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Uncovering novel pathogenicity-associated loci among *Yersinia enterocolitica* species by subtractive hybridization

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A. INTRODUCTION

1. *Yersinia*

Yersinia species are gram-negative bacteria belonging to the *Enterobacteriaceae* family. They are facultative non-sporulating anaerobes with optimal growth at 27 °C. Based on differentiating biochemical traits, 11 species have been identified to date which are further divided into different biotypes. On the basis of antibody reactions to different lipopolysaccharide structures, *Yersinia* species can also be divided into different serological groups. Typically, the serotypes are linked to geographical distribution, severity of human disease, and animal reservoir (Boyd and Cornelis, 2001, Salyers and Whitt, 1994).

Three species of *Yersinia* cause disease in humans: *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*. *Y. pestis* is the agent of the bubonic plague, the infamous black death that swept through the Middle Ages, killing millions of people. Today, isolated cases of *Y. pestis* infection are reported sporadically in the US, India and Madagascar (Perry, 1997). Human infection with *Y. pestis* can be acquired in two ways: by transmission from rats or other wild animals to humans by flea bite or by direct human-to-human transmission via aerosols. *Y. pestis* is an obligate parasite, in contrast to *Y. enterocolitica* and *Y. pseudotuberculosis*, which can survive outside animal hosts and are food-borne pathogens (Boyd and Cornelis, 2001, Black *et al.,* 1978). *Y. enterocolitica*, which is the most prevalent in humans, and *Y. pseudotuberculosis* (mainly isolated from animals such as pigs) cause a broad range of gastrointestinal syndromes, ranging from acute gastroenteritis to mesenteric lymphadenitis and, on rare occasions, they can provoke systemic infections such as septicemia and meningitis. In contrast to *Y. pestis* which is non-motile, the two enteropathogenic *Yersinia* species are motile at 27 °C (Cover and Aber, 1989; Bottone, 1997, Tauxe, *et al*., 1987).

Y. pseudotuberculosis and *Y. pestis* are closely related species that share nearly 97 % gene homology (Achtman *et al.,* 1999, Garcia, 2002, Trebesius *et al*., 1998). Recent studies strengthened by whole scale genome sequencing propose that *Y. pestis* is a recently emerged clone of *Y. pseudotuberculosis* (Radnedge *et al.,* 2002; Achtman *et al*., 1999). *Y. enterocolitica* on the other hand presents a more variable genomic arrangement with

only 60 - 65 % DNA homology with *Y. pestis / Y. pseudotuberculosis* (Bercovier *et al*., 1980).

1.1 *Y. enterocolitica*

Y. enterocolitica, which is the focus of this study, is widely distributed in nature in aquatic and animal reservoirs, with swine serving as a major reservoir for human pathogenic strains (Bottone, 1997). Inanimate reservoirs include lakes, streams, well water, soil, vegetables (Cover and Aber, 1989). Because *Y. enterocolitica* can also survive in cold environments, disease outbreaks have also been traced to infected blood samples stored at 4 °C (Tipple *et al*., 1990). Various biotyping schemes have been defined over the years for differentiation of the *Y. enterocolitica* strains. In the revised biotype scheme proposed by Wauters *et al*. (1987), *Y. enterocolitica* strains are divided into 5 biotypes (BT) based on their differing biochemical and ecological characteristics. In terms of pathogenicity, two broad groups are identified: the non-pathogenic isolates represented by biotype 1A strains, and the pathogenic group. The latter is further subdivided into two groups, namely the high-pathogenicity, mouse lethal group comprising biotype 1B strains (typified by serotypes O:8, O:13, O:20 and O:21) and the low-pathogenicity, non-mouse lethal group (represented by biotypes 2-5) (Carniel, 2002).

As a mouse virulence determinant, a high-pathogenicity island (HPI) has been identified, which codes for synthesis and uptake of the siderophore yersiniabactin, an iron-sequestering low molecular weight compound invaluable in the iron-limiting environment of the host (Carniel, 1992, Carniel *et al.,* 1996, Carniel, 2002, Heesemann, 1993, Heesemann, 1987, Pelludat *et al*., 1998). To date, the HPI defines essentially the differences in pathogenicity existing between the low and high-pathogenicity *Y. enterocolitica* strains, with the low-pathogenicity strains lacking this chromosomal locus. All other known virulence markers are however shared between the two groups. The presence of the HPI has also been demonstrated in *Y. pseudotuberculosis* and *Y. pestis* (Buchrieser *et al.*, 1998a, Buchrieser *et al.,* 1998b, Fetherston *et al*., 1999, De Almeida, *et al*., 1993). In terms of geographical distribution, the low and high-pathogenicity *Y. enterocolitica* species exhibit some preferences: the high-pathogenicity organisms are more frequently isolated in the US (so-called New World Strains), while the low-pathogenicity isolates (so-called Old World Strains) are predominantly isolated in

Europe and Japan (Aleksic and Bockemühl, 1990). Fig. 1 below presents a summary of the *Y. enterocolitica* strains in terms of pathogenicity profile.

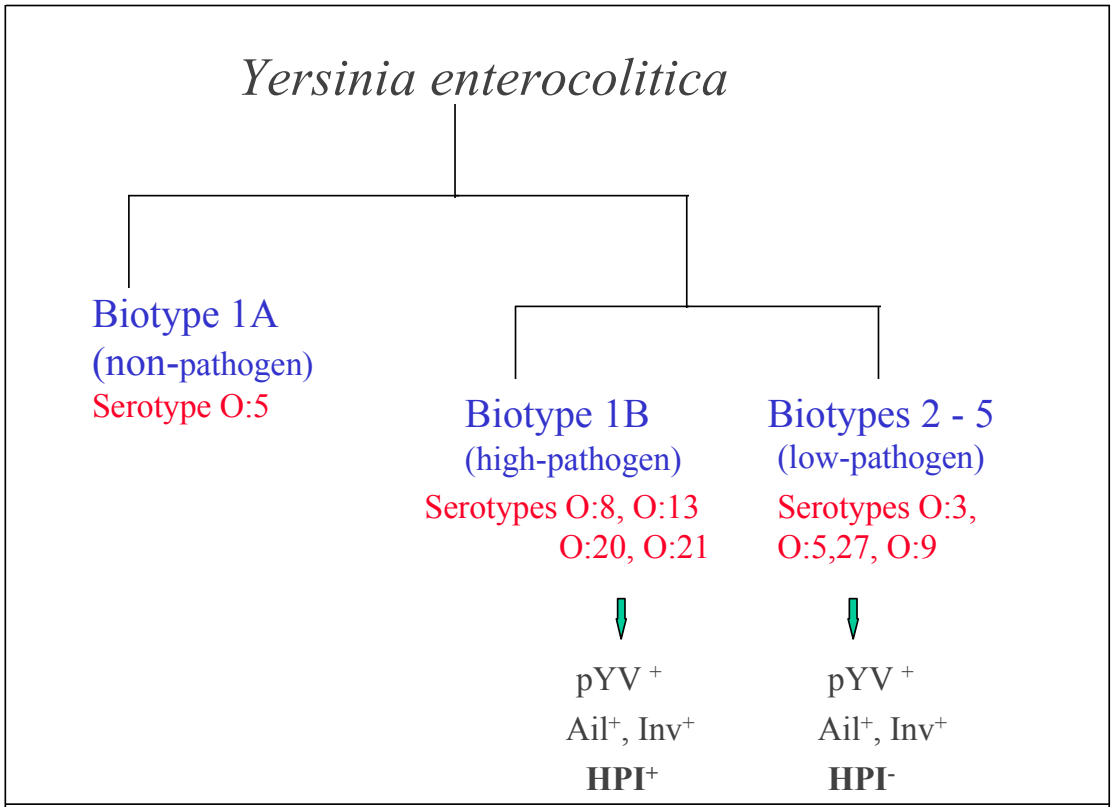


Fig. 1: An overview of the species diversity / variability existing among the *Y. enterocolitica* strains.

1.2 Representational difference analysis as a tool in the elucidation of the genetic variability among *Y. enterocolitica* strains.

The presence of chromosomal "pathogenicity islands" in several bacterial pathogens suggests that bacterial diversity may commonly involve horizontal acquisition of substantial blocks of chromosomal DNA encoding a series of related gene products which convey a new set of virulence properties on the recipient (Hacker *et al*., 1997). A variety of techniques have been developed to detect regions of DNA that differ between two closely related genomes that may be involved in genomic diversity. One such method is representational difference analysis (RDA), otherwise referred to as suppression subtractive hybridization (Diatchenko *et al*., 1996, Lisitsyn *et al*., 1993, Lisitsyn and Wigler, 1995). Originally developed as a tool in eukaryotic organisms to identify genetic polymorphisms in human neoplasia, this tool has since then been expanded to screen for genetic variability among different bacterial species, and even between closely related

strains of the same species. In the work by Tinsley and colleagues for example, the method of RDA was successfully applied to map out genetic differences between *Neisseria meningitidis* and *Neisseria gonorrhoeae,* two closely related bacteria which have developed two different pathogenicity strategies (Calia *et al*., 1998, Choi *et al.,* 2002, Perrin *et al*., 1999 and Tinsley and Nassif, 1996).

In another study by Morrow *et al*., 1999 and Emmerth *et al*., 1999, RDA was successfully applied to detect genomic differences responsible for the different host propensities of two closely related pathogenic *Salmonella enterica* serovars, *S. typhimurium* and *S. typhi*. *S. typhimurium* is a frequent cause of gastroenteritis in humans yet typically causes a lethal systemic infection in genetically susceptible mice. *S. typhi* on the other hand is the etiologic agent of human typhoid fever, capable of causing a potentially fatal systemic infection in humans, but is completely avirulent in nonprimate hosts such as mice. Their study revealed a novel fimbrial operon and a transcriptional regulator that were unique to the tested *S. typhimurium* strain but absent in the *S. typhi* genome.

The *Y. enterocolitica* species as depicted in Fig. 1 represent a highly heterogeneous group of bacteria ranging from the non-pathogenic 1A strains to the high-pathogenicity 1B and low-pathogenicity isolates. Although closely related, the 1A and 1B organisms differ significantly with respect to pathogenicity. 1A isolates are generally considered avirulent while the high-pathogenicity 1B strains cause a broad range of gastrointestinal syndromes. In this work, the method of representational difference analysis was applied to map out additional genomic differences that could contribute to the high virulence of the 1B strains.

1.3 State of the Art

1.3.1 Overview of the pathogenesis of *Yersinia* species.

Human clinical infections with *Y. enterocolitica* usually ensue after ingestion of the microbes in contaminated food (Black *et al*., 1997) or water (Keet, 1974), or by direct inoculation through blood transfusion (Stenhouse and Milner, 1982). In the gastrointestinal tract, *Y. enterocolitica* can cause acute enteritis (more common in children), enterocolitis, mesenteric lymphadenitis, and terminal ileitis. For effective colonization of the host, a medley of virulence factors comes into play (Bottone, 1997). Some of these factors are chromosomally encoded like the invasin InvA, the adhesive factor Myf (mucoid *Yersinia* factor), the enterotoxin Yst, and proteins involved in iron acquisition. In addition, all three pathogenic *Yersinia* species harbor a 70-kb virulence plasmid (designated pYV for *Yersinia* virulence plasmid by Portnoy and Falkow, 1981, Portnoy *et al.,* 1981) which codes for an array of tightly regulated and sophisticated antihost factors that guide the invading *Yersinia* pathogen past numerous host defence mechanisms. The pYV plasmid carries a number of important virulence genes whose products fall under four broad categories, namely, the adhesin protein (YadA), translocated antiphagocytic proteins called Yops (for *Yersinia* outer proteins), proteins dedicated to the processing and extracellular secretion of Yops designated Ysc (for "Yop secretion"), and a complex regulatory network (Lcr proteins for low calcium response) (Cornelis, *et al.,* 1998, Boyd and Cornelis, 2001).

1.3.2 Invasin (Inv)

Inv is a chromosomally encoded outer membrane protein that is expressed in pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* strains (Isberg *et al*., 1987, Pepe and Miller, 1990, Young *et al.,* 1990) while the *Y. pestis inv* gene is inactivated by an insertion sequence (Simonet *et al*., 1996). Inv promotes both attachment to and invasion into eukaryotic cells, including non-phagocytic cells, by *Yersinia* species (Miller and Falkow, 1988, Isberg and Leong, 1988). Although not absolutely required for the virulence of the species, it however favors efficient translocation of the bacteria from the intestinal tract into the Peyer's patches and subsequently to underlying tissues (Pepe and Miller, 1993, Han and Miller, 1997, Marra and Isberg, 1997). The *inv* gene is located within the flagellar biosynthetic gene cluster of *Yersinia* (Fauconnier, *et al*., 1997) but expression of

Inv is inversely related to that of flagella (Badger and Miller, 1998). The Inv receptor on eukaryotic cells are ß1 integrins (Isberg and Leong, 1990) and the Inv protein is maximally expressed at 23-27 °C (Isberg *et al.,* 1988).

1.3.2 Attachment invasion locus (Ail)

Ail is another chromosomally encoded membrane-associated protein that plays an important role in the attachment to and subsequent invasion of eukaryotic cells by *Yersinia* (Miller and Falkow, 1988, Miller *et al.,* 1989). Although not so powerful as the Inv adhesin (Miller *et al.,* 1990), Ail also confers resistance to killing by serum complement. (Bliska and Falkow, 1992, Pierson and Falkow, 1993). Like Inv, Ail has not been demonstrated to be absolutely essential for pathogenicity of *Y. enterocolitica* (Pepe *et al*., 1995, Wachtel and Miller, 1995), and its role in the virulence of *Y. pseudotuberculosis* species is unknown (Boyd and Cornelis, 2001). Like Inv, Ail is not functional in *Y. pestis,* where an insertion sequence interrupts the protein coding sequence. Maximal expression of Ail is at 37 °C (Pierson and Falkow, 1993).

1.3.3 Mucoid *Yersinia* factor (Myf)

Myf in *Y. enterocolitica* and its homolog pH 6 antigen in *Y. pestis* and *Y. pseudotuberculosis* are chromosomally encoded proteins which form a fibrillar structure of strands, bundles and aggregates that surround the bacteria (Iriarte *et al*., 1993, Lindler and Tall, 1993). Like Inv and Ail, expression of Myf is restricted to pathogenic strains (Iriarte *et al.,* 1993). They enhance thermoinducible binding of *Yersinia* species to eukaryotic cells and can bind intestinal luminal mucus (Marra and Isberg, 1997, Yang *et al*., 1996). Synthesis of this antigen has been found to be induced in the acidic environment of macrophages, thus it may play a role in the intracellular survival of the bacteria (Lindler and Tall, 1993). Its definite role in the virulence of *Y. enterocolitica* species remains however to be proven, unlike in *Y. pestis* where it is absolutely required for full virulence (Boyd and Cornelis, 2001).

1.3.4 *Yersinia* adhesin (YadA)

YadA is an outer membrane protein encoded by the 70-kb pYV plasmid in *Y. enterocolitica* and *Y. pseudotuberculosis* (Balligand *et al*., 1985, Bölin and Wolf-Watz, H., 1984). In *Y. pestis*, the *yadA* gene is inactivated by a single basepair deletion that

results in a reading frame shift and a mRNA with reduced half-life so that the YadA protein is not produced by this species (Rosqvist *et al.,* 1988). Unlike the previously described adhesion factors, YadA has been demonstrated to play a definite role in the pathogenicity of the *Y. enterocolitica* species (Pepe *et al.,* 1995, Roggenkamp *et al.,* 1995). Thus, *Y. enterocolitica yadA* mutant strains are attenuated in mouse virulence, compared to fully virulent wild type species (Roggenkamp *et al.,* 1996). The host cell receptors recognized by YadA are varied and include collagen, laminin, fibronectin and ß1 integrins, with interaction with the latter playing a crucial role in the internalization of the bacteria (Flugel *et al*., 1994, Schulze-Koops *et al*., 1992, and Schulze-Koops *et al*., 1993). Other roles that have been assigned YadA include conferring resistance to killing by serum and by PMNs (Balligand *et al*., 1985, Visser *et al*, 1996, Martinez, 1989).

1.3.5 Yst Enterotoxin

Pathogenic *Y. enterocolitica* strains produce a heat-stable enterotoxin called Yst which is responsible for the diarrhoea associated with *Yersinia enterocolitica* infection (Delor *et al*., 1990, Delor and Cornelis, 1992). Yst is similar to the heat stable enterotoxin (STI) produced by *E. coli* and to rat guanylin, an endogenous activator of the intestinal guanylate cyclase (Currie *et al*., 1992). Yst functions by stimulating cGMP synthesis in the intestinal brush area, resulting in fluid loss and lack of fluid absorption (Robins-Browne *et al*., 1979). Yst expression is maximal at 30 °C, but increased osmolarity and pH at 37 °C (to values normally present in the ileum lumen) have been shown to induce high expression of the protein (Mikulskis *et al*., 1994).

1.3.6 Yersinia outer proteins (Yops)

The Yops are a set of plasmid-encoded proteins that play a central role in antiphagocytosis. *In vitro* expression of Yops is triggered at 37 °C in the absence of calcium ions, a condition correlated with growth arrest and designated as calcium dependency. 14 Yops have been identified to date and they appear to be well conserved among the *Yersinia* species. Most of the Yops play definite roles in virulence (Mulder *et al.,* 1989, Straley and Bowmer, 1986, Galyov *et al.,* 1993, Ruckdeschel, 2001), while the functions of a few of them remain to be elucidated (Boyd and Cornelis, 2001).

Calcium depletion (or contact with a eukaryotic cell) and temperature both control transcription of the *yop* genes (Cornelis *et al.,* 1998). VirF, a transcriptional activator of the AraC family (the AraC protein regulates the transcription of the *ara* operon responsible for arabinose utilization and also autoregulates its own transcription) controls transcription of most of the genes involved in Yop synthesis and secretion (Michiels *et al*., 1991, and Cornelis *et al.,* 1989, Skurnik and Toivanen, 1992). Secretion of the Yop proteins relies on the type III secretion system (Cornelis *et al.,* 1998, Lee, 2001).

1.3.7 The Yersiniabactin iron acquisition system

Essential in the pathogenesis of the *Yersinia* species is an efficient iron-sequestration apparatus to obtain this important element from the iron-limiting environment of the host. Four TonB-dependent high-affinity iron transport systems have been identified to date among *Y. enterocolitica* species, namely, ferrichrome-, ferrioxamine-, yersiniabactin- and hemin uptake systems (Baümler and Hantke, 1992, Haag *et al*., 1993, Koebnik *et al*., 1993, Pelludat *et al*., 1998, Stojiljkovic and Hantke, 1992). More recently, the TonB-independent *yfu* system was identified as an additional iron-uptake system existing in these bacteria (Saken *et al*., 2000). Yersiniabactin is an endogenously produced siderophore whose synthesis and utilization is restricted to the high-pathogenicity *Yersinia,* notably *Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica* BG 1B (Carniel, 2002, Buchrieser *et al.,* 1998a, Buchrieser *et al.,* 1998b, Perry *et al.,* 1999). Low-pathogenicity and non-pathogenic *Y. enterocolitica* isolates do not produce yersiniabactin (Heesemann *et al*., 1993, Robins-Browne and Prpic, 1985, Boelert *et al.,* 1987, Pelludat *et al*., 2002). The genes involved in yersiniabactin synthesis are clustered on a chromosomal locus designated a high-pathogenicity island (HPI) as it carries traits usually associated with pathogenicity islands (PAIs). The genes of the HPI include *irp1-5* which account for the synthesis of the siderophore yersiniabactin (Rakin *et al.,* 1999, Carniel *et al.,* 1992, De Almeida *et al.,* 1993). *irp6-7* which encode ABC transporters (Brem *et al.,* 2001, Fetherston *et al.,* 1996) and *fyuA* which codes for the yersiniabactin-pesticin receptor together mediate transport of yersiniabactin. *ybtA* encodes the transcriptional regulator (Fetherston *et al.,* 1996) while *intB* codes for an integrase (Rakin *et al.,* 2000a). The function of *irp8* is not elucidated but it probably encodes a signal transducer, while *irp9* encodes a protein required for salicylate synthesis (Bearden *et al*., 1997, Pelludat *et al*., 1998, Rakin *et al*., 1999).

Like the pYV plasmid among the *Yersinia* species, the HPI has been shown to be necessary for mouse virulence. Thus, it was recently demonstrated by Pelludat *et al*. that transfer of the Yen O:8 HPI to a low-pathogenicity *Y. enterocolitica* strain (Yen O:9) accentuated the mouse virulence of the species. The HPI, though initially discovered in pathogenic yersiniae, is quite promiscuous and recent studies have detected this pathogenicity island among various pathogenic *E. coli* pathotypes, *Klebsiella, Enterobacter*, and *Citrobacter* (Rakin *et al*., 1999).

2. Protein Secretion

A successful hallmark of bacterial pathogenesis lies not only in the synthesis of a plethora of virulence factors, but also in the efficient translocation or delivery of these antihost effector molecules into the target. Among pathogenic bacteria, six transport systems have been clearly elucidated to be involved in the transport of proteins to the extracellular milieu. These include the signal sequence independent pathway (type I), the main terminal branch of the general secretion pathway (type II or secreton), the contact-dependent pathway (type III), the type IV pathway, the *Bordetella pertussis* filamentous haemagglutinin secretion pathway (TPS, two-partner secretion) and the autotransporter pathway (Binet *et al.,* 1997, Burns, 1999, Collazo and Galan, 1997, He *et al*., 1991, Henderson *et al.,* 1998, Jacob-Dubuisson *et al*., 1999, Jahagirdar and Howard, 1994, Sandkvist *et al.,* 2001a).

2.1 Type III secretion systems in *Y. enterocolitica*

Among *Yersinia* species, four type III secretion systems (TTSS) have been elucidated. One is the TTSS encoded by the *ysc* (for *Yersinia* secretion) genes located on the pYV plasmid. This type III secretion system is dedicated to the delivery of Yops (*Yersinia* outer proteins) into target eukaryotic cells, a prerequisite for pathogenesis (Cornelis *et al*., 1998, Boyd and Cornelis, 2001). A second TTSS, designated the Ysa (*Yersinia* secretion apparatus) locus, was recently identified by Haller *et al.* (2000) (Foultier *et al.,* 2002). The Ysa TTSS has been implicated in the extracellular secretion of at least eight proteins denoted as Ysps (*Yersinia* secreted proteins) when the bacteria are challenged by high osmolarity at 27 °C. Although the role and biological significance of these proteins have not been defined, this TTSS has nevertheless been shown to be important for the virulence of the species as a Ysa TTSS mutant exhibited an attenuated virulence when

compared to wild type strains (Haller *et al*., 2000). The chromosomally encoded TTSS identified on the *Y. pestis* genome is another example (Parkhill *et al.,* 2001).

A fourth TTSS, functionally related to the Ysc and Ysa TTSSs operates during flagellar biosynthesis (Young *et al*., 1999, Young and Young, 2002) . The core components that form the flagellar apparatus are conserved in all studied type III secretion systems. Studies indicate that the proteins that form the export machinery assemble to form a base for the entire structure. This machinery functions to specifically locate other flagellar subunits to form an intermediate structure (designated as hook-basal body) which extends to the outside of the cell. This hook-basal body exhibits an architecture similar to the structure of contact-dependent type III secretion apparatus. During the last stages of flagellar biogenesis the hook-basal body serves as both the machinery for the export of filament subunits and the base for filament assembly.

2.2 Type II secretion systems

Although the type II secretion pathway has been described for various species (Sandkvist *et al*., 2001a, and Sandkvist *et al*., 2001b) belonging to the proteobacteria family, its presence among the *Yersinia* species has never been conclusively demonstrated. Large scale genome sequencing has however identified type II secretion homologues in several bacteria, including *Y. pestis* (Parkhill *et al*., 2001), suggesting that this pathway is widely distributed among the proteobacterial family. At least 12 genes encode a functional type II secretion apparatus. Typically the exported proteins possess a unique signal peptide that is cleaved before extracellular translocation. Protein translocation across the cytoplasmic membrane is usually effected by the Sec secretion system (or Sec translocase), following which the proteins are folded into a translocation competent conformation in the periplasm before export. The variety of proteins secreted by the type II pathway include proteases, pectinases, cellulases, lipases, toxins, etc. In general these proteins catalyze the destruction of various tissues in plant and animal hosts, leading to cell damage and disease (Sandkvist *et al*., 2001a and b, Stathopoulos *et al*., 2000).

The type II secretion pathway in *K. oxytoca*, exemplified by pullulanase (PulA) export has been extensively studied and this system serves as a good model for understanding the intricacies of the type II secretion machinery (Stathopoulos *et al*., 2000). Secretion of

PulA across the cell envelope involves the products of at least 25 genes. The second, terminal step of PulA secretion (otherwise referred to as type II secretion pathway) involves the products of 14 specific genes located in the *pul* gene cluster as well as the DsbA (disulphide oxidoreducatse) protein. Below is a brief summary of the components of the pullulanase secreton (Pugsley *et al.,* 1993, Stathopoulos *et al*., 2000).

i) Cytoplasmic protein PulE. PulE contains an ATP-binding site and a tetracysteine motif resembling the zinc-binding motif found in adenylate kinases.

ii) Integral IM proteins PulC, PulM, PulN, PulK, and PulF. All, with the exception of PulF, are predicted to be anchored to the inner membrane by a single N-terminal transmembrane spanning segment followed by a large C-terminal periplasmic domain.

(iii) PulG, PulH, PulI, and PulJ are type IV pilin-like proteins (pseudopilins) with prepilin peptidase cleavage and methylation sites. They are typically periplasmic and contain prepilin peptidase cleavage and methylation sites.

(iv) OM proteins PulD and PulS. PulD is a large integral OM protein that belongs to the secretin family. The N-terminal domain of PulD appears to be responsible for substrate recognition and possible binding to PulC. It has been suggested that the N-terminal domain may serve as a gated channel, allowing the secretion of PulA while preventing the nonspecific release of other periplasmic proteins.

A novel type II secretion apparatus was uncovered by this work that demonstrates significant homology to the type II secretion pathway Eps of *V. cholerae* which secretes the cholera toxin, a protease and a chitinase. A graphical overview of the Eps pathway in *V. cholerae* is presented in Fig. 2.

