathogenic *Yersinia enterocolitica* O:8 are complicated by the presence of an efficient *Pst*I-like *Yen*I restriction-modification (RM) system. We have characterised the *Yen*I RM system in *Y. enterocolitica* O:8, biotype 1B. A 5039-bp DNA fragment of the pSAK2 recombinant plasmid carrying the *yen*I locus was used to determine the nucleotide sequence. DNA sequence analysis identified a single 2481 bp open reading frame that encodes an 826 aa large polypeptide having an apparent molecular mass of 93 kDa. The N-terminal part of the *Yen*I ORF has 45% and 40% identity to *Pst*I and *Bsu*I methyltransferases (MTases), respectively; while the C-terminal part depicts 55% and 45% identity to endonucleases (ENases) of both isoschyzomeric enzymes. The *yen*I gene has been shown to encode a single polypeptide of expected molecular mass. A specific recognition sequence, typical to the type II RM systems and single peptide organization, typical to type IV RM systems, make *Yen*I unique among known restriction-modification systems. We have constructed a truncated recombinant variant of *Yen*I enzyme, which conserved only MTase activity, that can be applied to *Yen*I methylation of the DNA to be transformed into *Yersinia enterocolitica* O:8 biotype 1B.

F. REFERENCES

Achtman, M., Morelli, G., and Schwuchow, S. (1978) Cell-cell interactions in conjugating *Escherichia coli*: role of F pili and fate of mating aggregates *J.Bacteriol.* **135**: 1053-1061.

Anisimov, R., Brem, D., Heesemann, J., and Rakin, A. (2005) Transcriptional regulation of high pathogenicity island iron uptake genes by YbtA *Int.J.Med.Microbiol.* **295**: 19-28.

Antonenka, U., Nolting, C., Heesemann, J., and Rakin, A. (2005) Horizontal transfer of *Yersinia* high-pathogenicity island by the conjugative RP4 *attB* target-presenting shuttle plasmid *Mol.Microbiol.* **57**: 727-734.

Antonenka, U., Nolting, C., Heesemann, J., and Rakin, A. (2006) Independent acquisition of site-specific recombination factors by *asn* tRNA gene-targeting genomic islands *Int.J.Med.Microbiol.* **296**: 341-352.

Antonenko, V., Pawlow, V., Heesemann, J., and Rakin, A. (2003) Characterization of a novel unique restriction-modification system from Yersinia enterocolitica O:8 1B *FEMS Microbiol.Lett.* **219**: 249-252.

Bach, S., Buchrieser, C., Prentice, M., Guiyoule, A., Msadek, T., and Carniel, E. (1999) The high-pathogenicity island of *Yersinia enterocolitica* Ye8081 undergoes low-frequency deletion but not precise excision, suggesting recent stabilization in the genome. *Infect.Immun.* **67**: 5091-5099.

Beaber, J.W., Hochhut, B., and Waldor, M.K. (2002) Genomic and functional analyses of SXT, an integrating antibiotic resistance gene transfer element derived from *Vibrio cholerae J.Bacteriol.* **184**: 4259-4269.

Bercovier, H., Mollaret, H.H. (1984) *Yersinia*. In *Bergey's Manual of Systematic Bacteriology*. N.R.Krieg and J.G.Holt (ed). Baltimore, Md: The Williams & Wilkins, Co, pp. 498-506.

Bharathi, A., Polasa, H. (1991) Elimination of broad-host range plasmid vectors in *Escherichia coli* by curing agents. *FEMS Microbiol.Lett.* **68**: 37-40.

Bobrov, A.G., Geoffroy, V.A., and Perry, R.D. (2002) Yersiniabactin production requires the thioesterase domain of HMWP2 and YbtD, a putative phosphopantetheinylate transferase. *Infect.Immun.* **70**: 4204-4214.

Bolivar, F., Backman, K. (1979) Plasmids of *Escherichia coli* as cloning vectors. *Methods Enzymol.* **68**: 245-267.

Boltner, D., MacMahon, C., Pembroke, J.T., Strike, P., and Osborn, A.M. (2002) R391: a conjugative integrating mosaic comprised of phage, plasmid, and transposon elements. *J.Bacteriol.* **184**: 5158-5169.