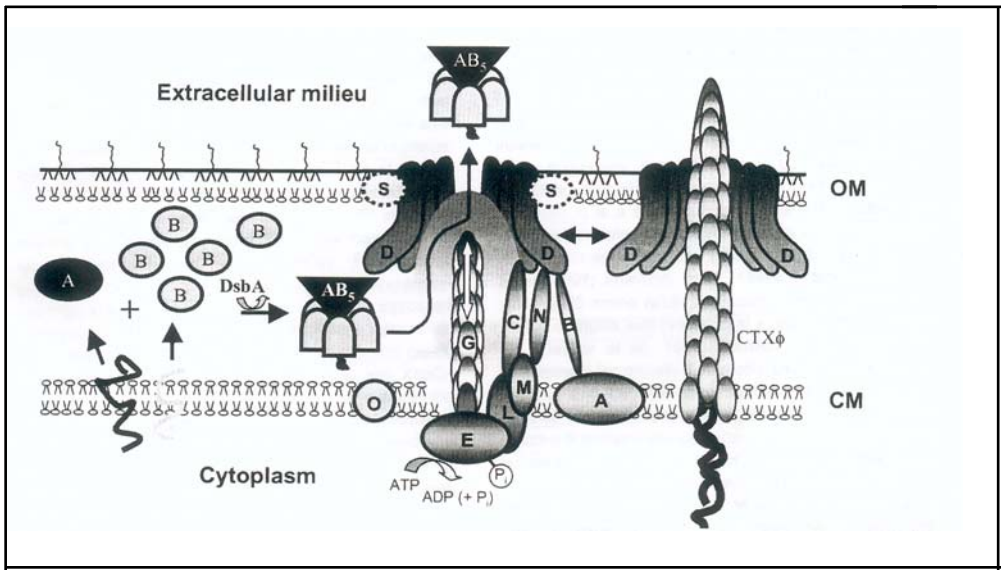


Fig.2: Assembly and secretion of cholera toxin via the type II secretion pathway Eps in *V. cholerae*. The cholera toxin A and B subunits are translocated as monomeric precursors across the cytoplasmic membrane (CM) via the Sec pathway. The subunits fold and assemble with the assistance of DsbA into the AB_5 toxin complex. The AB_5 then engages the type II secretion apparatus Eps via specific recognition of B_5, which carries the putative secretion signal. The complex is targeted to the secretion pore in the outer membrane (OM) followed by extracellular release. In this model, EpsD forms the secretion pore, and EpsE, EpsL, EpsM regulate extracellular secretion by communication of critical information through phosphorylation or ATP hydrolysis between the cytoplasmic membrane (CM) and the outer membrane (OM) pore, possibly via EpsC. Proteins G, H, I, J and K are processed by protein O and are likely to form a pilus-like structure, with protein G as the major component. The pilus may act as a piston to push the toxin out through the OM pore by repeated extension (polymerization) and retraction (depolymerization), as represented by the white double-headed arrow. The EpsD pore also supports extrusion of the filamentous phage CTXϕ. EpsD may shuttle between these two pathways (indicated by the black double-headed arrow) or may be present in excess of the other Eps components. Although not present in *V. cholerae*, protein S supports outer membrane insertion of protein D. Protein A forms a complex in the cytoplasmic membrane with protein B, which in turn appears to interact with protein D. Protein N can be immunoprecipitated with protein D. (Figure adapted from Sandkvist *et al*., 2001b).

3. Aims of this research study

The aim of this research study was to map out genomic differences between two *Y. enterocolitica* strains, namely *Y. enterocolitica* biotype 1A (non-pathogenic NF-O driver strain) and *Y. enterocolitica* biotype 1B (high-pathogenicity WA-314 tester strain) by means of representational difference analysis. This differential analysis approach should lead to the following goals:

1) Isolation of novel DNA sequences unique to the high-pathogenicity *Y. enterocolitica* 1B strain.

2) Sequence analysis of the tester specific fragments and prediction of putative virulence genes and functions.

3) Assessment of the impact of the genes on the virulence of the high pathogenicity *Y. enterocolitica* biotype 1B strains through reverse genetics of the tester specific genes and comparison of pathogenicity of isogenic pairs (parental and mutant strains) in the mouse infection model.

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	Biorad, München Mini-Protean -II Cell and Western Blot Apparatus
Electroporation-apparatus	Biorad, München Gene Pulser, II Pulse Controller II
Fluorescent activated cell sorter (FACScan)	Becton, Dickinson, Heidelberg
French Press	French Pressure Cell 40K SLM Aminco
Hybridization oven	Personal Hyb. Stratagene, Amsterdam
Incubator	Heraeus, Hanau Typ B20
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
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
Strains		
Y. enterocolitica		
WA-314	*Y. enterocolitica* O:8 with pYV virulence plasmid	Heesemann, 1987
WA-C	Plasmidless derivative of strain WA-314, serotype O:8 and spontaneously resistant to Nalidixic acid	Heesemann, 1987
WA-CS	Derivative of WA-C; Spontaneous Sm^r mutant	Pelludat *et al.,* 1998
8081	Clinical isolate, Serotype (ST) O.8	Portnoy & Falklow, 1981
WA-CS *yts1E*::Kan^r	Derivative of WA-CS, Nal^r, Kan^r, Sm^r (insertional inactivation of *yts1E*)	This research
WA-CS *chiY*::Kan^r	Derivative of WA-CS, Nal^r, Kan^r, Sm^r (insertional inactivation of *chiY*)	This research
WA-CS *hns*::Kan^r	Derivative of WA-CS, Nal^r, Kan^r, Sm^r (insertional inactivation of *hns*)	This research
Ye 1209-79	Clinical isolate, ST O:13 BG 1B	Heesemann, 1987
Ye 1223-75-1	Clinical isolate, ST O:20 BG 1B	Heesemann, 1987
Ye 737	Clinical isolate, ST O:21 BG 1B	Heesemann, 1987
Y-108	Clinical isolate, ST O:3 BG 1B	Heesemann, 1987
Y-96	Clinical isolate, ST O:9 BG 2	Heesemann, 1987
NF-O	Clinical isolate, ST O:5 BG 1A	Ratnam *et al.,* 1982
MRS-40	Clinical isolate, ST O:9 BG 2	Heesemann, 1987
Y. pseudotuberculosis		
Y. pstbc H141/84	ST O:1a	S. Aleksic
Y.pstbc H457/86	ST O:2a	S. Aleksic
Y. pstbc IP3295	ST O:1	E. Carniel
Y. pstbc 346	ST O:3	S. Aleksic
Y. pestis		
KIM	Biogroup (BG) A	R. R. Brubaker
KUMA	BG M	R. R. Brubaker
TS	BG O	R. R. Brubaker
E. coli		
ECOR strains	*E.coli* ECOR collection	Ochman and Selander, 1984
C-4441	Enteroaggregative, O:128	Schubert *et al.,* '98
12860	Enteroinvasive, O:124	Schubert *et al.,* '98
DH5α	*end*A1 *hsd*R17($r_{k-}m_{k+}$)*sup*E*44 thi*-1 *recA1*	Hanahan, 1983

	gyrA relA1 Δ(*lacZYA-argF*) U169 (ϕ80*lacZ*Δ*M15*)	
S17-1 λpir	*pir*$^{+}$ *tra*$^{+}$	Simon *et al.*, 1988
XLI Blue MR	Δ(*mcrA) 183 Δ(mcrCB-hsdSMR-mrr) 173 endAI supE44 thi-1 recA1 gyrA96 relA1lac*^{c}	Stratagene
Mos*Blue*	*end*A1 *hsd*R17 (r_{k12}-$m_{k12}$$^{+}$)*su*pE44 *thi*-1 *rec*A1 gyrA96 *rel*A1 lac [F' *pro*A^{+}B^{+} lacI^{q}ZΔM15:Tn10(Tc^{R})]	Amersham
BL21	F^{-} *ompT hsdS*($r_B$$^{-}$ $m_B$$^{-}$) *gal*	Studier & Moffat, 1986
HB101	*SupE*44 *hsdS*20($r_B$$^{-}$ $m_B$$^{-}$) *recA*13 *ara-14 proA*2 *lacY*1 *galK2 rpsL*20 *xyl*-5 *mtl*-1	Bolivar & Backman, 1979
Salmonella strains (SARC)		Boyd *et al.,* 1996
Plasmids		
Supercos1	Cloning vector Ap^{r} Neo^{r}	Stratagene
ScF2.2	*Bgl* II/*Bam*H I self-religated Supercos1 fragment (ScF) carrying the mutagenized *yts1E*	This research
ScF2.2*chiY*	*Bgl* II/*Bam*H I self-religated ScF carrying a kanamycin-inactivated *chiY* fragment	This research
ScF2.2*hns*	*Bgl* II/*Bam*H I self-religated ScF carrying the mutagenized *hns* gene	This research
pKAS32	Cloning vector with dominant *rpsL* gene	Skorupski & Taylor, 1996
pKAS32 *yts1E*::Kan^{r}	pKAS32 carrying the mutagenized *yts1E* gene fragment	This research
pKAS32 *chiY*::Kan^{r}	pKAS32 carrying the mutagenized *chiY* gene fragment	This research
pKAS32 *hns*::Kan^{r}	pKAS32 carrying the mutagenized *hns* gene	This research
pACYC184	Cloning vector, Cm^{r} Tet^{r}	New England Biolabs
pUC 4K	pUC vector carrying kanamycin cassette from Tn*903,* Kan^{r} Ap^{r}	Amersham
pMOS*Blue*	Cloning vector Ap^{r}	Amersham

2.2 Primers

Table 2. List of primers

Primer	5'-.....-3' Sequence	Description
YenInv.for	ATGATATGACTGGGCACAA	in *invA*, forward
YenInv.rev	TGGCAGCGCAGGTAAATAC	in *invA*, reverse
YenAil.for	ATGTGTACGCTGCGAGTG	in *ail*, forward
YenAil.rev	TGGCCCCATTGTTACTGA	in *ail,* reverse
yts1D.for	GCAGTAAAAGGCAACATCAGCG	in *yts1D* forward
yts1D.rev	AAACAACGCGCATGACGACTTC	in *yts1D* reverse
yts1E.for	GCTGCCGTTGGGTCAGTTGCA	in *yts1E* forward
yts1E.rev	GGCGTTGATCAGGCGAATAAT	in *yts1E* reverse
yts1F.for	GCGCCTCGTGGGTTTTCACTC	in *yts1F* forward
yts1F.rev	GGAAGCTGGCGAGTGCGTCAG	in *yts1F* reverse
yts1S.for	AACCGACAGCCCAGCAGCAGA	in *yts1S* forward
yts1S.rev	GCGCCTCATCCACAAAATCAA	in *yts1S* reverse
chiYgfp.f	GCTCTAGAATAGCGAATAACATTTTTTAAC	*chiY* promoter-forward with *Xba* I site
chiYgfp.r	GCGGATCCTTTCATGTTTATATCCTTT	*chiY* promoter-forward with *Bam*H I site
hns.for	GCGAATTCTAAAATACGTAAATTCGAG	in *hns* gene forward with *Eco*R I site
hns.rev	CGGCGGCCGCTCACGATTACAGCAGGAAAT	in *hns* gene forward with *Not* I site
aph 157	CTCACCGAGGCAGTTCCATAG	in Kanamycin cassette
irp 242	AAGGATTCGCTGTTACCGGAC	amplifies within the *irp1* gene
irp 505	TCGTCGGGCAGCGTTTCTTCT	amplifies within the *irp1* gene
f10	CTCGTCGACCGTTATCGCCATTCT	amplifies within the *fyuA* gene
f176	CAGAGGATAAAGCCGTGTCAT	amplifies within the *fyuA* gene
ybta 255	GCAAACGCAATCTGAAATCTC	amplifies within the *ybtA* gene
ybta 1012	GCCATAGACGCTGTTGTTGAA	amplifies within the *ybtA* gene
c 15-205	TACAGGCAGGTTCCCGATGAC	amplifies within the *int* gene
ybta 815	CATTGCGCTGGGTGCCTATC	amplifies within the *int* gene
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
ye 262	TTTTCCCCCGAGAGGCTGAGTAACC	amplifies a unique region specific to all *Y. enterocolitica* strains
3p 345	GAGGCGATCCCAGTCAGAG	amplifies a unique region specific to all *Y. enterocolitica* strains
aph 606	CGACCATCAAGCATTTTATCC	in Kanamycin cassette
D.for	GTAGGATATGCGGAGAACTTC	in *yts2D* gene forward
D.rev	CGTTTTGCGGGAATAACTTTT	in *yts2D* gene reverse

chit.for	AAATTGAATAAAATTATGTTA	in *chiY* gene forward
chitrev.new	CCGTAAAAAACCTGATAACCA	in *chiY* gene reverse
myf.for	TCGTTCTCAGTTGA	in *myf* gene, forward
myf.rev	GAGTCCGACATTAC	in *myf* gene, reverse
n41f.rna	GACCGGTGGCCAGTAACTT	in IS*1330* forward
n41r.rna	CGAGGTACGTAATGATCTT	in IS*1330* reverse
chiYf.eco	TAGAATTCATAGCGAATAACATTTTTTAAC	start of *chiY* gene with *Eco*R I site
chiYf.not	CGGCGGCCGCTGCCTCTTGTTATCATGCGG	end of *chiY* gene with *Not* I site
chiorf.f	GCGGATCCAAATTGAATAAAATTATGTTA	start of *chiY* with *Bam*H I site
chiorf.GST	GAGCGGCCGCTTAGCACGCGCCTTTATCTT	end of chiY with *Not* I site
n28orf.eco	TAGAATTCAGCTGCCAGGATGGTAAAGG	in *yts1D* gene, forward with *Eco*R I site
n28orf.rev	TTGCGGCCGCGTTGCAATTTGTCAGTGATG	in *yts1D* gene, reverse with *Not* I site

*The enzyme recognition sites are indicated in bold letters.

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

Sterilization of liquid media was 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	10 g Trypton 5 g Yeast 5 g NaCl H_2O_{dest} to 1 L pH set to 7.4 - 7.6 with NaOH
Nutrient Broth (NB) Medium	8 g Nutrient Broth 5 g NaCl ad to 1 L H_2O_{dest}
NBD Medium	NB-Medium with 200 µM dipyridyl (in 70 % EtOH) (Dipyridyl functions as an intracellular complexer of Fe^{2+})
Brain Heart Infusion (BHI) medium	37 g BHI powder in 1 L H_2O_{dest}

Minimal medium (M9)	Na_2HPO_4	6 g
	KH_2PO_4	3 g
	NaCl	0.5 g
	NH_4Cl	1 g
	Water	1 L
	pH adjusted to 7.4, autoclaved and cooled	
Further media components	1 M $MgSO_4$	2 ml
	20 % Glucose	10 ml
	1 M $CaCl_2$	0.1 ml

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	Kan	H_2O_{dest}	100
Nalidixic acid	Nal	0.5 N NaOH	100
Chloramphenicol	Cm	70 % EtOH	20
Streptomycin	Sm	H_2O_{dest}	100
Tetracycline	Tet	70 % EtOH	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 2 presents a summary of the strains and plasmids used in this study.

4. Molecular genetic methods

4.1 Isolation of Chromosomal DNA

High quality, undegraded chromosomal DNA was isolated according to the method described in Current Protocols (Ausubel *et al.,* 2000) as follows. Bacteria from a saturated liquid culture were lysed with SDS and proteinase K. Cell wall debris, polysaccharides, and remaining proteins were then removed by selective precipitation with CTAB (hexadecyl trimethyl ammonium bromide), and high molecular weight DNA was recovered from the resulting supernatant by isopropanol precipitation.

Reagents

Tris-EDTA Buffer	2 M Tris.HCl, pH 8 0.25 M EDTA, pH 8
CTAB / NaCl solution	10 % CTAB (hexadecyltrimethyl ammonium bromide) 0.7 M NaCl (4.1 g / 100 ml) Dissolve in 80 ml distilled water, heat to 65 °C and adjust volume to 100 ml

Procedure

50 ml LB medium (supplemented with antibiotics when appropriate) was inoculated with the bacteria of interest and grown overnight on a shaker at the 27 °C / 37 °C. The cells were then pelleted at 4000 x g for 5 min and gently resuspended in 9.5 ml TE buffer. 0.5 ml of 10 % SDS was added and 50 µl proteinase K (20 mg/ml in H_2O), followed by gentle mixing and incubation for 1 hr at 37 °C. 1.8 ml 5 M NaCl was added and mixed thoroughly. 1.5 ml CTAB / NaCl was then added, the suspension mixed and then incubated at 65 °C for 20 min. 1 volume (13.5 ml) of freshly prepared chloroform / isoamyl alcohol mixture (at a ratio of 24:1 respectively) was then added, and the suspension was centrifuged at 6,000 x g for 10 min at RT. The viscous upper phase was then drawn out with a tipless 5 ml pipette, mixed with 1 volume of Phenol / Chloroform / Isoamylalcohol (25:24:1) and subjected to centrifugation for at least 10 min at 6,000 x g at RT. This extraction step with phenol was repeated thrice, after which the last phase was collected in a fresh tube and treated with 0.6 volume isopropanol to precipitate the DNA. The solution was mixed carefully till the DNA self-precipitated. The DNA was then spooled out of the mix with a pipette tip and washed in 1 ml of 70 % ethanol. The DNA was allowed to air-dried and finally dissolved overnight in either 2 ml Tris-EDTA (TE) buffer or water.

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 of 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 30-60 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 -70 °C for 30 min.
- The mixture was centrifuged at 14,000 rpm for 30 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 TE buffer or water and stored at -20 °C.

4.3.2 Determination of DNA concentration and purity

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

1 A_{260} = 50 µg / ml for ds DNA

1 A_{260} = 33 µg / ml for ss DNA

For determination of DNA purity, the $A_{260/280}$ coefficient was photometrically determined. An $A_{260/280} < 1.8$ indicated contamination of the DNA preparation with protein or aromatic substances such as phenol, while an $A_{260/280} > 2.0$ 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 recation: 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 70 µl H_2O_{bidest}, cooked at 95 °C for 10 min and centrifuged (12,000 rpm for 5 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	DNA	5 µl
	Primer 1 (100 pmoles)	0.5 µl
	Primer 2 (100 pmoles)	0.5 µl
	dATP, dCTP, dGTP, dTTP	5 µl
	10 x Taq-Reaction buffer	5 µl
	Taq-polymerase (5 U / µl)	0.5 µl
	H_2O_{bidest}	ad to 50 µl
Cycling parameters	Denaturation 94 °C	5 min
	Denaturation* 94 °C	30 sec
	Annealing* x °C	30 sec
	Elongation* 72 °C	y min
	Final extension 72 °C	7 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.

4.5 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	40 mM Tris / HCl, pH = 8.2 20 mM Acetic acid 2 mM EDTA, pH 7.6
10 x Loading buffer for agarose gels	0.25 % (w/v) Bromophenol blue 25 % (v/v) Ficoll 400
Agarose gel	0.8 % - 2 % Agarose in 1 x TAE
Ethidium bromide staining solution:	1 µg Ethidium bromide pro ml H_2O_{dest}

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 $_{bidest}$	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 glyecrol concentration did not exceed 5 % final digestion volume.

Enzyme inactivation was either through heat treatment at 65 °C for 20 min or by employing 0.5 M EDTA, pH 8.0, to a final concentration of 10 mM (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 65 °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 1:3 vector to insert ratio was utilized for all ligations and the reaction proceeded at either 16 °C overnight or at RT for 1 - 3 hours.

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 1µg)	x µl
	DNase incubation buffer	1 µl
	DNase	1 µl
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^{TM} II RNase H^{-} Reverse Transcriptase (Gibco, Eggenstein) was used in all reverse transcription analyses as described below. 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.

Reaction components	RNA (50 ng to 1µg)	x µl
	Gene specific reverse primer	1 µl (2 pmol)
	DEPC-H_2O	ad 12 µl
Incubation	70 °C for 10 min, quick chill on ice	
1st strand synthesis		
Reaction components	5 x first strand synthesis buffer	4 µl
	0.1 M DTT	2 µl
	dNTPs (10 mM of each)	1 µl
Incubation	2 min, 42 °C	
	Reverse transcriptase	1 µl
	Incubation	50 min, 42 °C
	Inactivation of enzyme	15 min, 70 °C

The resulting cDNA was then probed in high-fidelity PCR reactions using 10 % (2 µl) of the reverse transcription reaction volume.

4.8.4 Reverse Transcription analysis from bacterial RNA isolated from an infected HeLa cell line.

HeLa cells 229 were cultured in RPMI 1640 medium, fortified with 10 % FCS (fetal calf serum) and 2 mM Glutamine at 37 °C in a 5 % CO_2 environment. The HeLa cells were seeded in 10 ml culture flasks one day before infection at a concentration of 5 x 10^6 cells per flask. The cultures were maintained with penicillin-streptomycin (100 units) before bacterial infection. For infection, *Y. enterocolitica* WA-314 was inoculated into Luria-Bertani Broth from a frozen stock, cultured overnight at 37 °C with aeration, and then diluted 1 : 100 into fresh medium and cultured an additional 90 - 120 min. Following centrifugation and resuspension of pellet in phosphate buffered saline (PBS), the HeLa cells were infected at a multiplicity of infection (MOI) of 1 : 50. After a 4 hr incubation, the HeLa cells were washed once with pre-warmed RPMI and incubated for an additional 2.5 hr in RPMI containing 10 % FCS and gentamicin at a concentration of 100 µg/ml to eliminate extracellular bacteria. Following this, the cells were washed twice with phosphate buffered saline (PBS) and then lysed with 5 ml of 1 % Triton to disrupt the HeLa cells and free the adherent *Yersinia* bacteria. After low gradient speed centrifugation to pellet the bacteria, bacteria were suspended in Trizol reagent and total RNA was isolated according to manufacturer's instructions (see

4.8.1). The RNA was incubated with RNase - free DNase and total RNA was reverse transcribed with the appropriate primers.

4.9 Bacterial transformation

Bacterial cells were either made electrocompetent or "calcium" competent 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 (1983) was employed. The procedure was carried out in the cold and under sterile conditions.

Procedure

100 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 in an ice-water bath (or on ice) for 10 - 15 min and transferred to a 1-liter prechilled centrifuge bottle.
- Cells were then centrifuged at 4200 rpm for 20 min in sterile centrifuge flasks or prechilled falcon tubes (at 4 °C).
- The supernatant was decanted and cells resuspended in 500 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 40 ml ice-cold 10 % glycerol (centrifuged in the cold at 4200 rpm for 10 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.

Electroporation with high voltage was achieved with the Gene Pulser II from Biorad (Dower *et al*., 1988). The principle relies on the fact that short electrical impulses directed at bacterial cells generates pores in the cell membrane that facilitates entry of foreign or exogenous DNA into the cell. The settings employed were 25 µF capacitance at 2.5 kV and 200 ohms resistance.

4.9.2 Production of calcium-competent cells

Calcium-competent cells were produced according to a simplified method from Current Protocols (Ausubel *et al*., 2000) as follows:

- 2 ml of an overnight culture was inoculated into 100 ml LB medium and incubated with vigorous shaking at 27 °C (for *Yersinia*) or at 37 °C (for *E. coli*) until an OD_{600} of 0.8 was realized.
- Cells were collected in 50 ml falcon tube, cooled for 15 min on ice and centrifuged in a pre-cooled centrifuge (4000 rpm for 10 min at 4 °C).
- The pellet was suspended in 20 ml ice-cold 50 mM $CaCl_2$ / 15 % glycerol solution, maintained on ice for 15 min and centrifuged again at 4000 rpm for 10 min at 4 °C.

Pellet was resuspended in 2 ml ice-cold 50 mM $CaCl_2$ / 15 % glycerol solution, kept on ice for 30 min and aliquoted in 400 µl volumes in Eppendorf tubes and stored at -80 °C until required.

Transformation of calcium-competent cells followed the procedure below:

Transformation Procedure

- Competent bacterial cells were briefly thawed and 200 µl of cells mixed rapidly with plasmid DNA in fresh, sterile Eppendorf tubes and maintained on ice for 30 min.
- The cell membranes were disrupted by subjection of cells to a heat-pulse for 90 sec at 42 °C.
- Cells were then mixed with 1 ml LB medium and incubated at 27 °C / 37 °C with shaking.
- Bacterial cells were then plated out in 100 - 200 µl aliquots on LB-agar plates containing the required antibiotics for selection of recombinants.

4.10 Southern Blot hybridization (Southern, 1975)

This method was originally described by Southern and the principle involves transfer of DNA from a gel to a membrane, fixation of transferred DNA with UV light and subsequent hybridization of the membrane with an appropriately labeled DNA probe. The objective is to locate DNA samples that share some level of homology with the labeled probe. DNA probes were typically labeled with digoxigenin according to manufacturers' instructions.

4.10.1 Preparation of DNA probe

4.10.1.1 Digoxigenin-labeling of DNA through PCR

(with DIG-dUTP –Roche, Mannheim):

Procedure

Reaction components	Template DNA	2.5 µl
	PCR Buffer	5 µl
	dNTPS (2 mMdATP/dCTP/ dGTP, 1.3 mM dTTP)	5 µl
	DIG dUTP	3.5 µl
	Primer 1 (100 pmol / µl)	1 µl
	Primer 2 (100 pmol / µl)	1 µl
	Taq-polymerase	0.3 µl
	H_2O_{dest}	31.7 µl
PCR amplification	35 PCR cycles of denaturation, annealing and elongation	

4.10.1.2 Random-primed method of DNA labeling

This method relies on the random labeling of a DNA sample with DIG-11-dUTP (Roche, Mannheim), catalyzed by the Klenow polymerase enzyme.

Procedure

Starting component	DNA cooked for 10 min rapid-chilled on ice	10 ng - 1µg
Other reaction components	Hexanucleotide mix (10x)	2 µl
	dNTP labeling mix (10x)	2 µl
	Klenow enzyme (2 U / µl)	1 µl
	H_2O_{bidest}	20 µl
Incubation	37 °C, overnight	
Further reaction components	0.2 M EDTA	2 µl
	4M LiCl	2 µl
	ice-cold $EtOH_{abs}$	50 µl
Incubation	-70 °C, 30 min	

The labeled DNA was then pelleted by centrifugation at 15,000 rpm at 4 °C for 15 min, the DNA pellet washed with ice-cold EtOH and in 50 µl H_2O_{bidest} suspended.

4.10.2 Southern (Vacuum) Blot

Basically the Southern blot was carried out by transferring DNA from an agarose gel onto a nylon membrane using a vacuumblot (LKB 2016 Vacu Gene^R, Pharmacia-LKB, Uppsala, Sweden). The blotting involved treating the gel consecutively with a depurinations solution, a denaturating solution, a neutralizing solution and finally 20 x SSC at a minimum pressure of 40 mbar for depurination and 50 mbar for the following steps. A successful DNA-transfer step was then followed by DNA fixation on a nylon membrane by crosslinking with UV light (0.12 J / cm^2).

Depurination solution	0.25 M HCl
Denturating solution	0.5 M NaOH; 1.5 M NaCl
Neutralizing solution	0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl
20 x SSC (pH 7.0)	0.3 M Na_3 citrate; 3 M NaCl

4.10.3 Hybridization and detection

Hybridization solution	SSC	5x
	SDS	0.02 %
	Blocking reagent	1 %
	N-Lauroyl sarcosine (Na salt)	0.1 %
Wash-buffer 1	SSC	2x
	SDS	0.1 %
Wash-buffer 2	SSC	0.1x
	SDS	0.1 %
Blocking solution	Blocking reagent in buffer 1	1 %
Buffer 1 (pH 7.5)	Tris-HCl	0.1 M
	NaCl	0.15 M

Substrate-buffer (pH 9.6)	NaCl	0.1 M
	Tris-HCl	0.1 M
	$MgCl_2$	0.05 M
Substrate-detection solution	NBT (1 mg/ml)	10 ml
	BCIP (5 mg/ml)	1 ml
	Substrate buffer	90 ml

Hybridization: The membrane blot was first prehybridized for a minimum of 3 hr at 68 °C, followed by hybridization with the appropriate DIG-probe at 68 °C overnight. Stringency washes then followed comprising two consecutive washes of the blot at RT for 15 min with wash buffer 1, followed by another round of two stringency washes at 68 °C for 30 min each with wash buffer 2 (see below for buffer compositions).

Blocking and incubation of the blot with Anti-Digoxigenin antibody: The membrane blot was then incubated with blocking buffer for 1 hr at RT and then washed briefly with buffer 1. This step prevented unspecific binding of the DNA probe to non-homologous DNA regions. The membrane was then washed briefly with buffer 1, followed by incubation for 45 min of the membrane blot in a solution containing the Anti-Digoxigenin antibody conjugated with alkaline phosphatase (the Anti-Dig antibody was diluted 1:5000 in buffer 1). The membrane was then washed twice with buffer 1.

Detection: Detection followed, by incubation in a freshly mixed substrate-detection solution at 37 °C in the dark. On visible detection of the bands, the reaction was stopped by the addition of water.

4.11 Cosmid gene bank of *Y. enterocolitica* WA-314

The cosmid gene library was prepared with the Supercos 1 cosmid vector (Stratagene) as cloning vehicle. This is a vector specially engineered to contain bacteriophage promoter sequences (*cos* sites) flanking a unique cloning site, thus allowing the *in vitro* packaging of DNA into phage heads.

4.11.1 Preparation of cosmid vector DNA

- 25 µg of the cosmid vector DNA (7.6 kb) was digested with 9 U / µg of *Xba* I in a total volume of 200 µl according to standard digestion procedures (see 4.6.1) for 1 hr at 37 °C.

Digested vector was purified once with phenol-chloroform-isoamyl alcohol (25:24:1). The *Xba* I digested cosmid DNA was resuspended in distilled water at a concentration of 1 µg/µl and subjected to dephosphorylation according to previously described protocols (see 4.6.2).

- The dephosphorylated DNA was phenol-purified and digested with 5 U / µg of *Bam*H I endonuclease in a total volume of 200 µl at standard buffer conditions. Complete digestion was verified by loading DNA sample on 0.8 % agarose gel. Two cosmid bands were observed running at 1.1 and 6.5 kb respectively.
- The X*ba* I / *Bam*H I digested cosmid DNA was purified with phenol-chloroform, resuspended in deionized water at a concentration of 1 µg/µl and stored at -20 °C.

4.11.2 Preparation of genomic DNA

Procedure

- Isolation of chromosomal DNA was according to the CTAB / NaCl procedure previously described (see 4.1).
- The chromosomal DNA was partially digested with *Sau*3A I in order to clone into the *Bam*H I site of the Supercos 1 cosmid vector. Preliminary digests prior to the main one were carried out to identify the optimal incubation time to achieve an insert size range of 30 - 42 kb, essential for cloning into the supercos1 vector.

Preliminary digest: 10 µg of genomic DNA in a total reaction volume of 100 µl was digested at 37 °C with 0.5 U of *Sau*3A I. 15 µl aliquots of the digest was then removed at various time intervals: 0 -, 5 -, 10 -, 15 -, 20 -, 30 -, and 45 minutes time points, and checked by pulsed-field gel electrophoresis on a 0.6 - 0.7% (w/v) agarose gel. The time interval that yielded a large proportion of the digested fragments running within 35 - 50 kb range was chosen as the desired digestion time.

- **Main digest:** After the optimal time interval was determined (15 min), a partial *Sau*3AI digest of 100 µg of genomic DNA in a 1 ml total reaction volume was carried out. The reaction was scaled up to best mimic the test partial digest including enzyme concentration, temperature and reaction time. The reaction was stopped with 15 µl of 0.5 M EDTA (pH 8). 10 µl aliquot of the reaction mix was loaded on a 0.7 % gel to ensure appropriate size distribution.The DNA was resuspended in 50 µl of TE buffer.

- Dephosphorylation of the partially digested genomic DNA with SAP (see 4.6.2) after which it was phenol-chloroform purified. For dephosphorylation, the following were added to the 50 µl of DNA from above:

10x SAP buffer	10 µl
Distilled water	38 µl
SAP	2 µl (1 unit / µl)

The reaction volume was made up to 100 µl, and incubated at 37 °C for 1 hr.

- 3 µl of 0.5 M EDTA was added to stop the reaction followed by heat inactivation of the enzyme at 65 °C for 20 min.
- The DNA was extracted once with phenol-chloroform saturated with 50 mM Tris-HCl (pH 8.0) and once with chloroform. The aqueous phase was then adjusted to 0.3 M sodium acetate (pH 5.5) and ethanol-precipitated by adding 2.5 volumes of 100 % (v / v) ethanol.
- The DNA was resuspended to a 1µg/µl concentration in TE buffer and a sample run on 0.7 % agarose gel.

4.11.3 Ligation and packaging of DNA

- The ligation reaction was set up by adding the following reaction components to a microcentrifuge tube:

  2.5 µg of partially digested SAP genomic DNA

  1.0 µl of Supercos 1 DNA (*Xba* I-SAP and digested with *Bam*H I, 1 µg/µl)

  2.0 µl of 10x ligase buffer

  Water to a final volume of 20 µl

- A negative control ligation was set up by adding the following components to a microcentrifuge tube:

  1.0 µl of Supercos 1 DNA (*Xba* I-SAP and digested with *Bam*H I, 1 µg/µl)

  2.0 µl of 10x ligase buffer

  Water to a final volume of 20 µl

- A 1 µl aliquot was removed from each reaction and stored at 4 °C for later gel analysis.
- 1 µl of T4 DNA ligase was added to the remaining 19 µl of the reaction and incubated at 4 °C overnight.

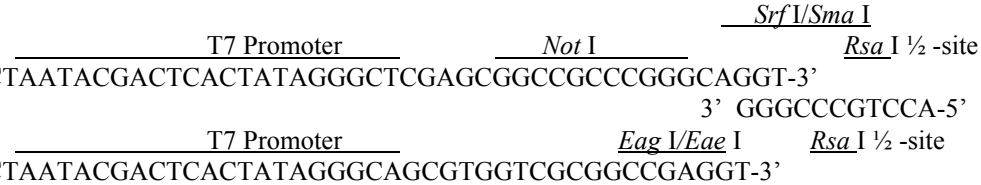
- A further 1 µl aliquot was removed from each reaction and loaded on a 0.7 % agarose gel for analysis.

Packaging

The packaging reaction was carried out with the Gigapack II gold packaging extract (Stratagene) according to manufacturers' instructions. *E. coli* XL 1- Blue MR was the bacterial host strain utilized for titration of the cosmid packaging reaction.

4.12 Representational difference analysis (suppressive subtractive hybridization)

Genomic differences between *Y. enterocolitica* 1A (NF-O, non-pathogen) and *Y. enterocolitica* 1B WA-314 (high-pathogenicity, mouse lethal strain) were mapped out by employing the PCR-Select Bacterial Genome Subtraction System from Clontech. An overview of the PCR-Select method is shown in Fig. 3 (pg. 39) while Fig. 4 (pg. 40) details the molecular basis of the procedure. Genomic DNA (1.5 - 2 µg) was first isolated from the two strains of bacteria to be compared (*Y. enterocolitica* 1A (NF-O), the driver strain and *Y. enterocolitica* 1B (WA-314), the tester strain). The chromosomal DNA was then digested with *Rsa* I, a four base cutter. This step generated DNA fragments ranging in size from 0.1 to 3 kb. The tester DNA was then subdivided into two portions, each of which was ligated with a different oligonucleotide adaptor (adaptor 1 & 2R at 10 µM concentration each). The adaptors employed with their unique restriction sites are shown below. After adaptor ligation, the DNA was purified by phenol / chloroform extraction.



4.12.1 Hybridization

4x hybridization buffer	200 mM HEPES-HCl (pH 8.0), 2 M NaCl 0.2 mM EDTA (pH 8.0).

- For the first round of subtractive hybridization, approximately 0.6 µg of *Rsa* I-digested driver DNA was mixed in two separate reactions with 0.012 µg each of i) adaptor 1-ligated tester DNA and ii) adaptor 2R-ligated tester.
- The reaction volume was made up to 4 µl using a 4 x hybridization buffer. The samples were covered with a drop of mineral oil, centrifuged briefly and incubated in a thermal cycler (Perkin Elmer) at 98 °C for 1.5 min (initial denaturation).
- Incubation followed at 63 °C for 1.5 hr. The two samples from this first hybridization were then mixed together and 0.3 µg freshly denatured driver DNA was added to further enrich for tester-specific sequences.
- This DNA mixture was then subjected to a second hybridization reaction, with overnight incubation in the thermal cycler at 63 °C.

4.12.2 PCR amplification

Prior to thermal cycling, a preincubation step was carried out at 72 °C to fill in the missing strands of the adaptors and thus create binding sites for the PCR primer. This would provide binding sites for PCR Primer 1 whose nucleotide sequence corresponds to the first 22 nucleotides of both adaptors 1 & 2R. PCR amplification to selectively amplify for tester-specific DNA was done in two stages. In the first amplification, only ds DNAs with different adaptor sequences on both ends are sequentially amplified. In the second round of amplification, nested PCR, employing nested primers 1 & 2, was used to further reduce the background and enrich the DNA pool for tester specific sequences. The nucleotide sequences of the two nested primers 1 & 2 corresponded to the last 22 and 20 nucleotides respectively of Adaptors 1 & 2R utilized in the DNA ligation reaction.

Cycling parameters

Primary PCR	Denaturation	94 °C	30 sec
	*Denaturation	94 °C	30 sec
	*Annealing	66 °C	30 sec
	*Elongation	72 °C	1.5 min
	* 25 cycles		
Secondary nested PCR	Denaturation	94 °C	30 sec
	*Denaturation	94 °C	30 sec
	*Annealing	68 °C	30 sec
	*Elongation	72 °C	1.5 min
	* 10-12 cycles		

Analysis of the subtracted fragments on a 2 % agarose gel revealed a smear with distinct bands running from about 300 bp to 1.5 kb. The subtracted fragments were subsequently cloned into pMOS*Blue* vector (Amersham), followed by transformation into the highly efficient MOS*Blue* (Amersham) competent cells (see section 4.9.2 for transformation procedure). The pMOS*Blue* vector allows for blue-white screening with recombinant clones appearing white when plated on X-gal and IPTG indicator plates.

4.12.3 Preparation of X-gal / IPTG plates for blue-white screening of recombinants

- 35 µl of 50 mg / ml X-gal and 20 µl 100 mM IPTG was spread on LB agar antibiotic plate.
- The plates were left to soak for at least 30 min prior to plating.
- 10 - 50 µl of each transformant was then spread on to the LB agar plates.

Inverted plates were incubated overnight at 37 °C.

The pMOS*Blue* vector allows for blue-white screening, with recombinant colonies appearing white when plated on X-gal and IPTG indicator plates.

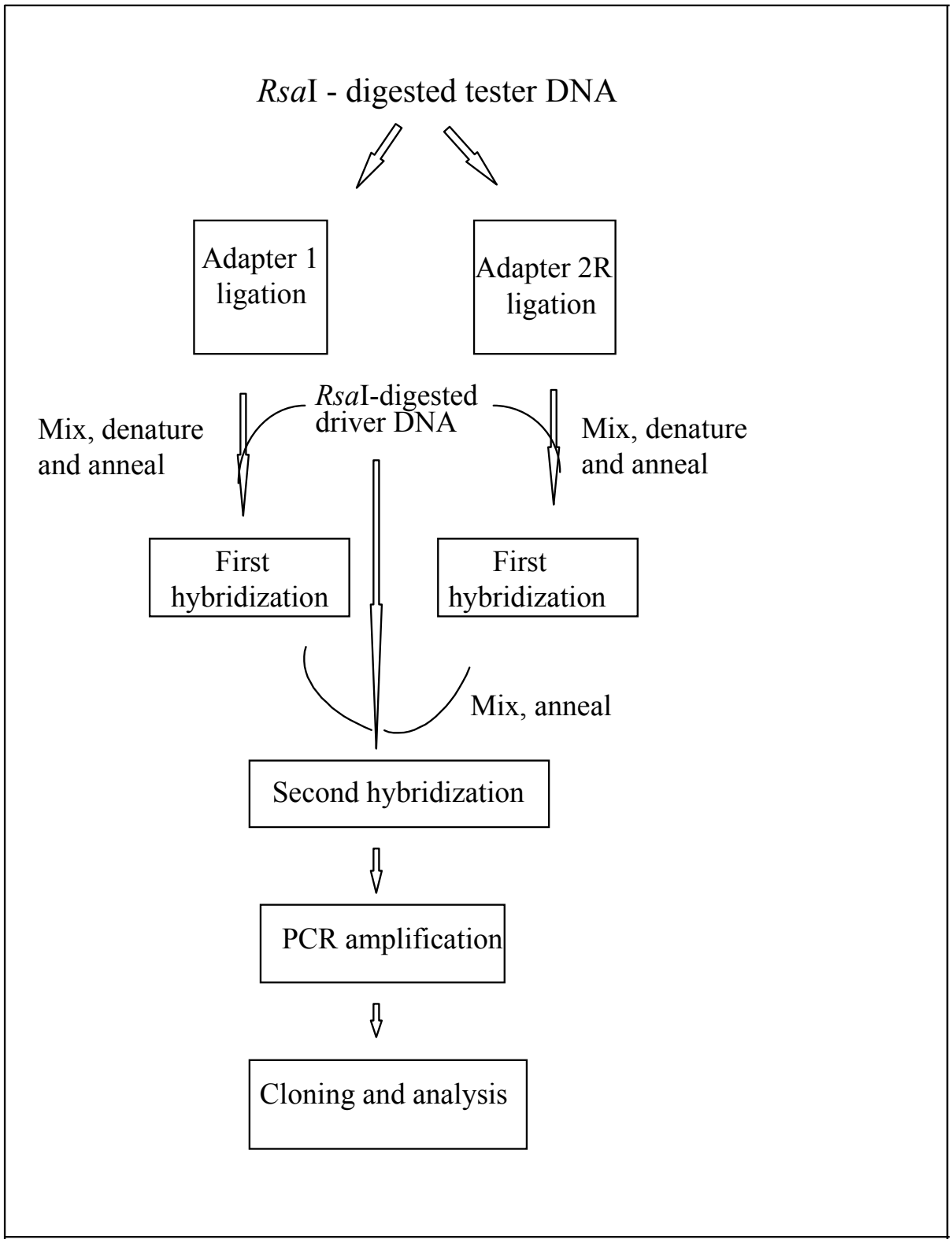


Fig. 3. Overview of the CLONTECH PCR-Select procedure. The cDNA in which specific transcripts are to be found is the ”tester” and the reference cDNA is the ”driver”.

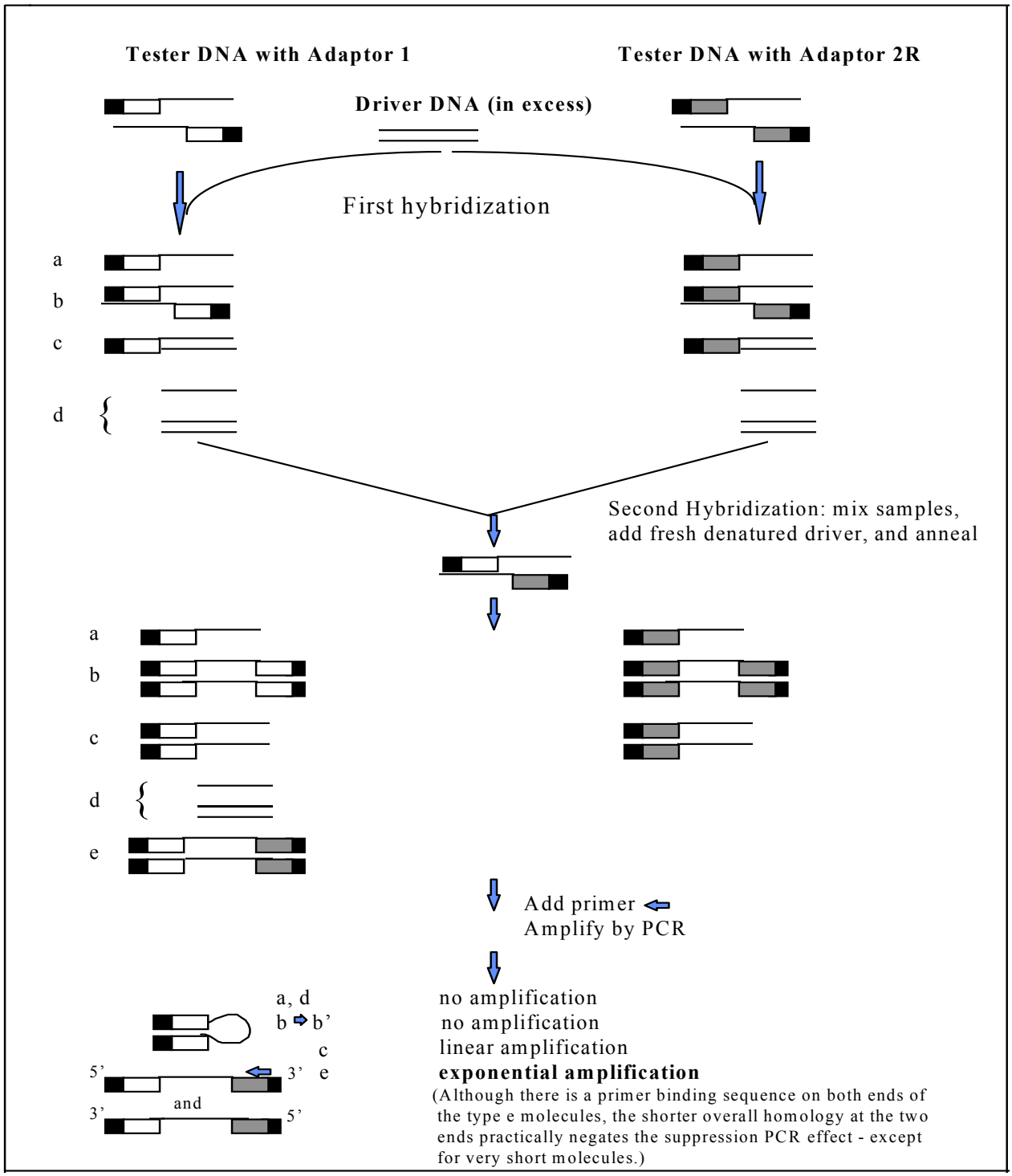


Fig. 4. Schematic diagram of PCR-Select subtraction. Type e molecules are formed only if the sequence is present in the tester DNA, but absent in the driver DNA. Single lines represent the *Rsa*I-digested DNA. Solid boxes represent the outer part of the adaptor 1 and 2R longer strands and corresponding PCR primer 1 sequence. Clear boxes represent the inner part of adaptor 1 and the corresponding Nested Primer 1 sequence; shaded boxes represent the inner part of adaptor 2R and the corresponding Nested Primer 2R sequence.

5. Enzyme activity assays

The following enzyme activity assays were carried out to determine the potential of the *Y. enterocolitica* 1B cells to degrade chitin or cellulose as alternative carbon sources *in vitro*.

5.1 Chitinase assay

LB agar plates containing the following supplements were prepared for the chitinase assays: 16 ml agar, 160 µl of 1 % trypan blue and 1.2 ml of ethylene glycol chitin solution (5 mg/ml in water). The plates were incubated at 27 °C / 37 °C overnight. Colonies of enzyme-positive transformants developed clear white halos on the blue background of the medium, because ethylene glycol chitin was hydrolyzed with the enzyme liberated from the cells due to natural autolysis (Ueda et al., 1994). Alternatively, colonies were streaked on LB agar containing 0.1 % (w/v) colloidal chitin, the plates incubated at 27 / 37 °C and the formation of halos around the colonies monitored at regular time intervals.

5.2 Detection of chitinolytic activity with Methylumbelliferyl (MU)-diacetyl-chitobioside and MU-triacetyl-chitotrioside (Sigma Aldrich, Taufkirchen)

Principle

The substrates MU-diacetyl-chitobioside and MU-triacetyl-chitotrioside comprise 1,4-N-acetyl-β-D-Glucoseamine-Dimer or Trimer bonded to the fluorescent molecule methylumbelliferyl. Chitinolytic activity results in the breakup of this bond, thus liberating the methylumbelliferyl moiety from the chitin-substrate, a reaction which is detectable by UV irradiation (Transilluminator) at 302 nm.

Procedure

4-Methylumbelliferyl-diacetyl-chitobioside (or 4 MU-triacetyl-chitotrioside)	
0.01 M Stock-solution dissolved in DMF	0.5 µl
1 x TE-Buffer	9.5 µl
Protein probe (Cell supernatant)	10 µl
0.01 N NaOH	10 µl

The reaction was carried out in a microtiter plate at RT for 20 min. Detection of chitinase activity was by UV illumination and the reaction was photographed with a video-documentation system (Sigma).

5.2 Cellulase assay

Bacterial colonies were streaked out on LB agar and following incubation at 27 °C / 37 °C, the plates were overlaid with 4 ml of soft agar (5 g/liter agarose) containing a 1 mg/ml concentration of carboxymethylcellulose. After a further incubation for 18 - 36 hours, plates were flooded with an aqueous solution of Congo red (1 mg/ml concentration) and incubated for 20 min. The Congo red solution was poured off and plates were further flooded with 1 M NaCl for 15 min. The visualized zones of hydrolysis were stabilized for up to two weeks by flooding the plates with 1 M HCl.

6. Protein biochemical studies

To analyze putative secreted proteins under various growth conditions, bacterial cells were subjected to different growth media (BHI or LB), temperatures (27 / 37 °C), acidic pH (pH 5 - 5.5) and high osmolarity (0.4 M NaCl). The bacteria were removed from cultures by centrifugation, supernatants filtered through 0.45 µm filters, and proteins precipitated with trichloroacetic acid (10 % end concentration). Analysis of total proteins was by SDS-PAGE according to standard procedures.

6.1 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 differ 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
H_2O	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 (Serova)	250 mg
50% Methanol	100 ml
10% Acetic acid	20 ml
H_2O	ad 200 ml

Destaining solution

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

The electrophoresis system from Biorad 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 cooked briefly at 95 °C for 5 - 10 min and then applied on the gels. Electrophoresis proceeded at an applied voltage of 150 V (or at 20 mA) for 1 - 2 hr.

6.2 **Western Blot** (Towbin et al., 1979)

Principle

Western blot relies on the principle of the specificity of the interaction between a protein and its cognate antibody which is visualizable by means of chemiluminiscence or autoradiography.

Reagents

1x Western-blot buffer	0.025 M Tris 0.19 M Glycine
Wash buffer	0.5 % Tween 20 (v/v) in 1x PBS
Blocking solution	5 % BSA (w/v) in wash buffer

Procedure

- Following separation of proteins in a conventional SDS-PAGE, the proteins were electrophoretically transferred to a nitrocellulose membrane (in 1x Western blot buffer) at a constant voltage of 150 - 200 V (or 350 mA) for 1 hr.
- The membrane was then incubated in blocking solution overnight in the cold and subjected to three rounds of washing with the wash buffer.
- The membrane was then incubated for 1.5 hr with the first antibody directed against the protein of interest, following which the blot was washed three times.
- The secondary antibody, conjugated with either alkaline phosphatase (AP) or peroxidase (POD) was then added (usually at a 1:5000 dilution), incubation for 1.5 hr followed, after which the membrane blot was washed three times.
- The detection of protein-antibody interaction or binding was by means of the ECL kit (Amersham Pharmacia) for peroxidase-coupled second antibody, or the BCIP / NBT-Blue liquid substrate system (Sigma) for alkaline phosphatase-coupled secondary antibody.

The Amersham Biosciences ECL Western blotting system is a chemiluminescent, non-radioactive method that makes use of a horseradish peroxidase (HRP) conjugated secondary antibody that, in conjunction with a chemiluminescent substrate, luminol, generates a signal that can be captured on film. In contrast, the BCIP / NBT system is a colorimetric antigen detection method. NBT and BCIP are two colorless substrates which form a redox system. BCIP is oxidized by alkaline phosphatase to indigo by release of a phosphate group. In

parallel, NBT is reduced to diformazan. The reaction products form a water insoluble brownish precipitate on nylon membranes.

6.3 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 26 kDa 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-ChiY fusion was constructed (using the pGEX-4T-3 vector) to facilitate the purification of the ChiY protein for the production of ChiY antibodies (see 15.1).

6.3.1 Cultivation and induction of bacteria

E. coli BL21 carrying the pGEX*chiY* fusion 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 0.1 mM IPTG, incubated further at 27 °C for 6 hr. The cells were then pelleted by centrifugation at 6000 rpm for 10 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).

6.3.2 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).

6.4 Preparative SDS-PAGE, electroelution and protein recovery

6.4.1 Preparative SDS-PAGE

The fusion protein was mixed with SDS-loading gel buffer (Laemmli) and loaded on a preparative SDS-Polacrylamide gel. The run conditions were as described previously. Following the SDS-PAGE run, the gel was stained with a 0.3 M $CuCl_2$ solution (negative staining) and the required protein band was excised from the gel with a clean scalpel.

6.4.2 Electroelution

Gel electroelution is based on the principle of migration of negatively charged protein macromolecules in an electric field to an anodic pole. Following the preparative SDS-gel described above, the excised gel band was cut into fine pieces with a clean scalpel and the gel pieces applied to the Schleicher & Schuell Biotrap electroelution equipment. Under an applied electric field, the proteins are eluted from the gel and migrate to the anodic pole. The Biotrap equipment is assembled with two membrane types, an impermeable BT1 membrane aligned at both cathodic and anodic poles, and a semi-permeable BT2 membrane inserted before the 2nd BT1 membrane at the anodic pole. As migrating proteins eluted from the gel pieces pass through the semi permeable membrane, they are trapped on the BT1 membrane, and a quick reversal of the voltage for 15 to 20 sec detaches the protein molecules from the membrane into the run buffer.