Bottone, E.J. (1997) *Yersinia enterocolitica*: the charisma continues. *Clin.Microbiol.Rev.* **10**: 257-276.

Brem, D., Pelludat, C., Rakin, A., Jacobi, C.A., and Heesemann, J. (2001) Functional analysis of yersiniabactin transport genes of *Yersinia enterocolitica*. *Microbiology* **147**: 1115-1127.

Buchrieser, C., Brosch, R., Bach, S., Guiyoule, A., and Carniel, E. (1998) The high-pathogenicity island of *Yersinia pseudotuberculosis* can be inserted into any of the three chromosomal *asn* tRNA genes. *Mol.Microbiol.* **30**: 965-978.

Burrus, V., Pavlovic, G., Decaris, B., and Guedon, G. (2002) Conjugative transposons: the tip of the iceberg. *Mol.Microbiol.* **46**: 601-610.

Carniel, E. (1999) The *Yersinia* high-pathogenicity island. *Int.Microbiol.* **2**: 161-167.

Carniel, E. (2001) The *Yersinia* high-pathogenicity island: an iron-uptake island. *Microbes.Infect.* **3**: 561-569.

Carniel, E., Guilvout, I., and Prentice, M. (1996) Characterization of a large chromosomal "high-pathogenicity island" in biotype 1B *Yersinia enterocolitica*. *J.Bacteriol.* **178**: 6743-6751.

Carniel, E., Mazigh, D., and Mollaret, H.H. (1987) Expression of iron-regulated proteins in *Yersinia* species and their relation to virulence. *Infect.Immun.* **55**: 277-280.

Cho, E.H., Gumport, R.I., and Gardner, J.F. (2002) Interactions between integrase and excisionase in the phage lambda excisive nucleoprotein complex. *J.Bacteriol.* **184**: 5200-5203.

Cox, C.D., Rinehart, K.L., Jr., Moore, M.L., and Cook, J.C., Jr. (1981) Pyochelin: novel structure of an iron-chelating growth promoter for *Pseudomonas aeruginosa*. *Proc.Natl.Acad.Sci.U.S.A* **78**: 4256-4260.

Datsenko, K.A., Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc.Natl.Acad.Sci.U.S.A* **97**: 6640-6645.

de Almeida, A.M., Guiyoule, A., Guilvout, I., Iteman, I., Baranton, G., and Carniel, E. (1993) Chromosomal *irp2* gene in *Yersinia*: distribution, expression, deletion and impact on virulence. *Microb.Pathog.* **14**: 9-21.

Deng, W., Burland, V., Plunkett, G., III, Boutin, A., Mayhew, G.F., Liss, P., Perna, N.T., Rose, D.J., Mau, B., Zhou, S., Schwartz, D.C., Fetherston, J.D., Lindler, L.E., Brubaker, R.R., Plano, G.V., Straley, S.C., McDonough, K.A., Nilles, M.L., Matson, J.S., Blattner, F.R., and Perry, R.D. (2002) Genome sequence of *Yersinia pestis* KIM. *J.Bacteriol.* **184**: 4601-4611.

Dimopoulou, I.D., Jordens, J.Z., Legakis, N.J., and Crook, D.W. (1997) A molecular analysis of Greek and UK *Haemophilus influenzae* conjugative resistance plasmids. *J.Antimicrob.Chemother.* **39**: 303-307.

Dobrindt, U., Agerer, F., Michaelis, K., Janka, A., Buchrieser, C., Samuelson, M., Svanborg, C., Gottschalk, G., Karch, H., and Hacker, J. (2003) Analysis of genome plasticity in pathogenic and commensal *Escherichia coli* isolates by use of DNA arrays. *J.Bacteriol.* **185**: 1831-1840.

Dobrindt, U., Hentschel, U., Kaper, J.B., and Hacker, J. (2002) Genome plasticity in pathogenic and nonpathogenic enterobacteria. *Curr.Top.Microbiol.Immunol.* **264**: 157-175.

Dobrindt, U., Hochhut, B., Hentschel, U., and Hacker, J. (2004) Genomic islands in pathogenic and environmental microorganisms. *Nat.Rev.Microbiol.* **2**: 414-424.