Electroelution Buffer	1x SDS-PAGE electrophoresis run buffer
Run conditions	200 V overnight

6.4.3 Protein Precipitation

After electroelution the collected proteins in solution were precipitated by a mixture of methanol, chloroform and water as outlined below.

Reaction components	200 µl protein eluate
	+ 800 µl methanol, vortexing
	+ 200 µl chloroform, vortexing
	+ 600 µl H_2O_{dest}, vortexing.

The reaction mix was then centrifuged (14,000 rpm for 10 min) and the solution separated into an interphase and an upper and lower phase. The upper and lower phases were carefully

removed and the interphase comprising the eluted proteins was washed twice with methanol. The resulting pellet was air-dried, dissolved in 8 M urea diluted to a 2 M concentration with PBS.

6.5 Rabbit immunization

The rabbit was injected three times subcutaneously, with a time period of three weeks between each immunization. Typically, 100 - 500 µg of protein was used per immunization. For the first immunization, the protein was mixed with an equal proportion of complete Freund's adjuvant. Further immunizations utilized Freund's incomplete adjuvant also in a 1:1 ratio with the protein. Ten days after every immunization, blood samples were collected from the animal. The blood was allowed to coagulate at RT for 3 - 5 hours and centrifuged for 30 min at 6000 rpm. The serum (supernatant) was then collected and stored at -20 °C.

7. Mouse experiments

Six weeks old female BALB/c mice in groups of five were infected with yersiniae orogastrically at a dosage of 5 x 10^9 bacterial colony forming units (cfus). For intravenous inoculation, 10^4 bacteria were inoculated into mice and the mice sacrificed after 2 days. Mice were monitored twice daily and forty-eight hours after infection, they were sacrificed and the average bacterial loads from infected organs (liver, spleen and Peyer's patches) extrapolated. For the intestinal lavage, 5 ml of ice-cold sterile phosphate buffered saline (PBS) was flushed through the small intestine, recovered and bacterial loads determined from it.

7.1 Mobilization of the pYV plasmid into recipient *Y. enterocolitica* strains

A prerequisite for infection by the *Yersinia* strains is the presence of the pYV plasmid. The *Y. enterocolitica* strain WA-314, commonly manipulated in our laboratory has been cured of this virulence feature and so before the mouse experiments could take off, the pYV virulence plasmid was mobilized into the strains to be monitored in mice. To achieve this, the cointegrate (comprising vector pRK290B8-5 and the pYV virulence plasmid from *Y. enterocolitica* O:8) was introduced through conjugation into the appropriate strains required for the mouse experiments (Heesemann and Laufs, 1983). For conjugation, an additional helper plasmid (pRK2013) containing *tra* genes was necessary. Conjugation was carried out with *E. coli* HB101 *E. coli* HB 101 (pRK290B8-5::pO:8) and the different *Y. enterocolitica* recipient strains (WA-314, WA-314 *yts1E* and WA-314 *chiY*).

7.2 Confirmation of the presence of the pYV plasmid

The verification of pYV plasmid into the various recipient *Y. enterocolitica* strains was accomplished as follows: PCR reactions with the primers YopP8+60F and YopP9 500R that amplify a region within the *yopP* gene (localized on the pYV plasmid) were carried out. Additionally, the existence of the pYV plasmid was confirmed by means of agglutination tests for YadA and presence of secreted Yops as described below.

7.2.1 Agglutination test

YadA is a pYV-plasmid encoded protein critical for the adhesion of *Yersinia* to host cells. YadA secretion was investigated by streaking out bacteria on blood agar, incubating plate at 27 °C overnight and then shifting to growth at 37 °C for a further 4 - 5 hours. A colony was then selected and rubbed into 10 - 15 µl of YadA antiserum previously dropped on a glass slide. Agglutination was observed as clumping of the cells into fine particles. *Y. enterocolitica* WA-314 which contains the pYV plasmid was utilized as the positive control, while WA-C, a plasmidless derivative of WA-314, was the negative control.

7.2.2 Proof of Yops - secreted proteins encoded by the pYV plasmid

Yops are known to be encoded by the pYV plasmid, hence their secretion by *Yersinia* is a confirmation that the pYV plasmid is present in the bacteria. The presence of Yops was investigated as follows:

The bacterial strains to be tested were grown overnight at 27 °C in Brain-Heart-Infusion (BHI) medium with moderate shaking. 1 ml of this overnight culture was then inoculated into 40 ml BHI-medium and then incubated with shaking at 37 °C for 90 min - 2 hr. 5 mM EGTA (a calcium chelator), 0.2 % Glucose and 15 mM $MgCl_2$ were then added and the culture incubated for a further 2 hr at 37 °C. The culture was then centrifuged (6000 rpm for 10 min at 4 °C) and the supernatant mixed with trichloroacetic acid to precipitate the released proteins (RPs). The protein pellet was washed thrice with ice-cold acetone, suspended in SDS - loading buffer, and denatured by heating at 95 °C. The protein sample was then subjected to an SDS-PAGE run.

8. Quantitative Tissue Culture Invasion (TCI) Assay

HeLa cells 229 were grown in RPMI 1640 medium, supplemented with 10 % FCS (fetal calf serum) and 2 mM Glutamine at 37 °C in a 5 % CO_2 environment. The HeLa cells were seeded in 24 - well microdilution plates one day before infection. Prior to infection, *Y. enterocolitica* WA-314 cells were inoculated into LB broth from a frozen stock, cultured overnight at 27 °C with aeration, and then diluted 1:100 into fresh medium and cultured for an additional 90 - 120 min. Following centrifugation and resuspension of the bacterial pellet in PBS, the HeLa cells were infected with approximately 10^7 bacteria. After a 2-hr incubation, the tissue culture medium was removed and the cells washed three times with PBS to remove non-adherent bacteria. Fresh tissue culture medium containing 100 µg gentamicin per ml of medium was added and the plates reincubated for another two hours. Thereafter, the medium was removed and the cell monolayers were washed twice with PBS to remove the gentamicin. The tissue cultures were then lysed with 0.2 ml of 1 % Triton X-100 per well to release intracellular bacteria. After 5 min incubation, 0.8 ml LB medium was added bringing the final concentration of Triton X-100 to 0.2 %. The suspension was then diluted and plated on Blood agar for viable cell count determination. The bacterial cfus in the initial bacterial cultures were also determined. Results were expressed as % invasion = 100 x (number of bacteria surviving gentamicin treatment / number of bacteria added).

9. Bioinformatics (Westend *et al.,* 2002 and Lesk, 2002)

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 auspices of the National Center for Biotechnology (NCBI) and is an official Sequence data bank which contains protein and nucleotide sequences from more than 55,000 different organisms. 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.

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.

10. Nucleotide sequence accession numbers: The sequences of the *Y. enterocolitica* WA-314 *yts1C-S* gene cluster and the IS*1330* gene have been submitted to the EMBL database under the accession numbers AJ344214 and AJ344215 respectively.

RESULTS

1. Construction of a library of subtracted fragments unique to *Y. enterocolitica* WA-314 and absent from the genome of NF-O

Total genomic DNA of *Y. enterocolitica* NF-O O:5 (non-pathogen) was subtracted from the genome of its high-pathogenicity counterpart, Yen WA-314 (O:8) using subtractive hybridization (see 4.12). The secondary PCR products generated were cloned into pMOS*Blue* vector and a total of 200 subtracted clones were recovered. These subtracted fragments, ranging in size from 200 - 1,500 bp were sequenced and compared at both the nucleotide and protein levels for homologies with sequences in Databanks (provided by NCBI, Sanger center and TIGR) (see 9.0). The fragments could be divided into the following categories:
1) fragments with sequence similarities to known genes from the *Yersinia* species and other organisms (67 %) 2) fragments with similarities to phages and mobile genetic elements (transposases, etc.) (13 %) and 3) fragments with unknown sequences (20 %). The summary of some significant homologies is shown in Table 4 (pg. 55).

The efficiency of the subtraction was verified by DIG-labeling of the entire pool of subtracted fragments and using this to probe *Not* I digested chromosomal DNA (Saken *et al.,* 1994) from WA-314 (tester) and NF-O (driver) in a first high stringency hybridization reaction (Fig. 5). Hybridization to a minimum of 7 *Not* I bands, ranging in size from 48 kb to 340 kb was detected for the WA-314 tester strain, while the non-pathogenic NF-O strain was negative for the presence of the subtracted fragments. The distribution of the subtracted fragments was also uniform along the chromosome of *Y. enterocolitica* WA-314 (tester), as vastly different *Not* I macrorestriction fragments of the genomic DNA hybridized to the subtracted library.

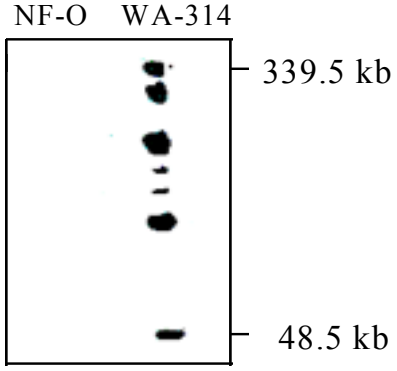


Fig. 5: Southern blot analysis confirms that the subtracted fragments are unique to *Y. enterocolitica* WA-314. Chromosomal DNA was isolated from *Y. enterocolitica* strains WA-314 and NF-O, *Not* I-digested and probed in a high stringency Southern blot reaction with DIG-labeled subtracted fragments.

In a second Southern blot reaction, chromosomal DNA from the non-pathogenic NF-O serotype was labeled and used to probe 50 (representing 25 % of the entire subtracted library)

purified subtracted DNA fragments. Five of the 50 subtracted fragments hybridized weakly to the non-pathogenic NF-O genomic DNA probe.

From the results of these high stringency Southern blots, the method of suppressive subtractive hybridization is shown to have successfully selected for tester-specific subtracted fragments, judging from the high percentage of tested fragments (45 from 50) that did not hybridize with genomic DNA from the non-pathogenic driver strain (NF-O).

2. Analysis of the subtracted library for tester-specific sequences

In order to confirm that the subtracted library was enriched for tester-specific fragments, the DNA generated from the 3rd round of subtraction was probed in high stringency PCR reactions with primers specific for the high-pathogenicity island (HPI), encoding the yersiniabactin iron-uptake system, a pathogenicity hallmark or virulence-associated locus absent in the non-pathogenic NF-O driver DNA (see Table 2 for list of primers). PCRs for four HPI genes (*irp1, intB, ybtA,* and *fyuA*) were carried out, of which all excluding *intB* gave positive signals for the expected PCR products, showing that 3 out of the 4 tested gene products were detected by subtractive hybridization. For the primer pair ye262 and 3p345, the subtracted library (SL) was negative for this PCR product. This was expected as this primer pair amplifies a 300bp locus unique to all *Y. enterocolitica* strains (in the pathogenic strains harboring the HPI, this region lies downstream of the *fyuA* gene). An efficient subtractive hybridization procedure would thus exclude this fragment from the pool of subtracted clones that are truly tester-specific. In contrast, PCR reactions probing the *irp1* (with primer pair irp 242 and irp 505) , *fyuA* (with primer pair f10 and f176) and *ybtA* (with primer pair ybtA 255 and ybtA 1012) genes, all resident on the HPI, were positive, indicating enrichment of the subtracted library for tester-specific clones.

However, in Fig. 6 it is observable that in lanes 10 & 11 the subtracted library tested negative for the PCR-product amplified with primer pair c15-205 and ybtA 815, which is specific for the HPI-*int* gene, a marker for the high-pathogenicity *Y. enterocolitica* strains. Absence of this fragment in the subtracted library points to some loss of tester-specific genes as a consequence of the subtraction procedure. In order to determine to what extent gene loss had occurred, three more tester-specific genes were examined. The subtracted library was probed with primers specific for the chromosomally encoded *ail, myf* and *inv* genes, which are known potential virulence loci unique to pathogenic *Y. enterocolitica* strains. The PCR reactions were

positive, indicating that although some gene loss may have occurred as a result of the subtraction procedure, the pool of subtracted fragments was still sufficiently enriched for tester-specific clones that could include novel virulence markers.

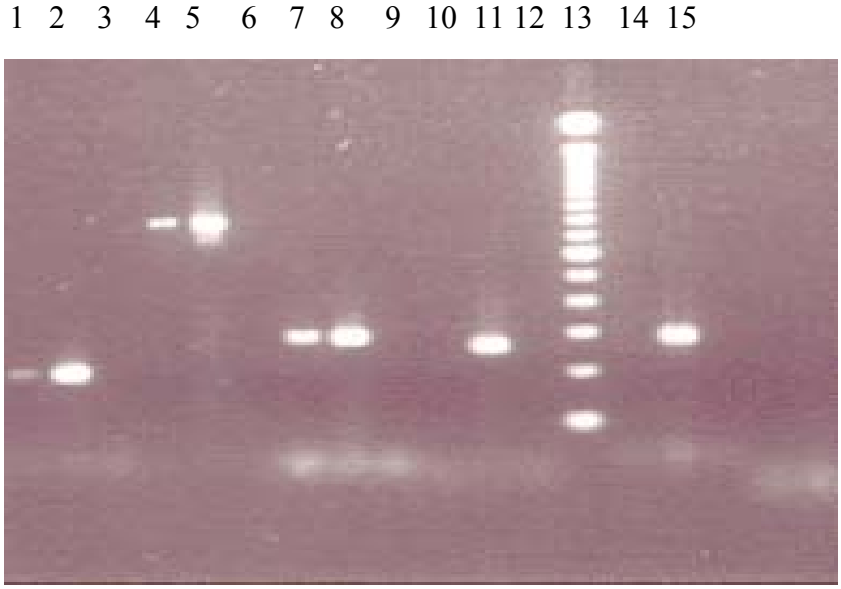


Fig. 6: PCR reaction to check for specificity of the subtracted library (SL).
In lanes 1 and 2 SL and DNA from WA-314 (+K) were probed respectively with primers f10 and f176 (specific for *fyuA* on the HPI). In lanes 4 and 5, SL and WA-314 DNA were respectively probed with primers ybtA 255 and ybtA 1012 (specific for *ybtA* on the HPI). In lanes 7 and 8, SL and WA-314 DNA were probed respectively with irp 242 and irp 505 (specific for HPI-*irp1*). In lanes 10 and 11, SL and WA-314 DNA were probed respectively with c15-205 and ybtA 815 (specific for HPI-*int*). Here the SL in lane 10 tested negative for the presence of this fragment. Lane 13: Marker. Lanes 14 and 15: SL and WA-314 DNA probed respectively with primers ye262 and 3p345 (specific for all *Y. enterocolitica* strains).

3. Sequence analysis of the subtracted fragments

The subtracted clones generated were sequenced and subjected to homology searches, at both the nucleotide and protein levels, to previously described gene and gene products in the various databanks available (Sanger, NCBI, TIGR, etc.). Some of the fragments demonstrated significant homology with sequences on the *Y. pestis* genome (75 - 90 %). Others demonstrated homology with genes encoding mobile genetic elements notably IS elements and transposases, also phage related sequences. Additionally, a number of the sequences were homologous to varying degrees with genes encoding putative virulence factors (see subtracted clones a82 and a92 below). The summary of some significant blast searches are depicted in Table 4.

Table 4: Profile of translated subtracted fragments of interest and their corresponding similarities to translated nucleotide sequences

Clone	Appr. Insert size (bp)	Homology	Identity/ Similarity
o2	650	YhgA Protein homologue in *Y. pestis* plasmid pPMTI; Probable Transposase in *Y. pestis* plasmid	150 / 214 (70%)
n35	1100	Putative transporter, permease protein in *Y. pestis*	156 / 173 (90%)
n1	400	ISRS011-Transposase ORFB protein (*Ralstonia solanacearum*) IS*150* transposase in *E. coli* Transposase for IS*1661* in *Y. pestis*	75 / 170 (44%) 75 / 172 (43%) 60 /153 (39%)
n5	650	Probable transcriptional regulator in *Pseudomonas aeruginosa* Transcriptional regulator LuxR family in *Vibrio cholerae*	26 / 73 (35%) 28 / 75 (35%)
n16	700	Putative chitinase in *V. cholerae*	75 / 145 (51%)
n27	480	Putative replication protein for prophage CP-933 T in *E. coli*	92 / 254 (40%)
n28	850	General Secretion pathway protein in *V. cholerae*, *Aeromonas hydrophila*, *Erwinia carotovora*, and *E. coli*	137 / 226 (60%)
m90	650	LysR family of transcriptional regulators (*Xylella fastidosa*)	80 / 148 (54%)
a82	500	Putative exported protein in *Y. pestis* Hemolysin related protein in *N. meningitidis*	77 / 145 (53%) 64 / 137 (46%)
a90	450	Putative flagellar assembly regulatory protein, Flk in *Y. pestis* Couples flagellar P-& L-ring assembly to flagellar morphogenesis in *S. typhimurium*	117 / 169 (69%) 64 / 158 (40%)
n41	700	IS*10* ORF of *S. flexneri* IS*10* transposase of *S. typhi*	158 / 233 (68%) 84 / 233 (36%)
a92	650	Putative adhesin / invasin (EaeH) in *E. coli*	52 / 197 (26%)

4. A novel IS*10*-like element - IS*1330* uncovered by subtractive hybridization

The subtracted fragment n41 as shown in table 4 was highly homologous to different IS*10*-like elements described for various members of the *Enterobacteriaceae*. This subtracted fragment was subsequently labeled as a probe and hybridized with the WA-314 cosmid gene library to identify hybridizing cosmids. A cosmid designated n41 was isolated and sequenced. From the sequence analysis, the subtracted fragment n41 was identified as part of a novel IS*10*-like element. This element was then designated IS*1330*.

IS*1330* has two imperfect 19-bp inverted repeats (IR) and is flanked by a 10-bp duplication of the target sequence (Fig. 7). At least one GATC methylation site is present in the IS*1330* sequence following the transcriptional start site (another one was found 36 nucleotides before the ATG start codon), which could thus influence its expression (Rasmussen *et al.,* 1994, Kleckner, 1989). The putative polypeptide, which is predicted to be a 46 kDa protein, exhibited a highly basic p*I* of 10.6, common to transposases (Galas and Chandler, 1989). Reverse transcription analysis was performed in order to determine whether IS*1330* transposase is transcribed in *Y. enterocolitica* WA-314. The result was positive, also when bacterial RNA derived from an infected HeLa cell line (simulated *in vivo* conditions) was used as template indicating possible *in vivo* transcriptability of the mobile element.

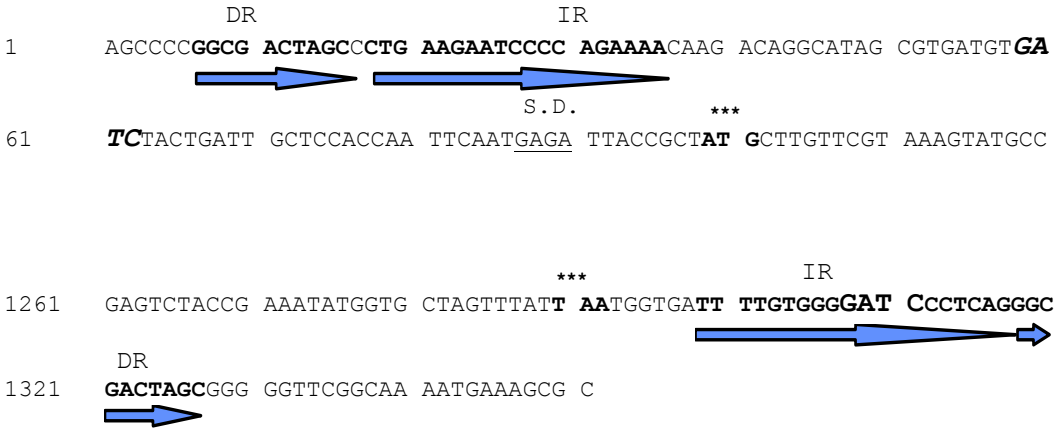


Fig 7: Partial nucleotide sequence of IS*1330*.
The positions of direct repeats (DR) and imperfect 19 bp inverted terminal repeats (IR) are indicated in bold letters and with arrows. Sites recognized by deoxyadenine methylase (GATC) are indicated in italics. Putative initiation (ATG) and termination codons (TAA) are indicated by asterisks and in bold letters, and the potential ribosome-binding site (S.D.) is underlined.

IS*1330* transposase demonstrated the highest homology (68 %) to IS*10* ORF of the large virulence plasmid pWR501 of *S. flexneri* (Venkatesan *et al.,* 2001) to IS*1999* (*tnpA*) of *P. aeruginosa* (36 %) (Stokes and Hall, 1991), and to IS*10* transposase of *S. typhi* (36 %) (Lawley *et al.,* 2000).

Southern blot analysis (Fig. 8) using the original n41 subtracted fragment (with homology to the putative IS element) as a probe against various representatives of *Yersinia* isolates showed a preponderance of this putative transposase gene among high-pathogenicity *Y. enterocolitica* strains: O:13 (6 copies), O:20 (6 copies), and O:21 (11 copies). Only a single copy of the IS*1330* gene was present in the low pathogenic isolate, *Y. enterocolitica* 108 BG 4 ST O:3, while *Y. enterocolitica* NF-O BG 1A ST O:5 (non-pathogen), and *Y. pestis* and *Y. pseudotuberculosis* strains of different serotypes were negative for this probe. Interestingly, among the two tested *Y. enterocolitica* O:8 strains WA-314 and Ye 8081, differences existed in both the copy number of the transposase fragment and their chromosomal locations. For example, WA-314 harbors four copies of this IS element with at least one copy 345 bp downstream of the chromosomal type III cluster described by Haller *et al*. (2000). *Y. enterocolitica* 8081 on the other hand carries six copies of I*S1330* sequence and no copy appears to be associated with this chromosomally encoded type III cluster, shedding interesting light on intraspecies differences that could exist between very closely related *Y. enterocolitica* strains in terms of genomic organization.

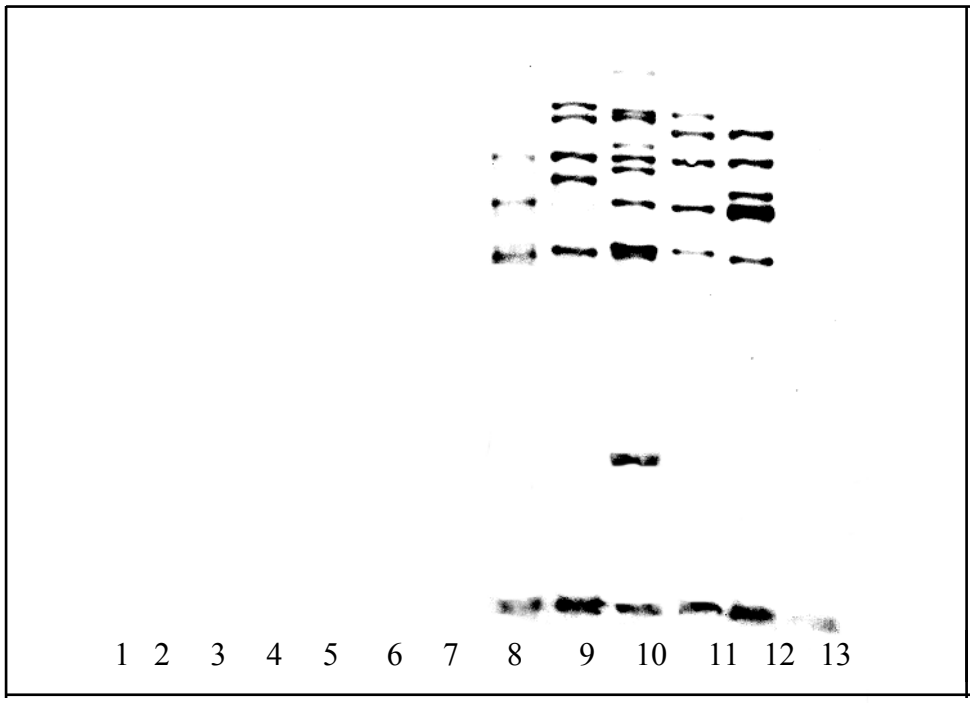


Fig. 8: Southern blot hybridization of *Eco*R I-digested bacterial chromosomal DNA with DIG-labeled IS*1330* probe.

1.*Y. pseudotuberculosis Y. pstbc* H141/84 (ST O:1a)
2. *Y. pstbc* H457/86 (ST O:2a)
3. *Y. pstbc* 346 (ST O:3)
4. *Y. pestis* (KIM)
5. *Y. pestis* (KUMA)
6. *Y. pestis* (TS)
7. Ye MRS40
8. Ye WA-314
9. Ye 8081
10. Ye 737
11. Ye 1223-75-1
12. Ye 1209-79
13. Ye 108

The result of colony blot hybridizations to identify sequences homologous to IS*1330* among other members of the *Enterobacteriaceae* family is depicted in Table 5. The results confirm that of the previous Southern blot showing apparent exclusivity of IS*1330* among high-pathogenicity *Y. enterocolitica* strains. *Y. enterocolitica* O:3 serotypes also moderately reacted with the probe but as evident from the Southern Blot may harbor only one or two copies of IS*1330* in contrast to the high-pathogenicity serotypes that harbored 4 - 11 copies.

Table 5: **Result of colony blots to determine the presence of IS*1330* among various representatives of *Enterobacteriaceae*. The *E. coli* strains are representatives of the ECOR collection.**

Organism	IS*1330* probe
Yersinia spp.	
Y. enterocolitica spp.	
Ye O:8 (WA-314)	+
Ye O:8 (8081)	+
Ye O:13	+
Ye O:20	+
Ye O:21	+
Ye O:3	+
Ye O:1,2a,3	+
Ye O:9	-
Y. frederiksenii	-
Y. intermedia;	-
Y.mollaretti	-
Y. pseudotuberculosis spp.	
Y. pstbc O:1a	-
Y.pstbc O:2a	-
Y. pstbc IP3295 (ST O:1)	-
Y. pstbc 346 (ST O:3)	-
Y. pestis spp.	
KIM (BG A)	-
KUMA (BG M)	-
TS (BG O)	-
Salmonella spp.	
S. typhimurium	-
S. typhi	-
S. arizonae	-
S. bongori	-
Shigella spp.	
S. sonnei,	-
S. flexneri	-
S. boydii	-
Proteus spp.	
P. vulgaris	-
P. mirabilis	-
Klebsiella spp.	
K. pneumoniae	-
K. oxytoca	-
E. coli	
14 ECOR strains	-

5. IS*1330* is associated with a novel *ars* operon in *Y. enterocolitica* 8081

Previously Neyt *et al*. (1997) reported on the occurrence of an arsenic resistance operon encoded by the pYV plasmids of low-virulence *Y. enterocolitica* strains. Generally, genes encoding Yop synthesis and secretion are clustered in three quadrants of the pYV plasmid. Neyt and colleagues (1997) however showed that the fourth quadrant of the plasmid of the low-virulence strains contained a new class II transposon, Tn*2502*. Tn*2502* encodes a defective transposase, suggesting that the transposition event was not a recent one, but transposition could be complemented *in trans* by Tn*2501*, another class II transposon. This transposon confers arsenite and arsenate resistance which is mediated by four genes designated *arsRBC* (homologous to the chromosomal *arsRBC* in *E. coli*) and *arsH*.

In contrast, the pYV plasmid of the high-virulence American serotypes lacks this transposon and the resident *ars* cluster. The authors therefore concluded that the *ars* genes are probably present in all serotypes of the low-virulence but not the American strains. Through analysis of the almost completed sequence genome of *Y. enterocolitica* 8081, an *ars* resistance cluster was uncovered in association with IS*1330*. This gene cluster differs considerably from that described by Neyt *et al.* (1997) both in terms of gene content and similarity. In contrast to the *ars* cluster encoded by Tn*2502*, the chromosomal *ars* cluster comprises 7 genes arranged in two divergent operons. The first operon comprises five genes, here designated *arsR, arsD, arsA*, *arsB* and *arsC*. The second comprises three genes *arsH*, a *ycjA* homologue of *S. typhimurium*, and IS*1330*. Interestingly, IS*1330* divides these two divergently transcribed operons and shares the same transcriptional direction as the *arsH* and *ycjA* homologue.

The figure below compares the arrangement of the genes on this chromosomal *ars* gene cluster associated with IS*1330* and that encoded by Tn*2502*, unique to low-virulence *Y. enterocolitica* strains. The *ars* cluster described by Neyt *et al.* shared the greatest similarity with the chromosomally encoded *ars* genes of *E. coli.* In contrast the *ars* cluster in 8081 described here, alternates in highest homology between the chromosomally encoded *ars* cluster in *E. coli* and the system encoded by the R64 plasmid of *S. typhimurium*. Generally, the 8081 *ars* genes encode proteins homologous to previously described arsenic resistance operons in gram negative bacteria. The ArsA protein which is missing in the low-virulence *Y. enterocolitica ars* operons, comprises 2 anion-transporting ATPases spanning residues 13 to 302 and 335 to 588 respectively of the predicted polypeptide.

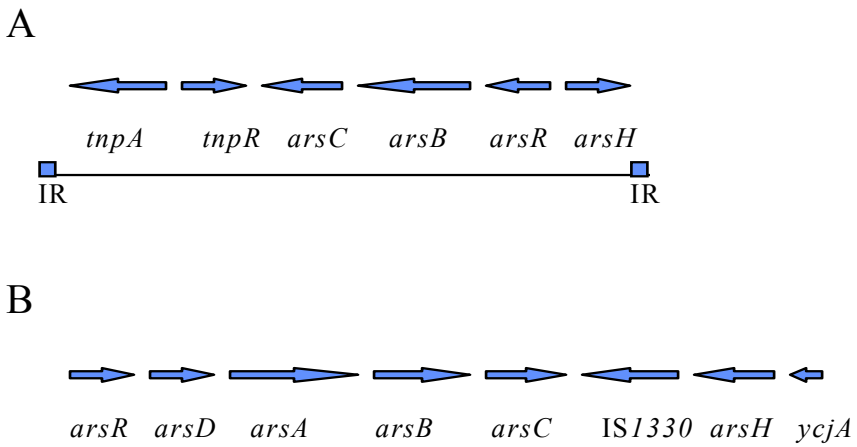


Fig. 21: **Comparison between the arsenic resistance cluster (*ars*) on the pYV plasmids of low-pathogenicity *Y. enterocolitica* isolates (A) and the chromosomally encoded *ars* cluster (B) in the high-pathogenicity 8081 strain**.

Interestingly, an IstB-like ATP binding protein spans residue 16 to 50 of ArsA. IstB is found associated with IS*21* family insertion sequences, and although its function has not been described, it may perform a transposase function. Considering that IS*1330* is also found in association with this *ars* cluster, one may be tempted to propose horizontal acquisition of these genes.

The ArsR regulatory protein encoded on the 8081 chromosome is highly homologous to the ArsR regulatory protein encoded by *E. coli*, both sharing 94 % homology with only 6 residues differently represented. This contrasts with the *ars* cluster on the low-virulence strain which shares only 70 % identity with its *E. coli* counterpart on the N-terminus, while the C-terminus of the protein is even less conserved. ArsR contains the typical DNA-binding helix-turn-helix domain and the metal binding site (ELCVCDL) which are both well conserved among this family of regulators. The 8081 *ars* cluster differs remarkably from its pYV-encoded counterpart in the low-virulence strains in harboring two additional arsenite resistance genes namely *arsA* (an ATPase) and *arsD* (a trans-acting repressor that could complement the *arsR* cis-acting repressor protein). In addition, a gene sharing 96 % homology with the *ycjA* gene encoded on the R64 plasmid of *S. typhimurium*, flanks downstream the *arsH* gene of 8081, which has no counterpart on the Tn*2502*-encoded *ars* cluster of the low-virulence strains. IS*1330* shares the same transcriptional direction as *arsH* and the *ycjA* gene homologue.

The YcjA protein shares 50 % identity with a hypothetical protein encoded on a transposable element of *Pseudomonas stutzeri*, designated ISPs1, that is a member of the ISL3 family. In *Pseudomonas stutzeri*, ISPs1 is an active transposable element which mediates activation and inactivation of methylbenzene catabolism pathways by precise transpositions or excisions from the corresponding catabolic genes. In the 8081 *ars* cluster, only vestiges of this ISPs1 transposon are remaining, and the associated IS*1330* element does not appear to inactivate any nearby genes. The 8081 *ars* cluster thus appears to be functional and its associated IS*1330* element might serve a positive regulatory function, for example activation of the downstream *arsH* gene which immediately flanks it.

Interestingly, in the work by Neyt *et al*. (1997), the high-virulence American serotype 8081 showed a level of arsenic resistance comparable to that demonstrated by the low-virulence strains, suggesting that they may harbor an *ars* operon in their chromosomes. In this work, sequence analysis of the 8081 genome identified a single copy of a chromosomally encoded arsenic resistance operon (in association with IS*1330*) that could explain the arsenite resistance phenotype of this strain observed by Neyt and colleagues.

6. Subtractive Hybridization uncovers a novel type II secretion apparatus unique to highly pathogenic *Y. enterocolitica* strains

The subtracted fragment n28 is highly homologous to the *epsE* gene of *V. cholerae*, part of the type II secretion apparatus in this organism (Sandkvist *et al.,* 1997). To determine whether this high homology was continuous and represented a novel and closely related gene cluster in *Y. enterocolitica*, a cosmid carrying the n28 fragment was isolated and subsequently sequenced. A unique genetic locus was uncovered, spanning approximately 14 kb and containing 13 open reading frames, which encodes a putative type II secretion apparatus. The genes were designated *yts1C - S* (*Yersinia* Type II Secretion 1), these letters corresponding to the generally accepted nomenclature for the type II pathway identified in different *Enterobacteriaceae* (see Fig. 9).

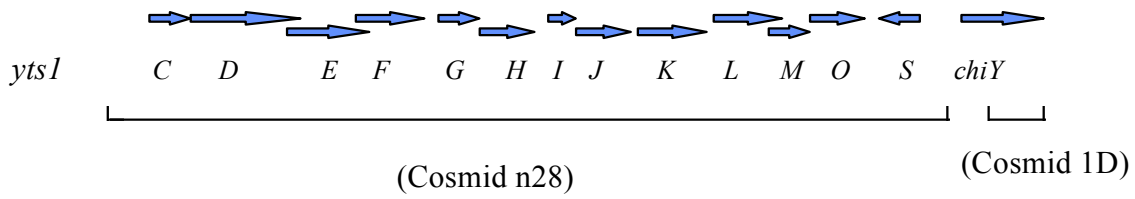


Fig. 9: Organization of the *yts1* gene cluster

Table 6 summarizes the characteristics of the genes identified in this study, which appear to be arranged in an operon module with several of the genes overlapping. In the accompanying Table 7, the location and characteristics of predicted reading frames in the sequence of the *yts1* gene cluster are detailed. The Yts1 secretion apparatus demonstrates highest homology (for example 59 % homology of Yts1E to the EpsE protein of *V. cholerae*) to the secreton of *V. cholerae* (*epsC - N*) required for outer membrane biogenesis and secretion of cholera toxin, along with a protease and a chitinase. Fig. 11 compares the organization of genes on the *yts1* secretion cluster with the homologous cluster predicted from the sequence of the *Y. pestis* genome and that encoded by *V. cholerae*. The similarity to other homologous secretons was highest for *yts1D - G*, an observation that confirms previous findings (Sandkvist *et al*., 2000) that the *D, E, F* and *G* genes typically share the highest identity among the species (60 to 80 %) with comparatively less homology (25 to 40 %) among the other genes. The putative *yts1* cluster here described lacks an *N* gene, in contrast to its counterpart in *V. cholerae*. However many functional type II clusters described to date do not possess an N gene, which several studies have shown not to be essential for protein secretion (Sandkvist *et al*., 2000).

Table 6: Genes of the *yts1* type II secretion cluster of *Y. enterocolitica* O:8 identified in this study

yts1 gene	Homology[x]	Potential location[y]	Putative function[y]
C	*epsC* (27 %)	im/om	putative energy transducer
D	*yheF* of *E. coli* (54 %)	om	om channel
E	*epsE* (59 %)	c	energy transducer
F	*epsF* (38 %)	im	unknown
G	*hofg* of *E. coli* (70 %)	im/om	pseudopilin
H	*xcpU* of *P. aeruginosa* (28 %)	im/om	pseudopilin
I	*epsI* (28 %)	im/om	pseudopilin
J	*epsJ* (30 %)	im/om	pseudopilin
K	*epsK* (28 %)	im/om	unknown
L	*yheK* of *E. coli* (23 %)	im/om	unknown
M	*xcpZ* of *P. aeruginosa* (32 %)	im	unknown
O	*PilD* (36 %)	om	leader peptidase
S	*outS* of *E. chrysanthemi* (35 %)	om	chaperone for Yts D

x. Unless otherwise indicated, the genes *epsC-M*, *PilD* comprise the secreton of *V. cholerae*.
y. Based on homology to known secreton proteins.
Inner membrane (im), Outer membrane (om), and c (cytoplasm)

Table 7: Location and characteristics of predicted Yts1 reading frames in the sequence of the *yts1C - S* cluster.

Protein	ORF (bp)	No. of amino acid residues	Predicted mol wt.
Yts1C	3785-4327	180	20.1
Yts1D	4396-6372	658	71.6
Yts1E	6369-7865	498	55.4
Yts1F	7862-9079	405	45.2
Yts1G	9111-9578	155	17
Yts1H	9578-10147	189	21
Yts1I	10153-10515	120	13.7
Yts1J	10512-11111	199	22.9
Yts1K	11101-12138	345	38.8
Yts1L	12140-13339	399	44.6
Yts1M	13326-13820	164	18.5
Yts1O	13817-14614	265	30
Yts1S*	1960-2361	133	15.1
ChiY	15455-16939	494	53.4

* The coding sequence is on the antiparallel strand.

The Southern blot as shown in Fig. 10 was carried out to determine the distribution of this putative transport cluster among different yersiniae. As seen in the blot, the *yts1* genes appear to be exclusive to the high-pathogenicity *Y. enterocolitica* strains (Yen ST O:8, O:13, O:20

and O:21). The tested low pathogenic isolates (Yen ST O:3 and ST O:9) and several *Y. pestis* and *Y. pseudotuberculosis* strains did not hybridize to the *yts1E* probe. This observation was consistent with results from high stringency PCR reactions employing primers for several genes on the cluster: among the different yersiniae, only the high-pathogenicity *Y. enterocolitica* strains tested positive.

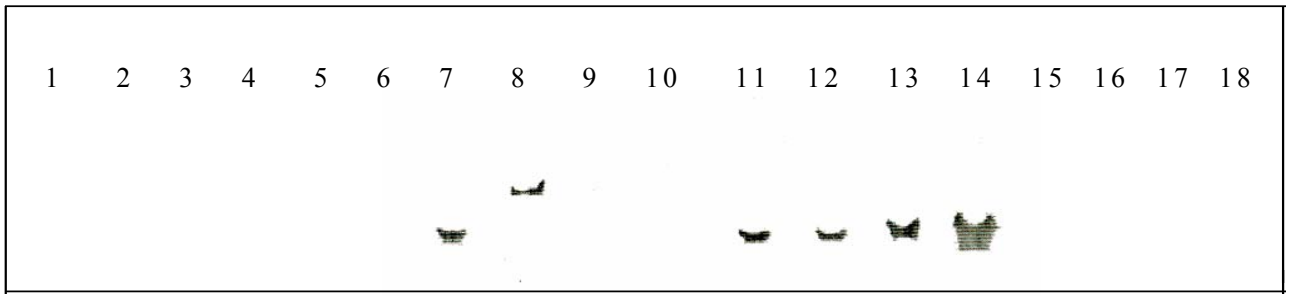


Fig. 10: The distribution of *yts1* secretion cluster among different *Yersinia* species determined by Southern Blot hybridization employing *yts1E* as a probe.

1. *Y. pestis* TS
2. *Y. pestis* KIM
3. *Y. pestis* KUMA
4. *Y. pseudotuberculosis* O:1a
5. *Y. pseudotuberculosis* O:2a
6. *Y. pseudotuberculosis* O:3
7. *Y. enterocolitica* O:8 (WA-314)
8. *Y.enterocolitica* WA-314 *yts1E*::Kan^r
9. *Y. enterocolitica* O:5 (NF-O)
10. *Y. enterocolitica* O:9
11. *Y. enterocolitica* O:8 (8081)
12. *Y. enterocolitica* O:21
13. *Y. enterocolitica* O:20
14. *Y. enterocolitica* O:13
15. *Y. enterocolitica* O:9
16. *Y. enterocolitica* O:3
17. *E. coli* C-4441
18. *E. coli* 12860

7. *yts2* - a species-specific type II secretion cluster

7.1 The *yts2* gene cluster

In order to identify further putative secretion clusters that would uniformly cut across the *Y. enterocolitica* strains without preference to the pathogenic species, the almost completed sequence genome of *Y. enterocolitica* 8081 was analyzed. A search in the Sanger center *Y. enterocolitica* GeneBank revealed a putative second secretion cluster designated here as *yts2* (*Yersinia* type II secretion 2, see Fig. 11). Genomic probing of representative *Yersinia* strains by PCR reactions revealed the presence of this putative type II secretion cluster among all tested *Y. enterocolitica* strains, namely non-pathogenic, low-pathogenicity and high-pathogenicity strains alike, while *Y. pseudotuberculosis* and *Y. pestis* tested negative for the presence of the genes of this cluster. Reverse transcription analysis of the *yts2D* and *E* genes revealed positive transcripts, indicating *in vitro* transcription of the genes on this second type

II secretion cluster. The ORFs encoded by this cluster bear homology to ORFs of previously described type II secretion clusters except for the absence of some proteins, notably homologues of the H, M and S proteins. Figure 11 presents an overview of the two type II secretion clusters identified in this study and their comparison with other closely related systems in *V. cholerae*, *E. coli* and *Y. pestis*.

7.2 Comparison of *yts1* and *yts2*

The G+C content of the *yts2* cluster differs from that of the *yts1* cluster (restricted to high-pathogenicity strains) having a 33 % versus a 48 % G+C content respectively. Strikingly, the *yts2* cluster shared the greatest homology (50 - 60 %) with the type II secretion genes (designated in Fig.11 as *typIIYP*) identified on the *Y. pestis* CO-92 genome. The G+C content of the *Y. pestis typIIYP* cluster was equally unusually low (34 %), however only the *M* gene was absent from this cluster, while the *H* and *S* gene homologues were, in contrast to the *yts2* cluster, present. A comparison of the novel *yts1* and *yts2* secretion clusters with other closely related secretons is shown in Fig. 11, while Fig. 12 shows the distribution of these two clusters among *Yersinia* species through high-fidelity PCR reactions. In Fig. 12a, the *yts1* secretion cluster is probed with the *yts1D* (chosen as representative of the *yts1* cluster) gene primers (primer pair yts1D.for and yts1D.rev). The expected 200 bp *D* gene-PCR product was observed to be present only in the high-pathogenicity *Y. enterocolitica* strains tested, confirming the result of the Southern blot presented earlier (Fig. 10). In contrast, in the second PCR reaction depicted in Fig. 12b, the *yts2D* gene was probed using primer pair 2D.for and 2D.rev. The expected 250bp PCR product was found to be present in all tested *Y. enterocolitica* strains, indicating that the *yts2* cluster is species-specific and is uniformly distributed among all *Y. enterocolitica* strains. The *Y. pestis and Y. pseudotuberculosis* strains probed tested negative for the presence of the *yts2D* gene.

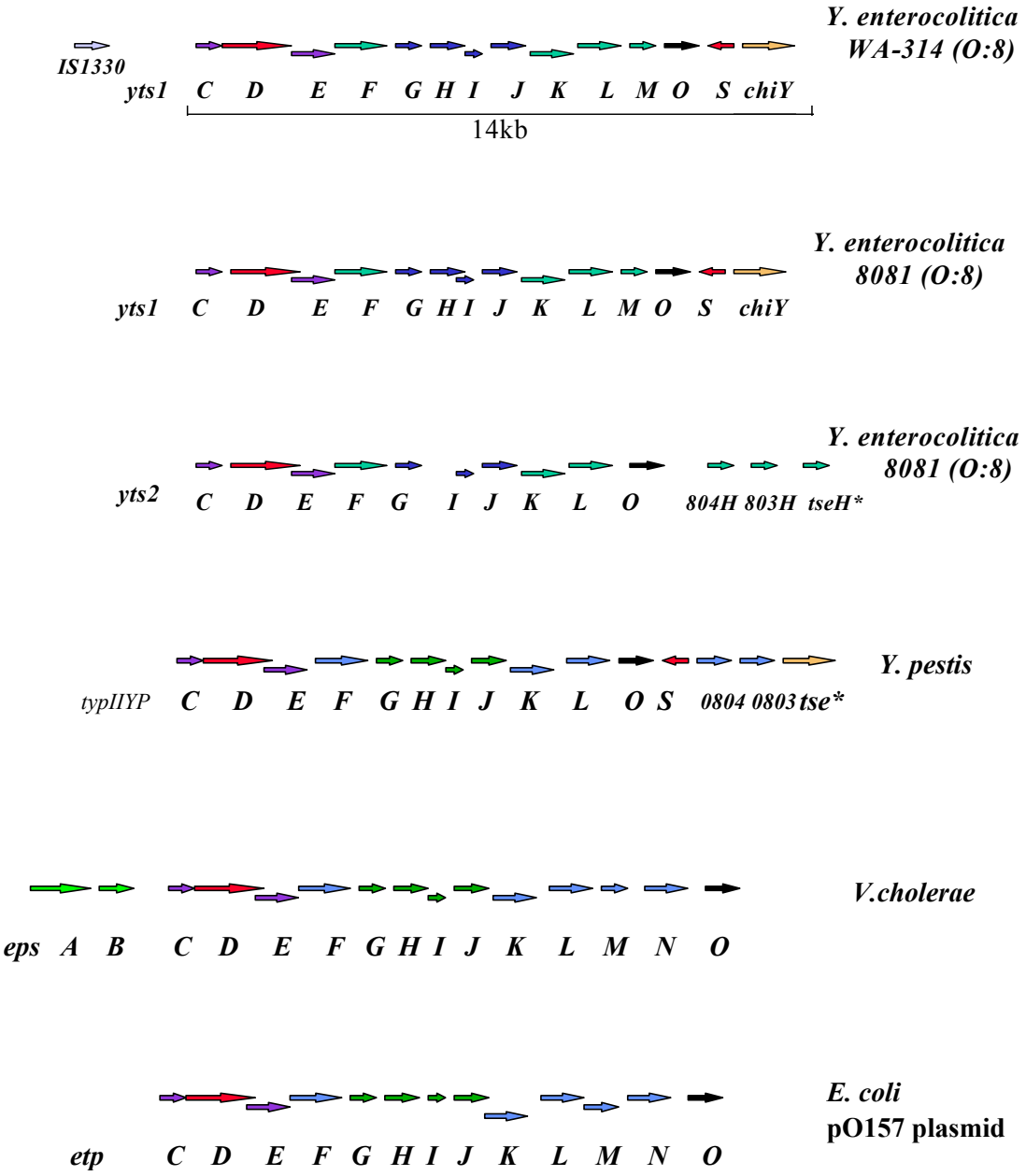


Fig. 11: The *yts1* gene cluster of the high-pathogenicity *Y. enterocolitica* biotype 1B strains and its comparison to related secretons: the *yts2* cluster of *Y. enterocolitica* species, the *eps* cluster in *V. cholerae*, the *etp* cluster in *E. coli*, and the secreton of *Y. pestis* (here designated *typIIYP*).

0804 & 0803 : correspond to ORFs predicted from the genome sequence of*Y. pestis.*
804H & 803H : Homologues of 0804 and 0803 on the*Y. enterocolitica* genome.
**tseH* : Homologue of the *Y. pestis tse* gene on the *Y. enterocolitica* genome.
**tse* : serine-sensor receptor.

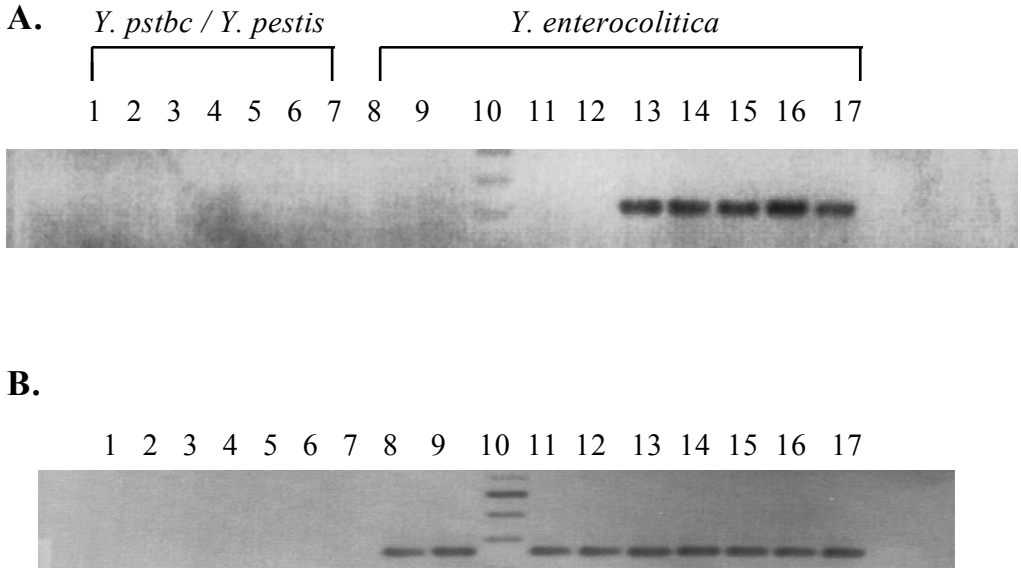


Fig. 12: Presence of A. the *yts1D* and B. the *yts2D* genes among different *Yersinia* representatives. PCR was performed on purified chromosomal DNA using oligonucleotide primers specific for the *yts1D* (primer pair yts1D.for and yts1D.rev) and *yts2D* (primer pair 2D.for and 2D.rev) genes respectively.

1. *Y. pstbc* H141/84
2. *Y. pstbc* H457/86
3. *Y. pstbc* IP3295
4. *Y. pstbc* 346
5. *Y. pestis* KIM
6. *Y. pestis* KUMA
7. *Y. pestis* TS
8. *Y. enterocolitica* NF-O (O:5)
9. *Y. enterocolitica* (O:9)
10. 100 bp ladder
11. *Y. enterocolitica* O:3
12. *Y. enterocolitica* O:9 (MRS40)
13. *Y. enterocolitica* O:8 (WA-314)
14. *Y. enterocolitica* O:8 (8081)
15. *Y. enterocolitica* O:13
16. *Y. enterocolitica* O:20
17. *Y. enterocolitica* O:21

8. Reverse transcription analysis of the *yts1* and *yts2* genes

Reverse transcription analysis was carried out to determine if the genes on the putative *yts1* and *yts2* clusters are transcribed. Because some virulence genes of the *Yersinia* species come under thermoregulation and are preferentially transcribed at 37 °C, bacterial cells were grown at two different temperatures, 27 °C and 37 °C and total RNA isolated from them was probed in reverse transcription analyses for the *yts1D* and *yts2D* genes. The *yts1D* and *yts2D* genes both gave positive transcripts indicating *in vitro* transcriptability of the genes. When bacterial RNA isolated from an infected HeLa cell line (see 4.8.4, page 28) was probed in the analysis, the tested genes also yielded positive transcripts, indicating possible *in vivo* transcription of the genes. However transcription of the genes showed some temperature dependency with regard to the abundance of the mRNA transcripts. For example, at 27 °C, the PCR product was very minimal for the *yts1D* gene following PCR amplification of cDNA corresponding to the *yts1* mRNA transcript. At 37 °C however, the *yts1D* gene was strongly transcribed as evident from Fig. 13 below. In contrast the *yts2D* gene was maximally transcribed at 27 °C while basal transcription occurred at 37 °C (Fig. 13). As positive controls, the *ail* gene (which is preferentially transcribed at 37 °C) and the *inv* gene (that is preferentially transcribed at 27 °C) were probed in parallel for transcription.

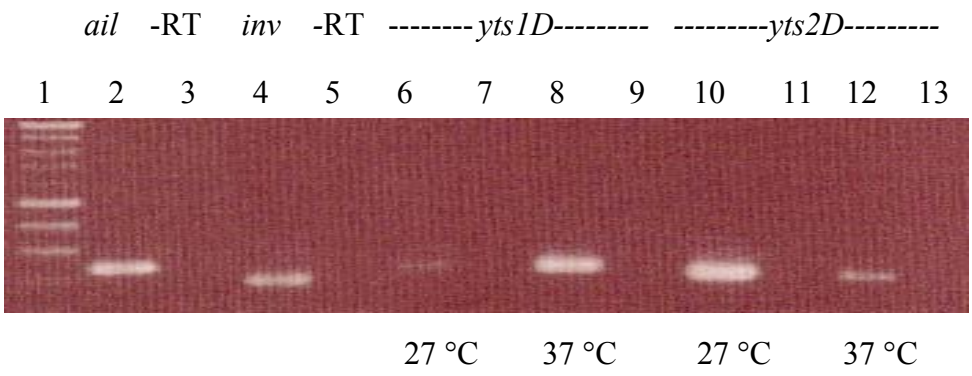


Fig. 13: Reverse transcription analysis of the *yts1D* (probed with the yts1D.rev primer) and *yts2D* (probed with the primer 2D.rev) genes at 27 °C and at 37 °C.