Dower, W.J., Miller, J.F., and Ragsdale, C.W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.* **16**: 6127-6145.

Fetherston, J.D., Bearden, S.W., and Perry, R.D. (1996) YbtA, an AraC-type regulator of the *Yersinia pestis* pesticin/yersiniabactin receptor. *Mol.Microbiol.* **22**: 315-325.

Finlay, B.B., Falkow, S. (1997) Common themes in microbial pathogenicity revisited. *Microbiol.Mol.Biol.Rev.* **61**: 136-169.

Gehring, A.M., DeMoll, E., Fetherston, J.D., Mori, I., Mayhew, G.F., Blattner, F.R., Walsh, C.T., and Perry, R.D. (1998) Iron acquisition in plague: modular logic in enzymatic biogenesis of yersiniabactin by *Yersinia pestis*. *Chem.Biol.* **5**: 573-586.

Guilvout, I., Mercereau-Puijalon, O., Bonnefoy, S., Pugsley, A.P., and Carniel, E. (1993) High-molecular-weight protein 2 of *Yersinia enterocolitica* is homologous to AngR of *Vibrio anguillarum* and belongs to a family of proteins involved in nonribosomal peptide synthesis. *J.Bacteriol.* **175**: 5488-5504.

Haag, H., Hantke, K., Drechsel, H., Stojiljkovic, I., Jung, G., and Zahner, H. (1993) Purification of yersiniabactin: a siderophore and possible virulence factor of *Yersinia enterocolitica*. *J.Gen.Microbiol.* **139 (Pt 9)**: 2159-2165.

Hacker, J., Bender, L., Ott, M., Wingender, J., Lund, B., Marre, R., and Goebel, W. (1990) Deletions of chromosomal regions coding for fimbriae and hemolysins occur *in vitro* and *in vivo* in various extraintestinal *Escherichia coli* isolates. *Microb.Pathog.* **8**: 213-225.

Hacker, J., Blum-Oehler, G., Muhldorfer, I., and Tschape, H. (1997) Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Mol.Microbiol.* **23**: 1089-1097.

Hacker, J., Carniel, E. (2001) Ecological fitness, genomic islands and bacterial pathogenicity. A Darwinian view of the evolution of microbes. *EMBO Rep.* **2**: 376-381.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J.Mol.Biol.* **166**: 557-580.

Hanahan, D., Meselson, M. (1983) Plasmid screening at high colony density. *Methods Enzymol.* **100**: 333-342.

Heesemann, J. (1987) Chromosomal-encoded siderophores are required for mouse virulence of enteropathogenic *Yersinia* species. *FEMS Microbiology Letters* **48**: 229-233.

Heesemann, J., Algermissen, B., and Laufs, R. (1984) Genetically manipulated virulence of *Yersinia enterocolitica*. *Infect.Immun.* **46**: 105-110.

Heesemann, J., Hantke, K., Vocke, T., Saken, E., Rakin, A., Stojiljkovic, I., and Berner, R. (1993) Virulence of *Yersinia enterocolitica* is closely associated with siderophore production, expression of an iron-repressible outer membrane polypeptide of 65,000 Da and pesticin sensitivity. *Mol.Microbiol.* **8**: 397-408.

Hentschel, U., Hacker, J. (2001) Pathogenicity islands: the tip of the iceberg. *Microbes.Infect.* **3**: 545-548.

Herrero, M., de,L., V, and Timmis, K.N. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J.Bacteriol.* **172**: 6557-6567.

Hinchliffe, S.J., Isherwood, K.E., Stabler, R.A., Prentice, M.B., Rakin, A., Nichols, R.A., Oyston, P.C., Hinds, J., Titball, R.W., and Wren, B.W. (2003) Application of DNA microarrays to study the evolutionary genomics of *Yersinia pestis* and *Yersinia pseudotuberculosis*. *Genome Res.* **13**: 2018-2029.

Hinnebusch, B.J. (1997) Bubonic plague: a molecular genetic case history of the emergence of an infectious disease. *J.Mol.Med.* **75**: 645-652.

Hochhut, B., Waldor, M.K. (1999) Site-specific integration of the conjugal *Vibrio cholerae* SXT element into prfC. *Mol.Microbiol.* **32**: 99-110.