1. Marker
2. *ail* 37 °C*
3. ail -RT (negative control, without reverse transcriptase)
4. *inv* 27 °C*
5. *inv* -RT (negative control, without reverse transcriptase)
6. *yts1D* at 27 °C
7. *yts1D* at 27 °C (negative control, without reverse transcriptase)
8. *yts1D* at 37 °C
9. *yts1D* at 37 °C (negative control, without reverse transcriptase)
10. *yts2D* at 27 °C
11. *yts2D* at 27 °C (negative control, without reverse transcriptase)
12. *yts2D* at 37 °C
13. *yts2D* at 37 °C (negative control, without reverse transcriptase).

*The *ail* and *inv* genes which are maximally transcribed at 37 °C and 27 °C respectively were probed in parallel as positive controls for the reverse transcription analyses.

9. A putative chitin-binding protein flanks downstream the Yts1 secreton

Flanking downstream the *yts1* gene cluster is a gene which could encode a putative chitin-binding protein (designated ChiY in this work). Two chitin-binding domains were identified on the predicted polypeptide. The first spans residues 7 - 199 and is also found associated with a variety of cellulose binding domains but is primarily a chitin-binding domain. The second is a short carbohydrate / chitin binding domain spanning residues 435 to 487 with six aromatic groups that may be important for binding (NCBI conserved domain search). Additionally, a truncated ATP / GTP-binding site motif A was identified between residues 393 - 400.

ChiY demonstrated significant homology (35 % amino acid identity) to a putative chitinase encoded by *V. cholerae*. No catalytic domain could however be identified on the putative ChiY protein. Thus, when wild type *Yersinia enterocolitica* WA-314 cells were subjected to chitinolytic and cellulolytic assays, no enzymatic activity was detectable. However, a

recombinant ChiY protein could bind chitin (see 15.2, pg. 87). ChiY is a likely substrate of the *yts1* secretion cluster because the encoding *chiY* gene is immediately downstream of *yts1*.

10. Construction *of Y. enterocolitica* mutants

The suicide vector pKAS32 was used for the generation of all mutants. Typically the gene to be mutagenized was ligated with a kanamycin cassette amplified from pUC4K and this construct was ligated with pKAS32. pKAS32 contains the *E. coli rpsL* gene which encodes the dominant ribosomal protein S12. Insertion of the suicide vector into the chromosome results in a Sm^s (streptomycin sensitive) phenotype of a formerly Sm^r (streptomycin resistant) strain. (Skorupski and Taylor, 1996). Kan^r (kanamycin resistance) Sm^r arose after an allelic exchange (double crossover) had taken place and the vector was lost.

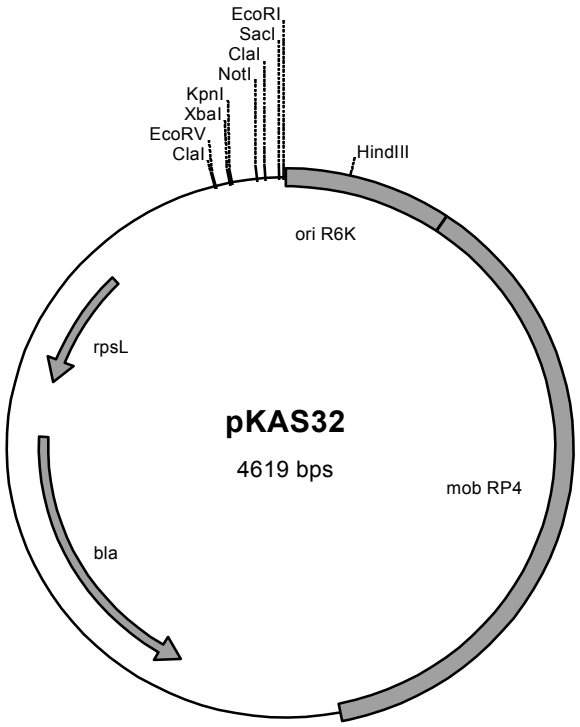


Fig. 14: Vector map of pKAS32 with recognition sites for restriction enzymes

E. coli S17-1 λpir cells were used for stable maintenance of the plasmid because they produce the π protein essential for the replication functions of pKAS32 (pKAS32 carries a π-dependent R6K *ori*). Transfer of recombinant pKAS32 was by conjugation between S17-1 λpir as donor with *Y. enterocolitica* WA-CS (a spontaneous streptomycin resistant strain) as recipient.

10.1 WA-CS *yts1E*::Kan^r

The *yts1C-S* gene cluster shares the highest homology to a type II secretion system identified in various members of the *Enterobacteriacea*e. This transport cluster has been implicated in protein secretion to the extracellular milieu. In order to determine if the *yts1* secretion cluster secretes extracellular proteins, a *yts1E* insertion mutant of the secretion apparatus was constructed. The profile of secreted proteins by the wild type and WA-314 *yts1E* mutant were then compared on SDS-PAGE gels.

Procedure for *yts1E* insertional inactivation

The *yts1E* gene encodes a putative energy transducer essential for protein secretion. To inactivate this gene, insertional mutagenesis was employed through kanamycin-inactivation. A plasmid derivative of Supercos1, designated ScF in this work, was used for initial cloning and mutagenesis of the desired gene. To generate ScF, the Supercos 1 cosmid vector was *Bam*H I - *Bgl* II digested, yielding two DNA fragments of 3.7 and 4 kb respectively. The 4 kb fragment, carrying the plasmid *ori* and the *bla* gene for ampicillin resistance, was purified from gel and self-ligated to yield plasmid ScF.

A 1.2 kb *yts1E* internal fragment was PCR-amplified from *Y. enterocolitica* WA-314 (primer pair n28orf.eco / n28orf.rev employed) with flanking *Eco*R I-*Not* I sites. This PCR product was ligated with plasmid ScF described above to yield ScF2. Utilizing the *Bam*H I site on the 1.2 kb *yts1E* insert, the ScF2 was *Bam*H I digested and ligated with a *Bam*H I-digested kanamycin cassette with transcriptional terminator (amplified from the pUC4K plasmid with primer pair aph 157 and aph 606, see Table 2) to yield ScF2.2. This ScF2.2 was then *Eco*R I-*Not* I digested to yield *yts1E*::Kan^r that was ligated in the next step with the *Eco*R I/*Not* I digested suicide vector pKAS32. The pKAS32 carrying the mutagenized *yts1E* insert was introduced into S17-1 λpir through electroporation and transconjugants selected on ampicillin-containing plates. The mutagenized *yts1E* gene was subsequently introduced into streptomycin resistant Yen WA-314 through conjugation. Selection of transconjugants on nalidixic acid, streptomycin and kanamycin, indicated strains that had excised the vector sequences. The mutation was confirmed through PCR, sequencing and Southern blot reactions. For the PCR reaction, the primer pair aph 157 and aph 606 (specific for the kanamycin cassette) were employed coupled with primer combinations aph 157 and the gene specific primers

n28orf.eco (forward) and n28orf.rev in two separate reactions and primer combinations aph 606 and the gene specific forward and reverse primers in two separate reactions. The primer combination n28orf.eco and aph 606 reverse, also primers n28orf.rev and aph 157 yielded products of approximately 1.2 and 1.7 kb respectively, as predicted from the sequence analysis. These products were subsequently purified and sequenced to confirm the DNA junctions between the kanamycin cassette and the *yts1E* insert.

For the confirmatory Southern blot reactions, chromosomal DNA from both wildtype WA-314 and mutant WA-314 *yts1E*::Kan^r cells were isolated, subjected to *Eco*R I digestion and the blotted DNA was hybridized with the 1.2 kb *yts1E* insert as probe. The results, depicted in Fig. 16 show a higher molecular weight DNA hybridizing with the probe in the case of the *yts1E* mutant when compared with the wild type strain - consistent with the fact that the kanamycin cassette introduced an additional 1 kb into the mutagenized locus.

To reconstitute the *yts1E* wild type allele, the original n28 fragment was cloned into pKAS32, and the construct was introduced into *Y. enterocolitica* WA-314 *yts1E*::Kan^r strain using the procedure described above. Selection of Str^r, Kan^s and Amp^s clones indicated strains where double allelic exchange had excised the kanamycin cassette and restored the parental wild type allele. This strain was subsequently designated as reconstituted wild type. For trans-complementation, cosmid n28 or cosmid 1D (both harboring complete copies of the Yts1 secreton) were introduced into electrocompetent *Y. enterocolitica* WA-314 *yts1E* mutant using standard procedures.

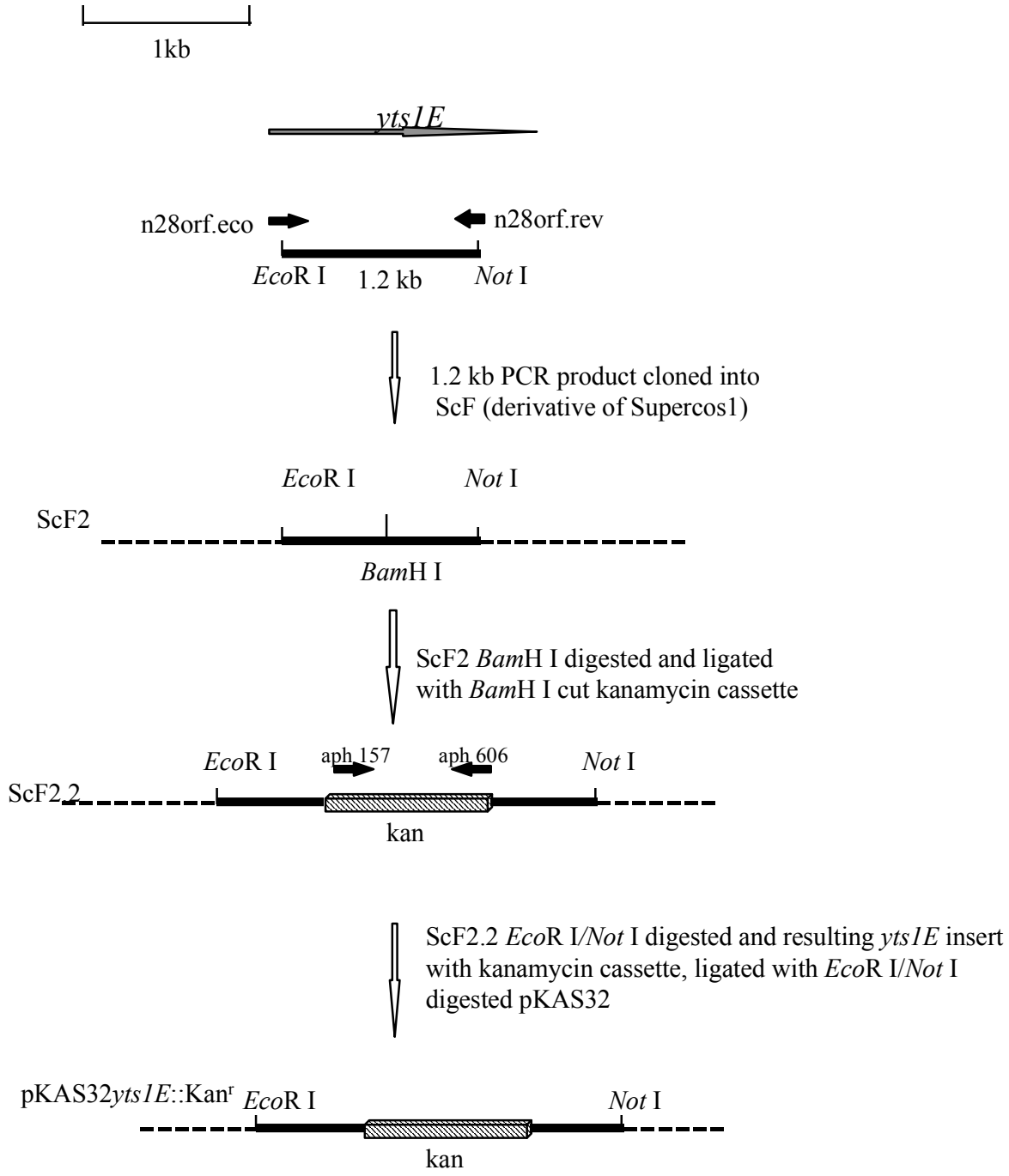


Fig. 15: Construction of the suicide vector pKAS32*yts1E*::Kan^r.
The small black arrows depict the position of the primers. The grey arrow depicts the *yts1E* gene fragment, while the striped rectangle depicts the kanamycin cassette used for gene inactivation. The solid black line denotes the subcloned *yts1E* fragment, while the vectors are displayed as dashed lines.

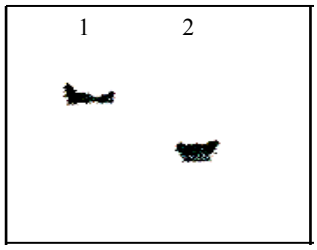


Fig. 16: Confirmation of kanamycin inactivation of the *yts1E* gene. Southern blot hybridization with the probe n28orf.eco and n28orf.rev (for the *yts1E* gene). 1.WA-C *yts1E* ::Kan^r (*Eco*R I digested) and 2. WA-C (*Eco*R I digested).

The wild type WA-314 and WA-314 *yts1E* mutant strain were then subjected to growth under different conditions:

1) LB medium

2) LB medium supplemented with salt (0.4 M NaCl)

3) BHI medium adjusted to an acidic pH of 5

The cultures were incubated either at 27 °C or at 37 °C.

No differences in protein profile were however observed between wild type and WA-314 *yts1E* mutant.

10.2 WA-CS *chiY*::Kan^r

Due to the fact that an ORF encoding a putative chitin-binding protein flanks the novel Yts1 secreton here described, it was hypothesized that the encoded ChiY protein is a likely substrate of the Yts1 secreton. In the work by Francetic *et al.,* (2000a) for example, an intact secreton was described in *E. coli* K-12 with a flanking putative chitinase as substrate.

In order to determine if the putative ChiY protein was a substrate of Yts1, the *chiY* gene was inactivated and secreted protein profiles for the wild type strain compared with the *chiY* mutant on SDS gels as was previously described for the WA-314 *yts1E* mutant (see 10.1).

For inactivation of the *chiY* gene, the sequence carrying the intact gene was PCR-amplified (with primer pair ChiYf.eco and ChiYr.not), *Eco*R I/*Not* I digested and ligated with the *Eco*R I/*Not* I digested ScF described previously (see 10.1). The resulting ScF2*chiY* plasmid was subsequently digested with *Swa* I, a blunt cutter, dephosphorylated and ligated with a *Hind* II-cut kanamycin cassette with transcriptional terminator (amplified from pUC4K) to yield ScF2.2*chiY*. *Eco*R I/*No*t I digestion of ScF2.2chiY yielded the *chiY* gene inactivated by the kanamycin cassette (*chiY*::Kan^r) which was subsequently ligated into *Eco*R I/*Not* I digested pKAS32 (yielding pKAS32*chiY*::Kan^r) and electroporated into S17-1 λpir. Conjugation with *Y. enterocolitica* WA-314 introduced the plasmid (carrying the mutagenized *chiY* gene) by homologous recombination into the chromosome, followed by double allelic exchange replacing the parental wildtype gene with the kanamycin-inactivated derivative. Confirmation of the inactivation of the *chiY* gene was by means of PCR, sequencing and Southern blot reactions. For the PCR reactions, chit.for and chit.rev specific for the *chiY* gene, and aph 157 / aph 606 for the kanamycin cassette were employed. The primer combination of chit.for and aph 606 gave a product of approximately 1.3 kb, predicted from sequence analysis.

As described in 10.1 above, the pattern of secreted proteins of the WA-314 *chiY* mutant were compared alongside the pattern for the wild type and WA-314 *yts1E* mutant. No significant differences were however observed in secreted proteins of all three strains.

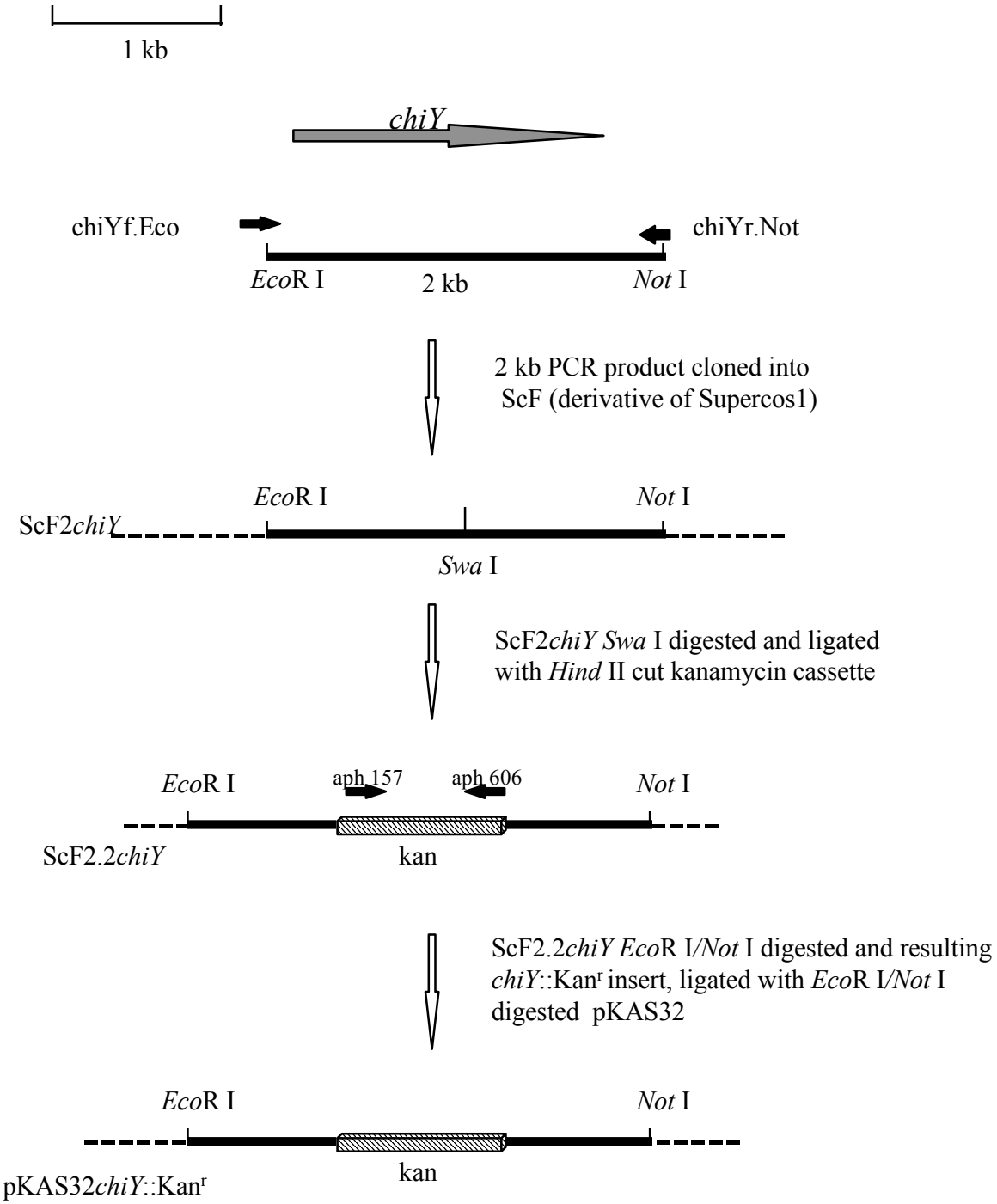


Fig. 17: Construction of the suicide vector pKAS32*chiY*::Kan^r.
The small black arrows depict the position of the primers. The grey arrow depicts the *chiY* gene fragment, while the striped rectangle depicts the kanamycin cassette used for gene inactivation. The solid black line denotes the subcloned *chiY* fragment, while the vectors are displayed as dashed lines.

10.3 WA-CS *hns*::Kan^r

Due to the fact that no significant differences in profile of secreted proteins were observed when wildtype WA-314 and WA-314 *yts1E* and WA-314 *chiY* mutants were compared (see 10.2 above), it was hypothesized that the type II secretion system might be functional only under some strict *in vivo* conditions. The *yts1* gene cluster shares reasonable homology with the generic *gsp* cluster in *E. coli*. In *E. coli* K-12, the common laboratory strain, a complete secreton was discovered which appeared to be functional. No extracellular protein secretion has however been demonstrated for these species. Recently Francetic and colleagues (2000a and 2000b) demonstrated that this secreton came under repression of the H-NS protein, a histone-like regulatory protein implicated in the global silencing of many genes involved among others in carbohydrate metabolism. In an *hns* mutant background, the genes of the secreton were upregulated and secretion of a putative chitinase was detected. It was therefore hypothesized that the *hns* gene in *Y. enterocolitica* could also play a regulatory role with regard to the *yts1* gene cluster. To test this hypothesis, the *hns* gene in *Y. enterocolitica* was inactivated.

In order to achieve this, a procedure similar to that employed for the mutants described in 10.1 and 10.2 above, namely kanamycin inactivation of the gene and its cloning into the pKAS32 suicide vector was employed. The *hns* gene (and its flanking 100 - 150 bases) was amplified with the primers hns.for and hns.rev that carry *Eco*R I and *Not* I recognition sites respectively. This PCR product was then subjected to an *Eco*R I/*Not* I double-digest and ligated with an *Eco*R I/*Not* I digest of the ScF plasmid described previously (see 10.1) to yield ScF2*hns*. Digestion of this plasmid construct with *Pma*C I, a restriction site existing naturally on the *hns* gene, and ligation with an *Hind* II-cut kanamycin cassette (amplified from pUC4K) yielded plasmid ScF2.2*hns*. *Eco*R I/*Not* I digestion of ScF2.2*hns* yielded the *hns* gene inactivated by the kanamycin cassette which was subsequently ligated into *Eco*R I/*Not* I digested pKAS32 and introduced into S17-1 λpir by electroporation. Conjugation of this S17-1 λpir transformant with *Y. enterocolitica* WA-314 transferred the mutagenized *hns* gene into the *Y. enterocolitica* cells and recombinants were screened for double allelic exchange on plates containing Nal, Tet and Sm. The inactivation of the *hns* gene was confirmed by PCR reactions and sequencing. For the confirmatory PCR reactions, primer pair aph 606 and hns.for yielded a product of approximately 1,050 bases, predicted from sequence analysis. This PCR product was subsequently purified and sequenced to confirm the ligation junction between the *hns*

gene and the interrupting kanamycin cassette. In the confirmatory Southern blot however, two bands reacted with the *hns* probe indicating that the kanamycin cassette had integrated elsewhere on the chromosome (see fig. 19).

A graphical representation of the generation of the suicide vector pKAS32*hns* is shown below.

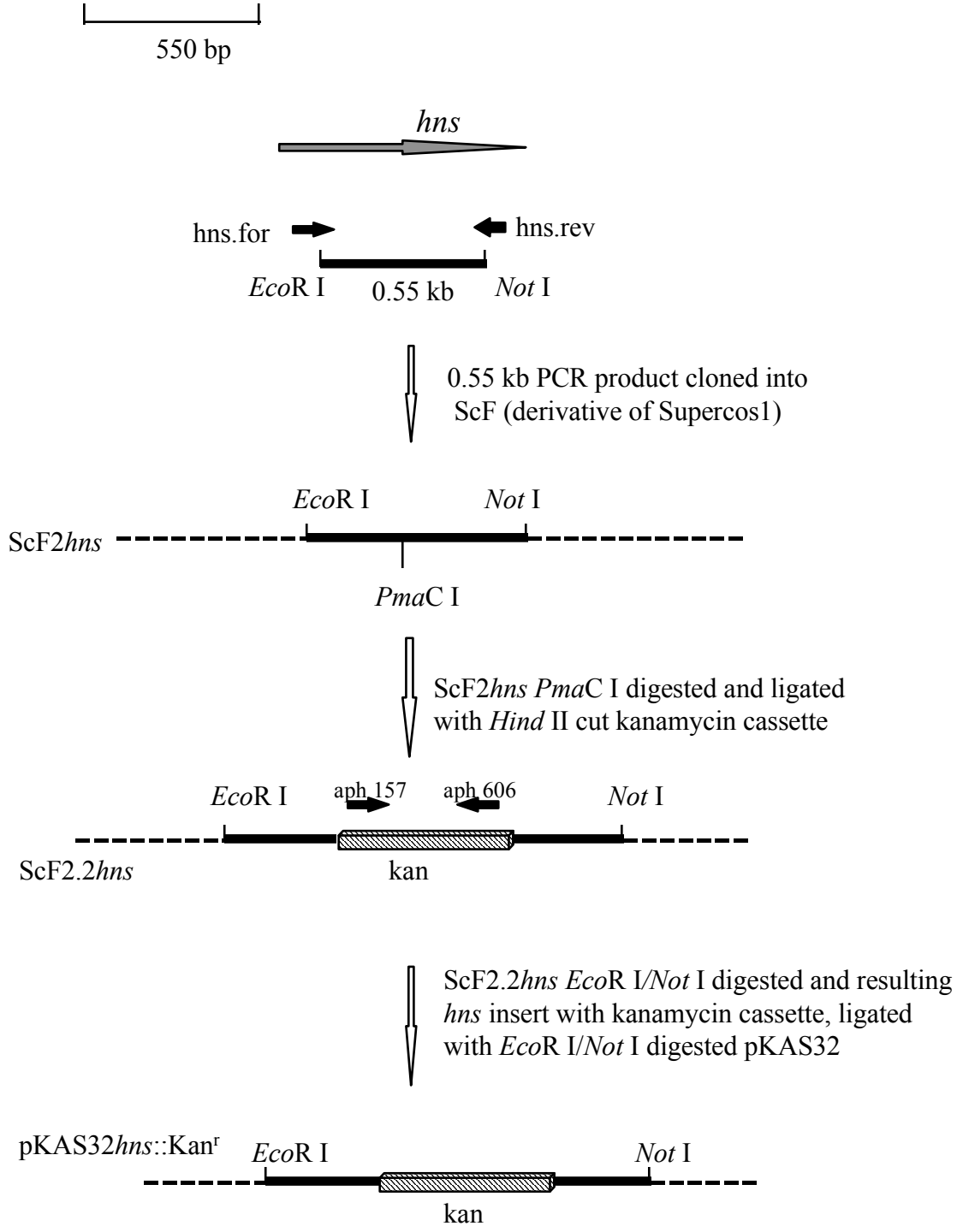


Fig. 18: Construction of the suicide vector pKAS32*hns*::Kan^r.
The small black arrows depict the position of the primers. The grey arrow depicts the *hns* gene, while the striped rectangle depicts the kanamycin cassette used for gene inactivation. The solid black line denotes the subcloned *hns* fragment, while the vectors are displayed as dashed lines.