Holloway, B., K.B. Low (1987) F-prime factors. In *Escherichia coli and Salmonella typhimurium.* F.C.Neidhardt (ed). Washington, D.C.: ASM, pp. 1145-1153.

Jacobi, C.A., Gregor, S., Rakin, A., and Heesemann, J. (2001) Expression analysis of the yersiniabactin receptor gene *fyuA* and the heme receptor *hemR* of *Yersinia enterocolitica* in vitro and in vivo using the reporter genes for green fluorescent protein and luciferase. *Infect.Immun.* **69**: 7772-7782.

Jaoua, S., Letouvet-Pawlak, B., Monnier, C., and Guespin-Michel, J.F. (1990) Mechanism of integration of the broad-host-range plasmid RP4 into the chromosome of *Myxococcus xanthus*. *Plasmid* **23**: 183-193.

Jelenska, J., Tietze, E., Tempé, J., and Brevet, J. (2000) Streptothricin resistance as a novel selectable marker for transgenic plant cells. *Plant Cell Rep* **19**: 298-303.

Kado, C.I., Liu, S.T. (1981) Rapid procedure for detection and isolation of large and small plasmids. *J.Bacteriol.* **145**: 1365-1373.

Karch, H., Schubert, S., Zhang, D., Zhang, W., Schmidt, H., Olschlager, T., and Hacker, J. (1999) A genomic island, termed high-pathogenicity island, is present in certain non-O157 Shiga toxin-producing *Escherichia coli* clonal lineages. *Infect.Immun.* **67**: 5994-6001.

Kawarabayasi, Y., Hino, Y., Horikawa, H., Jin-no, K., Takahashi, M., Sekine, M., Baba, S., Ankai, A., Kosugi, H., Hosoyama, A., Fukui, S., Nagai, Y., Nishijima, K., Otsuka, R., Nakazawa, H., Takamiya, M., Kato, Y., Yoshizawa, T., Tanaka, T., Kudoh, Y., Yamazaki, J., Kushida, N., Oguchi, A., Aoki, K., Masuda, S., Yanagii, M., Nishimura, M., Yamagishi, A., Oshima, T., and Kikuchi, H. (2001) Complete genome sequence of an aerobic thermoacidophilic crenarchaeon, *Sulfolobus tokodaii* strain7 *DNA Res.* **8**: 123-140.

Kidgell, C., Pickard, D., Wain, J., James, K., Diem Nga, L.T., Diep, T.S., Levine, M.M., O'Gaora, P., Prentice, M.B., Parkhill, J., Day, N., Farrar, J., and Dougan, G. (2002) Characterisation and distribution of a cryptic *Salmonella typhi* plasmid pHCM2. *Plasmid* **47**: 159-171.

Kim, S.R., Funayama, N., and Komano, T. (1993) Nucleotide sequence and characterization of the traABCD region of IncI1 plasmid R64 *J.Bacteriol.* **175**: 5035-5042.

Knapp, S., Hacker, J., Jarchau, T., and Goebel, W. (1986) Large, unstable inserts in the chromosome affect virulence properties of uropathogenic *Escherichia coli* O6 strain 536 *J.Bacteriol.* **168**: 22-30.

Kobayashi, I. (2001) Behavior of restriction-modification systems as selfish mobile elements and their impact on genome evolution. *Nucleic Acids Res.* **29**: 3742-3756.

Lesic, B., Bach, S., Ghigo, J.M., Dobrindt, U., Hacker, J., and Carniel, E. (2004) Excision of the high-pathogenicity island of *Yersinia pseudotuberculosis* requires the combined actions of its cognate integrase and Hef, a new recombination directionality factor. *Mol.Microbiol.* **52**: 1337-1348.

Lewis, J.A., Hatfull, G.F. (2001) Control of directionality in integrase-mediated recombination: examination of recombination directionality factors (RDFs) including Xis and Cox proteins. *Nucleic Acids Res.* **29**: 2205-2216.

Moran, N.A. (2002) Microbial minimalism: genome reduction in bacterial pathogens *Cell* **108**: 583-586.

Naderer, M., Brust, J.R., Knowle, D., and Blumenthal, R.M. (2002) Mobility of a restriction-modification system revealed by its genetic contexts in three hosts. *J.Bacteriol.* **184**: 2411-2419.

Nash, H.A., Robertson, C.A. (1981) Purification and properties of the *Escherichia coli* protein factor required for lambda integrative recombination. *J.Biol.Chem.* **256**: 9246-9253.

O'Shea, Y.A., Boyd, E.F. (2002) Mobilization of the *Vibrio* pathogenicity island between *Vibrio cholerae* isolates mediated by CP-T1 generalized transduction. *FEMS Microbiol.Lett.* **214**: 153-157.

Ochman, H., Moran, N.A. (2001) Genes lost and genes found: evolution of bacterial pathogenesis and symbiosis. *Science* **292**: 1096-1099.

Parkhill, J., Wren, B.W., Thomson, N.R., Titball, R.W., Holden, M.T., Prentice, M.B., Sebaihia, M., James, K.D., Churcher, C., Mungall, K.L., Baker, S., Basham, D., Bentley, S.D., Brooks, K., Cerdeno-Tarraga, A.M., Chillingworth, T., Cronin, A., Davies, R.M., Davis, P., Dougan, G., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Karlyshev, A.V., Leather, S., Moule, S., Oyston, P.C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S., and Barrell, B.G. (2001) Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature* **413**: 523-527.

Pelludat, C., Brem, D., and Heesemann, J. (2003) Irp9, encoded by the high-pathogenicity island of *Yersinia enterocolitica*, is able to convert chorismate into salicylate, the precursor of the siderophore yersiniabactin. *J.Bacteriol.* **185**: 5648-5653.

Pelludat, C., Hogardt, M., and Heesemann, J. (2002) Transfer of the core region genes of the *Yersinia enterocolitica* WA-C serotype O:8 high-pathogenicity island to *Y. enterocolitica* MRS40, a strain with low levels of pathogenicity, confers a yersiniabactin biosynthesis phenotype and enhanced mouse virulence. *Infect.Immun.* **70**: 1832-1841.

Perry, R.D., Fetherston, J.D. (1997) *Yersinia pestis* - etiologic agent of plague. *Clin.Microbiol.Rev.* **10**: 35-66.

Preston, G.M., Haubold, B., and Rainey, P.B. (1998) Bacterial genomics and adaptation to life on plants: implications for the evolution of pathogenicity and symbiosis *Curr.Opin.Microbiol.* **1**: 589-597.

Rakin, A., Noelting, C., Schropp, P., and Heesemann, J. (2001) Integrative module of the high-pathogenicity island of *Yersinia*. *Mol.Microbiol.* **39**: 407-415.

Rakin, A., Noelting, C., Schubert, S., and Heesemann, J. (1999a) Common and specific characteristics of the high-pathogenicity island of *Yersinia enterocolitica*. *Infect.Immun.* **67**: 5265-5274.

Rakin, A., Saken, E., Harmsen, D., and Heesemann, J. (1994) The pesticin receptor of *Yersinia enterocolitica*: a novel virulence factor with dual function. *Mol.Microbiol.* **13**: 253-263.

Rakin, A., Schubert, S., Pelludat, C., Brem, D., and Heesemann, J. The High-Pathogenicity Island of *Yersiniae*. Kaper, J. B. and Hacker J. 77-90. 1999b. Washington, D. C., ASM Press.

Rakin, A., Urbitsch, P., and Heesemann, J. (1995) Evidence for two evolutionary lineages of highly pathogenic *Yersinia* species. *J.Bacteriol.* **177**: 2292-2298.

Reim, P.Ed. (2003) *Roche molecular biochemicals Lab FAQS – find a quick solution.* Penzberg: Roche Applied Science, Roche Diagnostics GmbH.

Rigby, C.E., Fraser, A.D. (1989) Plasmid transfer and plasmid-mediated genetic exchange in *Brucella abortus*. *Can.J.Vet.Res.* **53**: 326-330.

Robertson, C.A., Nash, H.A. (1988) Bending of the bacteriophage lambda attachment site by *Escherichia coli* integration host factor. *J.Biol.Chem.* **263**: 3554-3557.

Ruzin, A., Lindsay, J., and Novick, R.P. (2001) Molecular genetics of SaPI1--a mobile pathogenicity island in *Staphylococcus aureus*. *Mol.Microbiol.* **41**: 365-377.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**: 487-491.