Figure 19 below reveals the result of a Southern blot reaction to confirm insertional inactivation of the *chiY* and *hns* genes respectively. DIG-labelled DNA probes specific for the *chiY* gene (amplified with primers chit.for and chit.rev) and the *hns* gene (amplified with primers hns.for and hns.rev) were employed in two separate hybridization reactions with *Eco*R I digested chromosomal DNA from the *chiY* and *hns* mutant strains respectively. As observed from Fig. A, the *chiY* probe hybridized with a higher molecular weight band in the mutant strain corresponding to insertional inactivation by the *chiY* gene of a 1.1 kb kanamycin cassette. In contrast, the *hns* probe hybridized with two bands in the proposed *hns* mutant strain, indicating insertion of the kanamycin cassette in another locus apart from the *hns* gene.

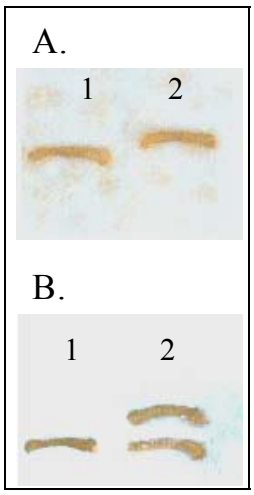


Fig. 19: Southern Blot to confirm inactivation of the *chiY* (A) and *hns* (B) genes respectively. Lane 1. *Eco*R I digested chromosomal DNA from wildtype WA-314. Lane 2. WA-CS *chiY*::Kan^r (in Fig. A) and "WA-CS *hns*::Kan^r" (in Fig. B).

11. The *yts1* secretion cluster may not involved in house-keeping functions of the harboring species

In order to determine if the genes of the *yts1* cluster are involved in some bacterial housekeeping functions, growth curves of wild type and *yts1E* mutant cells were analyzed. The result, depicted below, shows that in LB medium at 27 °C, the wild type and *yts1E* mutant were indistinguishable. At 37 °C however, the wild type was slightly more robust than the *yts1E* mutant, indicating a possible role for the Yts1 secreton when bacteria are cultured *in vitro* at 37 °C.

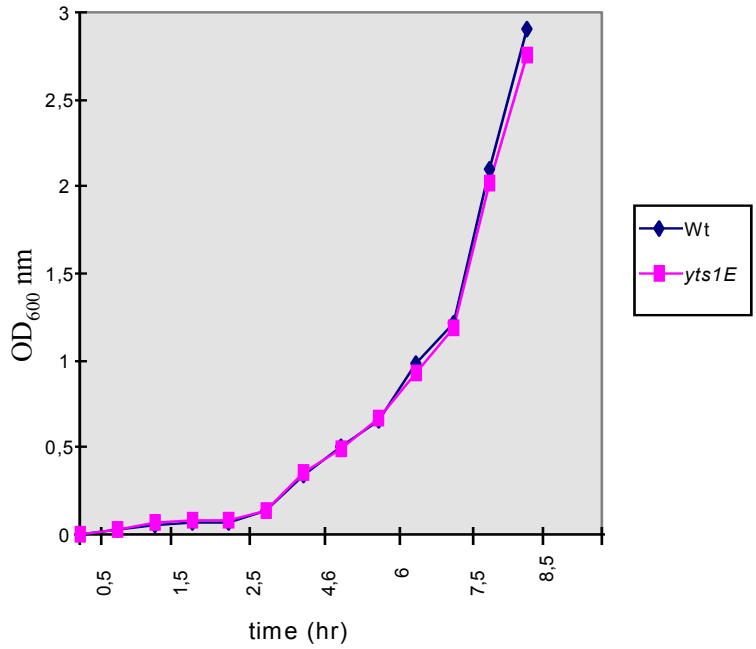


Fig. 20: Growth curve (OD_{600nm}) for WA-314 and WA-314 *yts1E* at 27 °C in LB-medium

12. Mouse virulence assays

In order to determine the impact of the Yts1 protein secretion apparatus on the virulence of strain WA-314, the average numbers of bacterial colony forming units (CFUs) recovered from infected organs were compared for wildtype and WA-314 *yts1E*::Kan^r mutant in perorally inoculated mice. Mice were orally inoculated (five per group) with 5 x 10^9 *Y. enterocolitica.* The infected mice were sacrificed forty-eight hours after infection and bacterial loads were determined by plating out the intestinal lavage (SI) and tissue homogenates from Peyer's patches (PP), liver (L), and spleen (SP). The results obtained are summarized in Fig. 20a. As shown in the figure, the bacterial CFUs recovered from the small intestine and Peyer's patches were only marginally different for wildtype WA-314 and the *yts1E* mutant. In contrast, the bacterial CFUs recovered from liver and spleen were 100 fold higher in WA-314 compared with the *yts1E* mutant, representing a significant attenuation in the *yts1E* mutant. For instance, the bacterial CFUs recovered from infected liver was 6.3 x 10^6 CFU for WA-314 compared with 5.2 x 10^4 cfu for WA-314 *yts1E*.

In a parallel experiment, survival of WA-314 *yts1E* mutant bacteria was compared with the reconstituted wild type strain (see 10.1) and the results are shown in Fig. 20b. As expected, the mutant strains presented with significant attenuation in spleen and liver, comparable to the results above. For example, 2.3 x 10^8 bacterial CFUs were recovered from infected liver for the reconstituted wild type strain compared with 1.9 x 10^6 CFUs for the *yts1E* mutant, representing a 120 fold attenuation in virulence by the mutant bacteria. In contrast, no significant differences in bacterial CFUs were observed when the intravenous route of inoculation was employed (see Table 8). For this experiment, Balb/C mice (five per group) were inoculated intravenously with 10^4 bacteria. Forty-eight hours after challenge, mice were sacrificed and bacterial load determined by plating out tissue homogenates from liver and spleen. Values are given as means ± standard deviations for five animals.

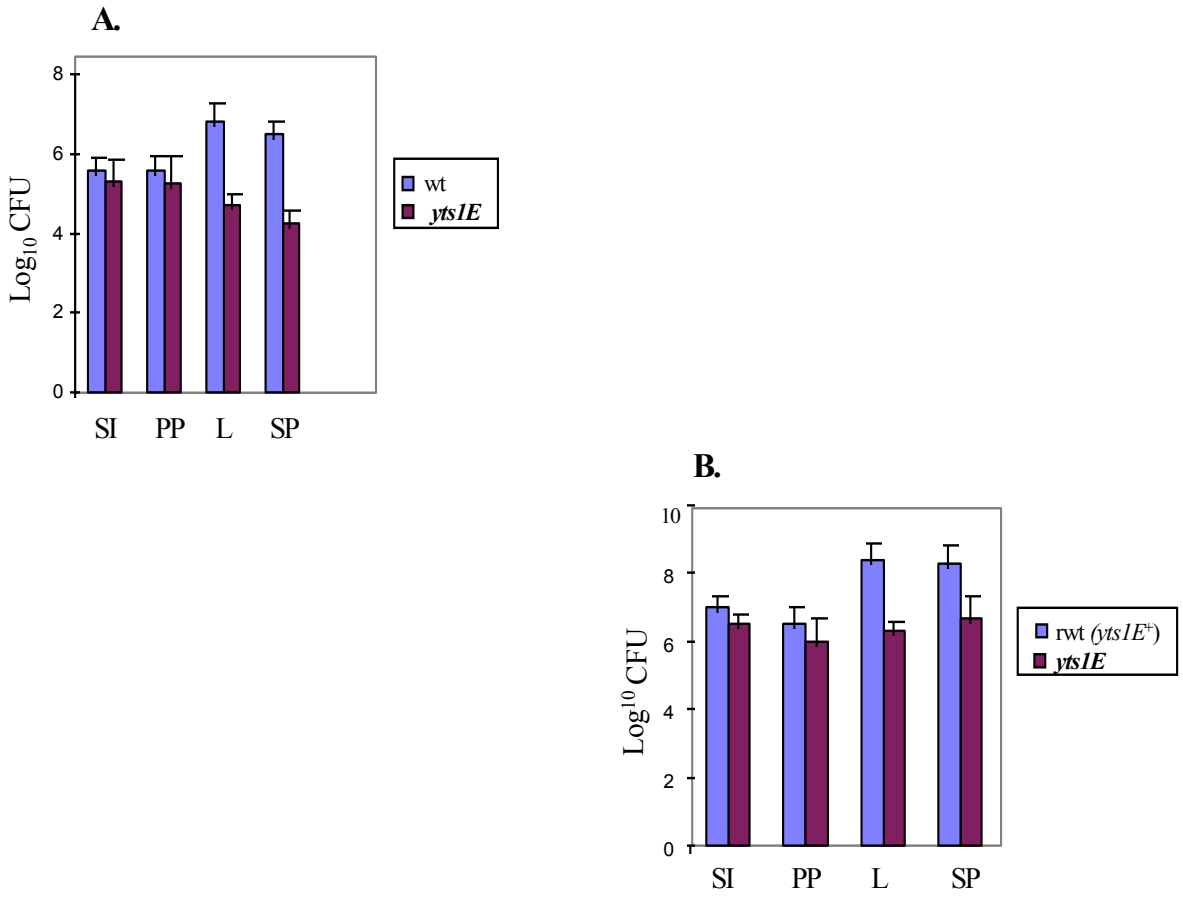


Fig. 21: Pathogenicity of the Yts1 secreton for mice (oral inoculation).
Balb/C mice (five per group) were orally challenged with 5 x 10^9 *Y. enterocolitica*. Forty-eight hours after infection, mice were sacrificed and bacterial load was determined by plating the intestinal lavage (SI) and tissue homogenates from Peyer's patches (PP), liver (L), and spleen (SP). A. Comparison of mice infected with strain WA-314 (wt) and *yts1E* mutant. B. Comparison of mice infected with reconstituted wild type strain (rwt) harboring an intact *yts1E* gene (following gene replacement of the kanamycin-inactivated *yts1E* gene with the wild type parental allele), and the *yts1E* mutant. Values are means for five animals, with standard error of the means indicated by error bars.

	WA-314	WA-314 *yts1E*
Liver	$(1.0 \pm 0.14) \times 10^7$	$(7.2 \pm 1.0) \times 10^6$
Spleen	$(7.5 \pm 0.7) \times 10^7$	$(4.5 \pm 0.2) \times 10^7$

Table 8: Mouse virulence assay result (intravenous inoculation).
Balb/C mice (five per group) were inoculated intravenously with 10^4 bacteria. Forty-eight hours after challenge, mice were sacrificed and bacterial load determined by plating tissue homogenates from liver and spleen. Values are means ± standard deviations for five animals.

13. Impact of ChiY on the virulence of *Y. enterocolitica* WA-314

In order to assess the impact of the ChiY protein, whose gene flanks the *yts1* secreton, mouse virulence assays were carried out, comparing wild type WA-314, the *yts1E* mutant, and the *chiY* insertion mutant (WA-314 *chiY*::Kan^r) in perorally inoculated mice. As was previously described, 5×10^9 bacteria were orally inoculated into female Balb/C mice. Mice were monitored twice daily and forty eight hours after infection mice were sacrificed. Preliminary results depicted below (Table 9), show significant attenuation in liver and spleen colonization by the *chiY* mutant - 1000 fold compared to the wildtype and 10 fold compared to the *yts1E* mutant.

	WA-314	WA-314 *yts1E*	WA-314 *chiY*
Small intestine	$(2.83 \pm 0.9) \times 10^5$	$(3.1 \pm 0.3) \times 10^5$	$(2.5 \pm 0.5) \times 10^5$
Peyer's patches	$(2.41 \pm 0.25) \times 10^6$	$(2.2 \pm 0.28) \times 10^6$	$(2.1 \pm 0.3) \times 10^6$
Liver	$(3.5 \pm 0.5) \times 10^5$	$(2.0 \times \pm 0.5) \times 10^3$	$(2.5 \pm 0.7) \times 10^2$
Spleen	$(3.7 \pm 0.2) \times 10^5$	$(5.3 \pm 0.4) \times 10^3$	$(4.0 \pm 0.3) \times 10^2$

Table 9: Number of bacteria in organs of infected mice three days after oral challenge.
Mice were perorally inoculated with 5×10^9 cfu bacteria. Three days after challenge, the bacterial loads in organs of infected mice were extrapolated. Values are means ± standard deviations for five animals.

In contrast, the number of surviving bacteria isolated from liver and spleen was not significantly different for the wild type and the *yts1E* and *chiY* mutants when the intravenous route of inoculation was considered, consistent with the results observed above when the wild type and *yts1E* secretion-deficient mutant were compared in intravenously inoculated mice (Table 10).

	WA- 314	WA-314 *yts1E*	WA-314 *chiY*
Liver	$(1 \pm 1.4) \times 10^7$	$(7.5 \pm 10) \times 10^6$	$(1.3 \pm 1) \times 10^7$
Spleen	$(7.5 \pm 0.7) \times 10^7$	$(4.5 \pm 1.3) \times 10^7$	$(3.8 \pm 0.9) \times 10^7$

Table 10: Numbers of CFU in organs of infected mice (intravenous inoculation).
Female Balb/C mice (five per group) were inoculated intravenously with 10^4 bacteria. Forty-eight hours after challenge, mice were sacrificed and bacterial load determined by plating tissue homogenates from liver and spleen. Values are means ± standard deviations for five animals.

14. Reporter fusion studies with *chiY-gfp*

In order to determine under what conditions the *chiY* gene product was expressed, the *chiY* promoter region was fused with the green fluorescent protein, *gfp,* reporter gene in a short translational fusion). To achieve this, 400 bases upstream of the ATG start, including the ATG start codon of *chiY* was amplified with primer pair ChiYgfp.for and ChiYgfp.rev. This PCR product, designated *chiY* promoter was *Bam*H I / *Xba* I digested and subsequently ligated with *Bam*H I / *Xba* I digested plasmid pCJFY5G3 (Jacobi, 1999). The plasmid derivative pCJFY5G3 carries a *fyuA-gfp* translational fusion. Digestion of this plasmid construct with *Bam*H I / *Xba* I and subsequent ligation of this construct with the *Bam*H I / *Xba* I digested *chiY*-promoter described above, effectively replaced the *fyuA* promoter (P*fyuA*) with the promoter region of *chiY* (P*chiY*). The resulting plasmid construct pGFP*chiY* was confirmed through PCR reactions (employing primer pair chiYgfp.for / chiYgfp.rev), restriction digestion (with *Bam*H I / *Xba* I) and through sequencing.

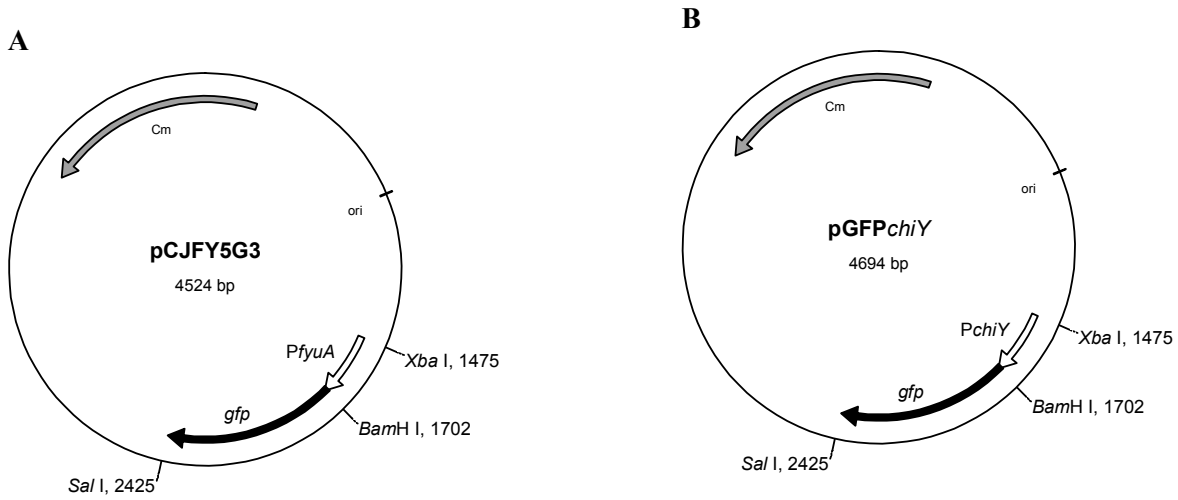


Fig. 22: Recombinant plasmids A. pCJFY5G3 (Jacobi, 1999) and B. pGFP*chiY*. The *gfp* gene is depicted by the black arrow, while P*fyuA* and P*chiY* are designated by unfilled arrows. The chloramphenicol cassette is here depicted as a grey arrow.

Plasmid pGFP*chiY* was then introduced into *Y. enterocolitica* WA-314 through standard electroporation procedures (see 4.9.1). The bacteria were cultivated under different physico-chemical conditions, listed below and subjected to GFP measurements:

- growth at 27 and 37 °C
- growth under minimal medium a) with and b) without chitin supplementation
- growth in LB medium a) with and b) without chitin supplementation
- growth under high salt conditions (0.4 M NaCl end concentration) a) with and b) without chitin supplementation
- growth in NB medium (with iron source) and in NBD (iron-deficient) medium
- growth at acidic pH of 5 (in BHI medium).

The GFP-measurement was minimal under all tested conditions, although a slightly elevated expression was observed when growth under minimal medium with chitin supplementation was considered. The very minimal GFP expression observed under all tested conditions seems to indicate that the optimal conditions for expression of the *chiY* gene product had not been met and future studies should elucidate the mechanism of regulation of the Yts1 secreton and the flanking chitin-binding protein ChiY, a putative substrate of the type II secretion system.

15. ChiY-antibody production and assessment of chitin-binding activity

When wild type and the *yts1E* secretion deficient mutant were cultivated in LB medium with or without supplementation with NaCl, a faint 55 kDa polypeptide was discernible in wild type strains when secreted proteins concentrated from culture supernanats were loaded on SDS-gels. This protein was sometimes absent and was detectable only by means of silver staining. Because this band size matches the predicted polypeptide from the *chiY* gene, which lies in close proximity to the *yts1* secreton, it was hypothesized that this protein band is the ChiY protein. However, secretion of this protein was always very minimal and could not be identified through mass spectrometry or n-terminal sequencing. Antibody production to the ChiY protein was therefore essential in identifying this protein band which sometimes was weakly expressed from culture supernatants of WA-314 and was always absent from culture supernatants of the *yts1E* and *chiY* mutant strains. To achieve this, a recombinant GST-ChiY fusion protein was generated for maximum expression of the ChiY protein, essential for antibody production. The initial western blot utilizing the produced ChiY antiserum showed

the antibody reacting unspecifically with other proteins. The result of affinity purification needed to improve specificity of the antiserum will be available later.

15.1 Recombinant fusion between GST and ChiY

The GST vector pGEX-4T-3 was employed for this fusion. 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*H I and *Not* I recognition sites present on the MCS of pGEX-4T-3, the *chiY* gene (also carrying compatible *Bam*H I/*Xba* I sites) was introduced into the vector generating plasmid pGEX*chiY*) so that an N-terminal GST-ChiY fusion was achieved. The vector was then introduced into the *E. coli* expression strain BL21 and the expression from *gst-chiY* induced with IPTG. The induced cultures presented with a strong 80 kDa band (absent in the non-induced culture), consistent with the mass predicted for the fusion, comprising 26 kDa of GST and 55 kDa from the ChiY protein.

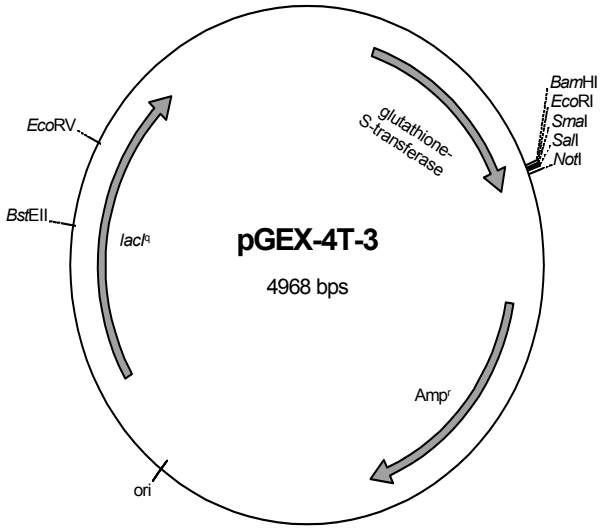


Fig. 23: Vector map of pGEX-4T-3

In order to recover considerably great amounts of the GST-ChiY fusion protein for antibody production, 2 x 2 L IPTG-induced cultures of BL21 (pGEX*chiY*) were subjected to French Press. After centrifugation of the cell lysates, the supernatant containing soluble protein fractions was purified with Glutathione Sepharose beads (Pharmacia) which have a strong affinity for the GST protein. The protein was then loaded on preparative SDS-gels and through electroelution, about 150 µg of GST-ChiY was obtained. The immunization was carried out three times, comprising the first immunization and two accompanying boosters, each time with 70 - 80 µg of purified fusion protein.

15.2 Chitin-binding activity of the ChiY protein

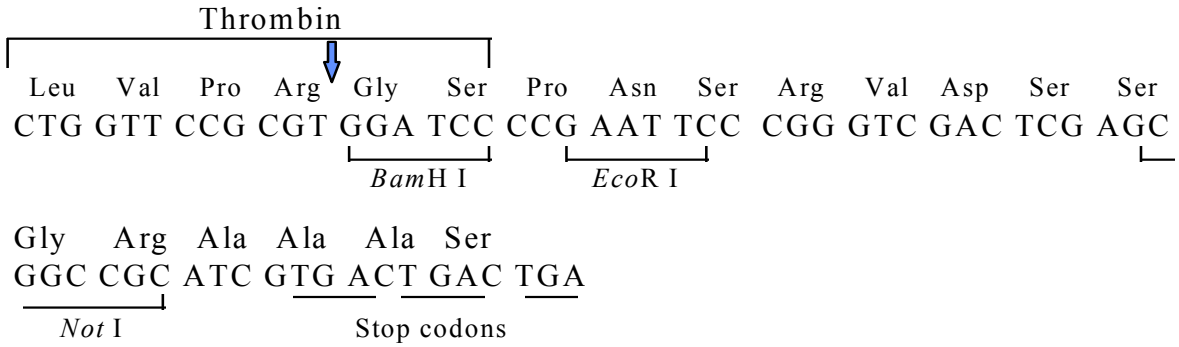
The ChiY protein is a 55 kDa protein with 2 predicted chitin-binding domains. In order to determine if ChiY did indeed bind chitin, the GST-ChiY fusion protein described above was utilized in chitin-binding assays. To achieve this, 1 L cultures of induced BL21 (pGEX*chiY*) cells were subjected to French Press and the resulting cell lysates incubated with chitin powder from crab shells (Sigma) at RT for 1 hour. Following several wash steps with physiological saline, the chitin powder was cooked with 2 x SDS loading buffer and loaded on SDS-gels. The results showed a single 80 kDa band protein reacting with the chitin. The intact GST protein (taken as a negative control), or proteins concentrated from a non-induced culture, failed to react with chitin in the chitin-binding assay. In order to verify if this 80 kDa protein was indeed the fusion protein and that the ChiY moiety was responsible for the chitin-binding activity, the GST-ChiY protein was incubated with thrombin which cleaves the desired protein from the fusion product. The cleavage products were then reacted with chitin in the chitin-binding assay. As was expected, a polypeptide running between 50 and 55 kDa, the MW range of the predicted chitin binding protein, was found to bind chitin (see fig. 25, pg 89). In contrast no protein band corresponding to the 55 kDa ChiY protein was discernible from proteins concentrated from the supernatant of the chitin-binding assay, indicating that all the ChiY in the protein suspension had bound chitin.

In parallel, the cleavage products were blotted on reinforced PVDF membrane, and the band corresponding to the ChiY moiety was excised from the membrane and subjected to N-terminal sequencing using the Edman degradation method. The result is depicted in figure A below. As observed in figure B, thrombin cleaves before the G S residue on the fusion protein, which then reads into the amino-acid sequence of the fused protein, in this case the ChiY protein. The **KLNKIMLAM** sequence revealed by N-terminal sequencing matches the first nine amino-acid sequence of ChiY, thus confirming ChiY as the chitin-binding moiety of the GST-ChiY fusion protein.

A.

G S K L N K I M L A M

B.



C.

KLNKIMLAMVVMSISGTAMAHGYIENPPSRNFLCNAQGGSLNKDCGGVQYEPQSSG ETADGFPQQGPVDGKLASGDNWVS

Fig. 24: Result of N-terminal sequencing to assess chitin-binding activity of ChiY-GST fusion protein. The first G S amino acid residues correspond to Glycine and Serine that immediately follow the thrombin cleavage site as depicted in Fig. B. The following KLNKIMLAM amino acid sequence match the first 9 amino-acid residues of the ChiY protein (Fig. C).

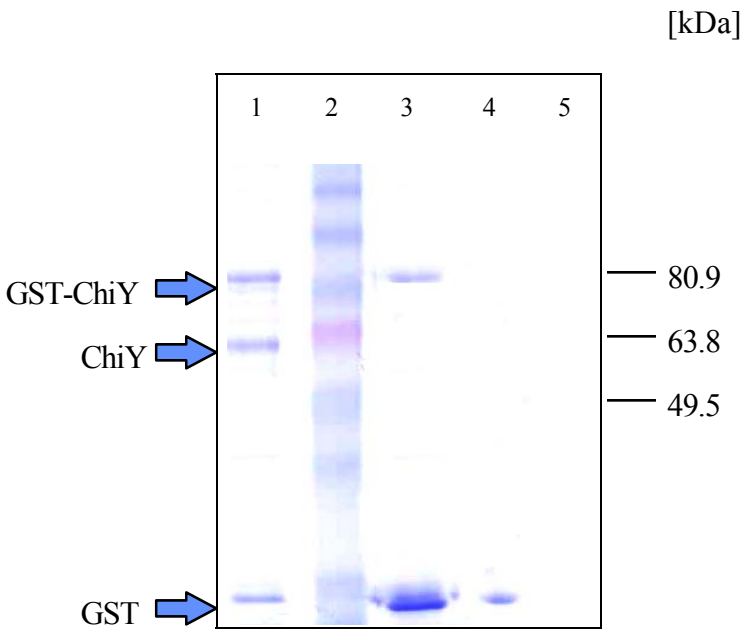


Fig. 25: Chitin-binding activity of the ChiY protein. The GST-ChiY fusion protein was cleaved with thrombin and the cleavage products reacted with chitin (see 15.2). 1. Chitin-bound proteins resolved on SDS-PAGE. 2. Protein ladder. 3. Proteins in the supernatant from the chitin-binding assay precipitated with 10 % TCA. 4. Purified GST. 5. GST reacted with chitin, and chitin-bound proteins loaded on SDS-PAGE.