Schmidt, H., Hensel, M. (2004) Pathogenicity islands in bacterial pathogenesis. *Clin.Microbiol.Rev.* **17**: 14-56.

Schubert, S., Rakin, A., Fischer, D., Sorsa, J., and Heesemann, J. (1999) Characterization of the integration site of *Yersinia* high-pathogenicity island in *Escherichia coli*. *FEMS Microbiol.Lett.* **179**: 409-414.

Schubert, S., Cuenca, S., Fischer, D., and Heesemann, J. (2000) High-pathogenicity island of *Yersinia pestis* in enterobacteriaceae isolated from blood cultures and urine samples: prevalence and functional expression. *J.Infect.Dis.* **182**: 1268-1271.

Schubert, S., Dufke, S., Sorsa, J., and Heesemann, J. (2004) A novel integrative and conjugative element (ICE) of *Escherichia coli*: the putative progenitor of the *Yersinia* high-pathogenicity island. *Mol.Microbiol.* **51**: 837-848.

Schubert, S., Rakin, A., and Heesemann, J. (2004) The *Yersinia* high-pathogenicity island (HPI): evolutionary and functional aspects. *Int.J.Med.Microbiol.* **294**: 83-94.

Schwartz, E., Henne, A., Cramm, R., Eitinger, T., Friedrich, B., and Gottschalk, G. (2003) Complete nucleotide sequence of pHG1: a *Ralstonia eutropha* H16 megaplasmid encoding key enzymes of H(2)-based ithoautotrophy and anaerobiosis. *J.Mol.Biol.* **332**: 369-383.

Simon, R., Pefer, V., and Puhler, A. (1983) A broad host range mobilisation system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Biotechnology* **1**: 784-791.

Smith, D.I., Blattner, F.R., and Davies, J. (1976) The isolation and partial characterization of a new restriction endonuclease from *Providencia stuartii Nucleic Acids Res.* **3**: 343-353.

Stedman, K.M., She, Q., Phan, H., Holz, I., Singh, H., Prangishvili, D., Garrett, R., and Zillig, W. (2000) pING family of conjugative plasmids from the extremely thermophilic archaeon *Sulfolobus islandicus*: insights into recombination and conjugation in *Crenarchaeota*. *J.Bacteriol.* **182**: 7014-7020.

Tabor, S., Richardson, C.C. (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes *Proc.Natl.Acad.Sci.U.S.A* **82**: 1074-1078.

Tillett, D., Burns, B.P., and Neilan, B.A. (2000) Optimized rapid amplification of cDNA ends (RACE) for mapping bacterial mRNA transcripts. *Biotechniques* **28**: 448, 450, 452-448, 453, 456.

Trebesius, K., Harmsen, D., Rakin, A., Schmelz, J., and Heesemann, J. (1998) Development of rRNA-targeted PCR and in situ hybridization with fluorescently labelled oligonucleotides for detection of Yersinia species *J.Clin.Microbiol.* **36**: 2557-2564.

Welch, R.A., Burland, V., Plunkett, G., III, Redford, P., Roesch, P., Rasko, D., Buckles, E.L., Liou, S.R., Boutin, A., Hackett, J., Stroud, D., Mayhew, G.F., Rose, D.J., Zhou, S., Schwartz, D.C., Perna, N.T., Mobley, H.L., Donnenberg, M.S., and Blattner, F.R. (2002) Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli*. *Proc.Natl.Acad.Sci.U.S.A* **99**: 17020-17024.

Williams, K.P. (2002) Integration sites for genetic elements in prokaryotic tRNA and tmRNA genes: sublocation preference of integrase subfamilies. *Nucleic Acids Res.* **30**: 866-875.

Wojciak, J.M., Sarkar, D., Landy, A., and Clubb, R.T. (2002) Arm-site binding by lambda -integrase: solution structure and functional characterization of its amino-terminal domain. *Proc.Natl.Acad.Sci.U.S.A* **99**: 3434-3439.

Xu, G.L., Kapfer, W., Walter, J., and Trautner, T.A. (1992) *Bsu*BI--an isospecific restriction and modification system of *Pst*I: characterization of the *Bsu*BI genes and enzymes: *Nucleic Acids Res.* **20**: 6517-6523.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors: *Gene* **33**: 103-119.

G. ABBREVIATIONS

dest	distilled water
Amp	Ampicillin
AP	Alkaline Phosphatase
APS	Ammonium persulphate
ATP	Adenosine Triphosphate
bp	base pair
CFU	Colony forming unit
Cm	Chloramphenicol
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP)
dsDNA	double stranded DNA
EDTA	Ethylenediamine triacetic acid
e.g.	for example
EtOH	Ethanol
FACS	Fluorescence activated cell sorter
Fig.	Figure
GEI	genomic island
GFP	Green fluorescent protein
hr	hour
Hepes	N-2-hydroxyethyl piperazine- N'-2-ethane sulfonic acid
HPI	High pathogenicity island
IHF	integration host factor
ICE	integrative and conjugative element
IPTG	Isopropyl ß-D-thiogalactopyranoside
kb	kilobase
Kan	Kanamycin
kDa	kilo Dalton
LB	Luria Bertani
Luc	Luciferase
µ	micro
m	milli
M	molar
MCS	multiple cloning site
min	minute
n	nano
Nal	Nalidixic acid
OD	optical density
PAGE	Polyacrylamide gel electrophoresis
PAI	Pathogenicity island
PCR	Polymerase chain reaction
RDF	Recombination directionality factor

RNA	Ribonucleic acid
rRNA	Ribosomal RNA
tRNA	Transport RNA
RNase	Ribonuclease
rpm	revolutions per minute
RT	Room temperature
RT	Reverse Transcriptase / Reverse transcription
SDS	Sodium dodecyl sulphate
sec	seconds
ssDNA	single stranded DNA
St	Streptothricine
Tab.	Table
TAE	Tris-Acetate-EDTA
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
Tet	Tetracycline
TRIS	Tris-(hydroxymethyl)-ammonium methane
V	Volt
Vol.	Volume
wt	wild type
X-gal	5-bromo-4-chloro-3-indolyl-ß-galactoside

Nucleic acids
A: Adenine
C: Cytosine
G: Guanine
T: Thymine

PUBLISHED ASPECTS OF THIS WORK

Antonenko, V., Pawlow, V., Heesemann, J., and Rakin, A. (2003) Characterization of a novel unique restriction-modification system from *Yersinia enterocolitica* O:8 1B *FEMS Microbiol.Lett.* **219**: 249-252.

Antonenka, U., Nolting, C., Heesemann, J., and Rakin, A. (2005) Horizontal transfer of Yersinia high-pathogenicity island by the conjugative RP4 *attB* target-presenting shuttle plasmid *Mol.Microbiol.* **57**: 727-734.

Antonenka, U., Nolting, C., Heesemann, J., and Rakin, A. (2006) Independent acquisition of site-specific recombination factors by *asn* tRNA gene-targeting genomic islands *Int.J.Med.Microbiol.* **296**: 341-352.

CURRICULUM VITAE

Uladzimir Antonenka

ACADEMIC BACKGROUND AND QUALIFICATIONS:

09/2001-09/2006:	**Doctorate research work** at Max von Pettenkofer Institute for Hygiene and Medical Microbiology of the Ludwig Maximilians University (LMU), Munich, Germany. Research theme: Mobility of the High Pathogenicity Island of *Yersinia*
2000-2001	**Graduate student,** Molecular Biology, Molecular Biology Educational Centre, Institute of Protein Research, Pushchino, Russia. Thesis: The search for a new efficient enhancers in wheat germ cell-free translation system.
1996-2001	**M.Sc. (High distinction) Biology/Biochemistry;** Gomel State University, Gomel, Belarus Thesis: Construction of genetic maps for *Picea abies* and *Picea obovata* as main structural components of spruce forests in Europe and Siberia.

AFFILIATIONS:

Member of the Gesellschaft für Biochemie
Und Molekularbiologie (GBM) e. V.
Mr. Tino Apel, Secretary
GBM e.V.
Mörfelder Landstr. 125
60598 Frankfurt/Main

Personal Data

Date of Birth:	Juli 02, 1979
Place of Birth:	Gomel, Belarus