DISCUSSION

1. Representational difference analysis to identify genomic differences among *Yersinia* species

The method of representational difference analysis (RDA) has been successfully applied in recent times to map out crucial genomic differences between closely related bacterial genomes, and even between isolates of the same species.

Previous applications of RDA to *Yersinia* species include the work of Radnedge *et al.,* 1999 who applied the technique to identify genomic differences among enteropathogenic strains of *Y. enterocolitica* and *Y. pseudotuberculosis*. Eleven novel DNA sequences were identified in their work which included a region unique to the *Y. enterocolitica* strains that shared homology with the response regulator PhoP in *S. typhimurium,* which is a virulence-associated factor. In another study, comparison of *Y. pestis* and *Y. pseudotuberculosis* revealed seven DNA regions in *Y. pestis* that do not occur in *Y. pseudotuberculosis*, with four of them mapping to the same region on the *Y. pestis* genome. The authors thus hypothesized that *Y. pestis* acquired this region while evolving from *Y. pseudotuberculosis* (Radnedge *et al.,* 2001).

Subtractive hybridization was also successfully applied by Radnedge *et al.,* 2002 for whole genome comparisons between different strains of *Y. pestis* with the goal to develop signatures for epidemiological studies. Six species-specific difference regions (DFRs) were identified between different biovars (Antiqua, Mediaevalis and Orientalis) of *Y. pestis*. The DFRs were mapped and four were flanked by insertion sequences. The appearance of these DFRs in eighty geographically diverse strains of *Y. pestis* representing all three biovars was determined and revealed genomic plasticity resulting from the acquisition and deletion of these DNA regions. Additionally *Y. pestis* biovar Orientalis was found to possess DFR profiles different from Antiqua and Mediaevalis biovars, reflecting most probably the recent origins of this biovar.

This work represents the first attempt to map out novel, potential virulence-associated sequences in high-pathogenicity *Y. enterocolitica* strains using subtractive hybridization.

1.1 Representational difference analysis applied to *Y. enterocolitica* strains.

As was previously elucidated (see Fig. 1) *Y. enterocolitica* species embrace an heterogeneous group of bacteria with different biochemical and antigenic properties, as well as geographical predispositions. Subtractive hybridization was applied to identify novel genetic markers that could account for the variability observed within this bacterial group. Three main groups are identifiable among *Y. enterocolitica* serotypes, namely the non-pathogenic biotype 1A organisms, the low-pathogenicity, non-mouse lethal serotypes (BT 2-5) and the high-pathogenicity, mouse-lethal serotypes (BT 1B). To date, the HPI (high-pathogenicity island) appears to define essentially the difference between the high-pathogenicity and low-pathogenicity strains, with the low-pathogenicity organisms lacking this locus.

Interestingly in a recent study by Pelludat *et al*., 2002, transfer of the HPI-core region to a low-pathogenic strain (MRS40, BT 2) led to a yersiniabactin biosynthesis phenotype and enhanced mouse virulence. In contrast, transfer of this HPI core to the non-pathogenic NF-O strain did not confer a yersiniabactin-positive phenotype, implying the existence of some additional genes that could be absent from the NF-O genome which play a role in yersiniabactin synthesis. Such studies imply important intraspecific differences within the *Y. enterocolitica* group that could account for significantly different phenotypes.

Recently, Grant *et al.* (1998 and 1999) showed that contrary to previously held conceptions, some non-pathogenic *Y. enterocolitica* 1A strains do harbor some potential virulence markers that confer an invasive phenotype to them. Their conclusion was that some clinical isolates of *Y. enterocolitica* which lack classical virulence markers may be able to cause disease via virulence mechanisms that differ from those previously characterized. Robins-Browne *et al.* for example reported on a BT 1A strain of serogroup O:6 that produced a novel heat-stable enterotoxin termed YST-II. This toxin differed from YST-a in a number of ways including its mechanism of action, which does not appear to involve activation of guanylate cyclase.

In the light of the foregoing, it becomes apparent that a better understanding of the genetic content and variability within *Y. enterocolitica* species will shed interesting light on the diversity that exists within this bacterial group and reasess our current definitions of

pathogenicity / virulence of these species. The aim of this research work was therefore to identify new genetic loci that differentiate the highly-pathogenic *Y. enterocolitica* serotypes from their "non-pathogenic" and low-pathogenic counterparts using the method of representational difference analysis.

2. IS*1330* is a novel insertion sequence unique to the assemblage of pathogenic *Y. enterocolitica* strains.

2.1 IS elements and genetic rearrangements

Of the 200 subtracted fragments analyzed through sequencing and homology search, fragments with similarities to phages and mobile genetic elements (transposases, etc.) constituted almost 13 % of the entire subtracted library. In particular was there a great preponderance of subtracted clones that were tester-specific, sharing significant homology with IS elements and putative transposases. In this work, a novel IS*10*-like element, designated IS*1330* was uncovered by genomic subtraction. This is in agreement with previous works where transposases specific for virulent strains were uncovered through subtraction. For example in the work by Sawada *et al.,* 1999, a novel insertion sequence designated IS*1598*, was identified in *Porphyromonas gingivalis*, a potent oral pathogen, through subtractive hybridization and shown to be peculiar only to virulent strains with the ability to form necrotic abscesses. The avirulent strains consistently tested negative for this IS element. This work furnishes a good example of the efficacy of the subtraction procedure as previous attempts based on biochemical profiling to define the differences between avirulent and virulent strains of this species failed to yield significant peculiarites. Interestingly, IS*1598* was shown to be present in multiple copies (up to 13 copies in some cases) in the highly virulent strains, which form pathological abscesses, compared to fewer copies (2-3) in the moderately virulent strains.

A similar result is presented in this work, where IS*1330* was shown to be present in multiple copies (See Fig. 7) in the high-pathogenicity American serotypes of *Y. enterocolitica* species while various low-pathogenicity (represented by serotype O:3) and non-pathogenic isolates tested negative for this mobile element. One might therefore be tempted to propose a possible virulence-enhancing function for IS*1330* in the harboring strains.

It has been reported that bacterial IS elements often cause mutations resulting in a number of genetic defects, including gene disruptions, polarity effects, and activation of nearby genes or DNA rearrangements, all of which may modify bacterial characteristics to varying degrees (Galas and Chandler, 1989). Previously Genco *et al.,* 1995, reported on the development of an efficient transpositional mutagenesis system employing the virulent *P. gingivalis* strain A7436 and a transposon (Tn*4351*). The Tn*4351*- generated mutants presented with increased infectious and virulent properties compared to the parent strain in mouse models. Furthermore, these authors reported morphological and structural alteration of the cell surface and overproduction of membrane vesicles as discernible through electron microscopy. Genco *et al*. therefore suggested that Tn*4351* may cause mutation in structural genes of outer membrane proteins (OMPs) and modify the virulence of *P. gingivalis* since bacterial OMPs were thought to be important virulence factors in the pathogenicity of periodontopathic bacteria. This corroborated with the work of Sawada *et al.* mentioned previously who showed that IS*1598* disrupted the structural gene for a 67 kDa outer membrane protein designated *pga67*, and at least two other genes.

The *Yersinia* chromosome is subjected to different types of genetic rearrangements, including deletions and insertions promoted by mobile genetic elements. In *Y. pestis* for example, the insertion elements IS*100* and IS*245* are present in multiple copies and are responsible for the inactivation of certain virulence factors (Parkhill *et al.,* 2001, Torosian and Zsigray, 1995). In *Y. enterocolitica*, IS*1328* has been implicated in possible deletions of the *fyuA / irp2* gene cluster that constitute part of the iron acquisition system in the *Yersinia* spp (Rakin *et al.,* 2000b). On the other hand, IS elements are involved in genetic mobility of chromosomal fragments, as is exemplified by antibiotic resistance cassettes harbored by a variety of the *Enterobacteriaceae.* Because IS*1330* shares some common features with previously described IS*10* elements including molecular weight in the 46 kDa range and possibly a related mechanism of regulation by Dam methylase, future studies will shed light on whether IS*1330* similarly mediates gene rearrangements and IS*10*-like transposition-related events in the *Yersinia* chromosome, thus enhancing the genetic plasticity of pathogenic *Y. enterocolitica* strains (Iwobi *et al.,* 2002).

2.2 IS*1330* flanks novel sequences in *Y. enterocolitica* 8081 that could have consequences on the pathogenicity of the species

The entire IS*1330* insertion sequence was used to probe the *Y. enterocolitica* 8081 sequence genome for locations of IS*1330* on the chromosome. Six locations were identified which correlates with the result of the Southern blot in Fig. 7 where IS*1330* was shown to be present in 6 copies in this strain (Iwobi *et al.,* 2002). IS*1330* does not appear to disrupt any gene sequences but it occurs intergenic of some operon-like structures on the 8081 genome and could therefore have modulatory effects on the neighboring encoded proteins (Galas, 1989). One of such clusters is the *ars* cluster described in section 5 of results.

Comparison of the other IS*1330*-associated sites on the 8081 chromosome does not indicate any insertion events. IS*1330* might therefore act as an activator for flanking genes or genetic clusters. Interestingly, however, IS*1330* is associated sometimes with genetic loci that share unusually high homology with the *Y. pestis* genome. For example, in one such locus, IS*1330* is flanked upstream by an ORF that shares 90 % homology with an hypothetical protein, designated YP02156 on the Y. pestis genome. Further upstream is an ORF that shares 84 % identity with YP02162 that encodes protease IV in Y. pestis. Downstream of IS*1330* on the antiparallel strand are several genes encoding ORFs mapping to the same cluster on the *Y. pestis* genome. One such ORF, sharing 88 % identity to YP02155 encodes a putative exported protein.

Since DNA homology between *Y. pestis* and *Y. enterocolitica* species is generally not greater than 60 - 65 %, such sequences with close to or greater than 90 % homology at the amino-acid level, could represent unique loci that have been jointly acquired by the two for a specific virulence-associated function or might represent sequences under strong selection pressure.

3. The *yts1* gene cluster

The potential to secrete proteins into the extracellular medium is generally regarded as an important attribute of pathogenic bacteria. Typically, six bacterial pathways for protein secretion to the extracellular milieu have been documented. These include the signal sequence independent pathway (type I), the main terminal branch (MTB) of the general secretion pathway (type II), the contact dependent pathway (type III), the type IV pathway related to bacterial conjugation systems, the two-partner secretion system (TPS) exemplified by *Bordetella pertussis,* and the autotransporter pathway (Binet *et al.,* 1997, Burns, 1999, Collazo and Galan, 1997, He *et al*., 1991, Henderson *et al.,* 1998, Jacob-Dubuisson *et al*., 1999, Jahagirdar and Howard, 1994, Sandkvist *et al.,* 2001a). Among pathogenic *Yersinia* species, four type III secretion systems (TTSS) have been discovered, namely the Ysc TTSS responsible for Yops secretion, the Ysa TTSS of *Y. enterocolitica* species required for the extracellular secretion of a set of proteins designated Ysps, the chromosomally encoded TTSS of *Y. pestis* and lastly the flagellar biosynthetic system (Haller *et al.,* 2000, Macnab, 1999, Parkhill *et al.,* 2001, Portnoy *et al.,* 1981, Young *et al.,* 1999).

Although the type II secretion pathway has been well described among many representatives of the *Enterobacteriaceae* (Sandkvist *et al.,* 2001a), it has not been previously demonstrated among *Yersinia* species. In the classical studies by Francetic and Pugsley (2000a), an intact secreton was even identified in the widely used laboratory strain *E. coli* K-12 (non-pathogen). Typically, the interaction of between 12 to 15 gene products have been shown to be essential for protein secretion through this pathway. The encoded proteins form a complex network of interactions spanning the cytoplasmic, inner and outer membranes (Sandkvist, 2001, Stathopoulos *et al.,* 2000).

In this work, a novel secreton, unique to highly pathogenic *Y. enterocolitica* species is described. This secreton, designated Yts1 is encoded by a set of thirteen genes, namely the *yts1C-O* genes that form an operon-like module, and the divergently transcribed *yts1S* gene. The encoded proteins have been designated Yts1C -Yts1S, corresponding to the encoding genes.

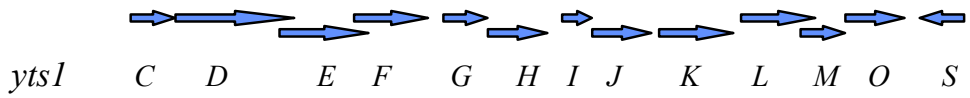


Fig. 22: The *yts1* secretion gene cluster

Immediately upstream of *yts1C* is a gene that encodes an ORF with low homology (31 %) to a hypothetical protein (YPO0818) on the *Y. pestis* CO-92 genome, 31 % identity to ORF1 encoded on the chromosomally encoded Ysa type III secretion system and 28 % identity to a putative response regulator in *S. typhimurium*. Further upstream of *yts1C* is a gene encoding a putative cytochrome oxidase. This gene is preceded by a **ggaa** nucleotide sequence that could represent a potential ribosome binding site for the immediately adjacent gene and the downstream *yts1* cluster.

The Yts1 putative secreton has well represented all the interacting partners for a functional secretion, namely the cytoplasmic, energy transducing Yts1E protein (which interacts with protein L); the integral inner membrane proteins comprising Yts1C, Yts1M, Yts1K and Yts1F; the four type 4 pilin-like proteins (pseudopilins), namely Yts1G, Yts1H, Yts1I and Yts1J; the outer member proteins or secretins, namely, Yts1D and Yts1S, and lastly the Yts1O protein, a putative prepilin-like peptidase. The encoded proteins showed varying homologies with previously described secreton proteins such as the secreton encoded by the plant pathogens *Erwinia chrysanthemi* and *E. carotovora*, the Xcp secretion apparatus in *Pseudomonas aeruginosa* and the Gsp proteins of *Escherichia coli*. The highest similarities though were to the Eps proteins (encoded by the *eps* type II secretion system) of *V. cholerae*, followed by the proteins of the putative type II secretion system of *Y. pestis*. For example Yts1E and Yts1F show 59 % and 38 % identities to the *V. cholerae* EpsE and F proteins respectively, while showing 55 % and 34 % identity to the corresponding proteins on the *Y. pestis* secreton (Sandkvist *et al.,* 1997, Parkhill *et al.,* 2001).

3.1 Expression of the *yts1* genes is temperature-enhanced

The *Yersinia* species furnish an excellent example of bacterial pathogens whose assemblage of virulence factors are exquisitely controlled by temperature. As was previously mentioned, *Y. enterocolitica (*and *Y. pseudotuberculosis)* can survive outside animal hosts (where the prevailing/ambient temperature is usually lower than

temperatures encountered in a suitable host) and are foodborne pathogens (Boyd and Cornelis, 2001). Early in the establishment of an infection, the bacterial factors that promote attachment to and invasion into eukaryotic cells are copiously produced, such as the chromosomally encoded Inv protein (Boyd and Cornelis, 2001, Bottone, 1997). As Inv is important at this initial stage of infection, it would be advantageous for the ingested *Yersinia* to have the adhesin already present on its surface to direct the bacterium to the Microfold (M) cells, and indeed Inv is maximally produced at 23 °C (Boyd and Cornelis, 2001, Isberg *et al.,* 1988). It has however been documented that under some conditions, namely reduction in pH to 5.5 and addition of sodium ions, Inv production can occur at 37 °C at levels comparable to those observed at 23 °C (Pepe *et al.,* 1994). Thus Inv expression may be possible inside the animal host at 37 °C with the appropriate environmental conditions, and indeed in harmony with these results, Inv can be detected on Peyer's patches 2 days after infection (Pepe *et al.,* 1994).

In contrast, the second chromosomally encoded protein that promotes attachment to and subsequent invasion of eukaryotic cells, namely the Ail protein, is preferentially expressed at 37 °C under aerobic conditions in the stationary phase (Pierson and Falkow, 1993). Myf is also a chromosomally encoded protein that enhances thermoinducible binding to eukaryotic cells and can also promote binding to intestinal luminal mucus (Marra and Isberg, 1997, Yang *et al*., 1996). The Yst enterotoxin of *Y. enterocolitica* also comes under thermal regulation with maximal production occurring at 30 °C during the stationary phase, although increased osmolarity and pH at 37 °C can also induce high expression of the protein (Mikulskis *et al*., 1994).

Transcription of many virulence plasmid genes, including all the *yop* genes, *sycE* which encodes SycE, a chaperone for YopE, *yadA* and the *virC* operon (part of the Ysc type III secretion machinery) is dependent on VirF/LcrF, an AraC regulator, which itself is regulated by temperature at both the transcriptional and posttranscriptional levels. Thus these pYV-encoded genes also come under exquisite thermoregulation (Michiels *et al*., 1991, and Cornelis *et al.,* 1989).

The *yts1* gene cluster might also come under thermoregulation as evident from the results of the reverse transcription analysis. The *yts1D* gene was probed in reverse transcription

analysis from total RNA isolated from bacterial cultures grown at varying temperatures (27 °C and 37 °C). The cultures grown at 27 °C (also to late stationary phase) gave very minimal mRNA transcripts in the transcription analysis, whereas clear transcripts were obtained from cultures grown at 37 °C (see Fig. 13). This correlated with results obtained from the *in vivo* infection of HeLa cell lines by *Yersinia* (see section 8 of results). When total RNA was isolated from the infected cell line and reverse transcribed using primers specific for the *yts1D* gene, it gave positive transcripts. This indicates that the *yts1* genes might play a role in the pathogenesis of the high-virulence *Y. enterocolitica* species.

3.2 Role of the Yts1 secreton in virulence

In order to determine the effect of this novel Yts1 secreton on the virulence of the species, mouse virulence tests were conducted which compared the wild type WA-314 species with its *yts1E* mutant. The results confirm that the chromosomally encoded Yts1 secreton does enhance the virulence of the *Y. enterocolitica* species as evident by a 100 fold attenuation in virulence observed in the *yts1E* mutant when mice were perorally inoculated. In contrast, the number of bacterial CFUs following intravenous inoculation of mice were not significantly different for wild type and secretion mutant. These results are strikingly similar to the observations made by Haller *et al*., 2000, with respect to the Ysa TTSS. The authors reported that the Ysa secretion system appears to play a role in the virulence of *Y. enterocolitica* strains when the bacteria are inoculated into mice perorally, while bacteria inoculated intraperitoneally appeared not to benefit from this transport system. The authors concluded that the Ysa TTSS possibly played a role in virulence after the initial invasion of the Peyer's patches but before spread to deeper tissues. In contrast, a possible mechanism for the Yts1 secreton might be its requirement for dissemination into deeper tissues such as the liver and spleen following an initial invasion of the host. The mouse-virulence data presented here confirms this hypothesis as the attenuation in virulence for the *yts1E* mutant was observed only with respect to colonization of liver and spleen, while the bacterial counts extrapolated from Peyer's patches were not significantly different between the wild type and secretion-deficient mutant. More detailed studies should shed light on the nature of the molecular interaction between the Yts1 secreton and the cognate host tissue it recognizes during the course of bacterial infection.

3.3 Is *yts1* a pathogenicity island?

The genes encoding certain secretion systems have been assumed to be products of horizontal gene transfer, evidenced by their different G+C content when compared to the average G+C content of the organism (Hacker *et al.,* 1997, Hueck, 1998, Mecsas and Strauss, 1996). The genes of the Yts1 protein secretion apparatus however have an average G+C content of 48 %, characteristic of the *Yersinia* species and a codon usage in line with the species. This could argue against horizontal acquisition of the transport system, or might point to transfer from an organism with similar G+C content. Interestingly, in WA-314, a copy of an IS*10*-like element, IS*1330*, flanks upstream the *yts1* cluster which might be indicative of an insertional event, and analysis of the sequences downstream of the flanking *chiY* gene will yield additional information in this regard. Future studies will therefore shed light on what mechanisms played a role in the acquisition of this additional type II secretion system by the high-pathogenicity *Y. enterocolitica* species.

3.4 The *yts2* gene cluster.

Sequence analysis of the genome of *Y. enterocolitica* 8081 revealed a second chromosomal locus with great similarity to a type II protein secretion locus (designated Yts2) that was confirmed to be uniformly distributed among all tested *Y. enterocolitica* strains - non-pathogenic, low-pathogenicity and high-pathogenicity strains alike (Fig. 12B). Future studies should reveal whether this common, species-specific Yts2 secreton is functional and will define the possible substrates that could rely on it for secretion. Recent studies (Miller, *et al.,* 2002, Personal communication) from the laboratory of Miller *et al.* however reveal that the recently discovered *Yersinia* transcriptional regulator RovA might act to regulate this putative Yts2 secreton (Revell and Miller, 2000).

Comparison of the *yts1* type II cluster with its counterpart on the *Y. pestis* genome reveals divergence between these two systems with respect to both gene arrangement and sizes of the putative encoded ORFs. The *Y. pestis* secreton however, shared greater homology with the *yts2* species-specific type II gene cluster common to all *Y. enterocolitica* strains both in terms of G+C content (a low 33 - 34%) and the arrangement of genes bordering the two clusters respectively (see Fig. 11). This could argue for a common origin for these

two secretion clusters, while the Yts1 transport system was independently acquired by the high-pathogenicity *Y. enterocolitica* 1B strains.

4. ChiY – a putative chitin-binding protein

The Yts1 secreton is flanked by ChiY, a putative chitin-binding protein with a predicted molecular weight of 55 kDa. Because of its proximity to the *yts1* secreton, ChiY was hypothesized to be secreted into culture supernatants of *Y. enterocolitica* strains by the *yts1* secreton. However, comparison of protein profiles from culture supernatants comparing wild type, WA-314 *yts1E* and the WA-314 *chiY* mutant failed to reveal significant differences when bacteria were cultured in LB broth at 27 °C or 37 °C. When bacterial cultures were grown under high osmolarity (0.4 M NaCl) at 27 °C a faint 50 - 55 kDa protein was sometimes discernible from culture supernatants of only the wild type strain. Addition of chitin powder to culture media did not induce secretion of the predicted ChiY protein, also when cells were grown in minimal medium with or without chitin supplementation. It was therefore hypothesized that the product of the *chiY* gene could come under some form of repression or its expression dependent on an unknown activator.

In the case of the common laboratory strain *E. coli* K-12, an apparently intact secreton was identified, which was flanked by a putative chitinase. Francetic *et al.,* (2000a and 2000b) showed in two classical experiments that expression of the genes on the secreton came under negative regulation of the histone-like H-NS protein, part of the global regulatory system of the bacterium. Mutation of the *hns* gene resulted in up-regulation of the genes of the secreton leading to detectable levels of secreted chitinase.

Several attempts were made at mutating the *Y. enterocolitica hns* gene in this work. The introduced kanamycin cassette however was consistently inserted into another chromosomal location, leaving the *hns* parental allele intact. Thus the impact of the *hns* gene could not be properly assessed in this work.

In *Y. enterocolitica* species, the YmoA protein has been implicated in the modulation of several virulence factors that come under thermoregulation (Cornelis *et al.,* 1991). According to the nucleic acid sequence, the product of *ymoA* is an 8064 Da protein rich in

aspartic acid, glutamic acid and lysine with properties similar to a histone-like protein. Recent studies indicate that the YmoA protein might form a complex with the H-NS protein to repress or regulate the expression of a variety of virulence genes (Nieto *et al.,* 2000, Nieto *et al.,* 2002). Since the *yts1* cluster is preferentially transcribed at 37 °C, the product of the *ymoA* gene might play a role in its expression. Thus future work utilizing an *hns* mutant, a *ymoA* mutant and an *hns ymoA* double mutant might shed light on the regulation of the *yts1* secretion cluster.

Interestingly, a recombinant GST - ChiY fusion demonstrated chitin-binding activity (see 15.2). Thus ChiY does indeed bind chitin but the expression and secretion of the protein might require some *in vivo* conditions that are yet to be identified.

4.1 The ChiY protein might play a virulence-enhancing role in *Y. enterocolitica* species

Preliminary results presented in this work indicate that a ChiY deficient mutant was attenuated in liver and spleen colonization of mice when the oral route of infection was utilized as opposed to the intravenous route of inoculation where no differences were observed (see Tables 9 and 10). More detailed studies should confirm this observation and shed additional light on the possible mechanisms of interaction between the ChiY protein and cognate host tissues that it recognizes.

Previous work with *Klebsiella pneumoniae*, a widely recognized urinary tract pathogen suggested that N-glycosylated proteins are involved in efficient internalization of *K. pneumoniae* by cultured human epithelial cells (Fumagalli *et al.,* 1997). In the cited work by Fumagalli *et al.,* pretreatment of HCT8 cell monolayers with tunicamycin which prevents N glycosylation of proteins in the endoplasmic reticulum, resulted in a reduction of internalization by more than 93 %. When lectins with known binding specificities for GlcNAc such as concanavalin and wheat germ agglutinin were added to the cell lines 1 hour before infection, bacterial uptake was inhibited by as much as 95 %. This was expected as the lectins would competitively bind to the GlcNAc host cell receptors, thus inhibiting bacterial uptake and internalization. Interestingly when bacteria were preincubated with chitin hydrolysate, a GlcNAc polymer and several other carbohydrates present on mammalian glycolipids and / or glycoproteins, chitin hydrolyaste showed a

greater inhibitory effect (98 %) than did GlcNAc on *K. pneumoniae* invasion. The other tested carbohydrates showed no effect on *K. pneumoniae* uptake.

In another study, Prasadarao and colleagues showed that the outer membrane protein A of *E. coli* interacts with the GlcNAcβ1 - GlcNAcβ4 epitopes of brain microvascular endothelial cells (Prasadarao et al., 1996). Perhaps the chitin binding ChiY protein described in this work acts as a lectin which interacts with GlcNAc residues or epitopes on host cell tissues. More detailed studies including invasion assays should shed light on the molecular nature of the interaction between ChiY and the host cell tissue it recognizes.

E. SUMMARY

Here we applied subtractive hybridization to map out novel virulence factors unique to the high-pathogenicity group of enteropathogenic *Yersinia enterocolitica*. Genomic DNA of the non-pathogenic *Y. enterocolitica* strain NF-O (biotype 1A) was employed as the driver DNA and DNA of the high-pathogenicity strain *Y. enterocolitica* WA-314 (biotype 1B) as the tester. The subtraction technique uncovered various tester-specific sequences that represented known as well as potential new virulence markers. In particular, a novel type II secretion gene cluster was uncovered and designated *yts1* (for *Yersinia* type II secretion 1). The *yts1* gene cluster comprises a 13 kb operon-like module spanning the *yts1C-S* genes. Significant transcription of the *yts1* genes could be demonstrated at 37 °C but not at 27 °C. Southern blot analysis and PCR reactions showed that the *yts1* gene cluster was unique only to the high-pathogenicity group of *Y. enterocolitica* strains of biotype 1B. The low-pathogenicity and non-pathogenic *Y. enterocolitica* strains as well as *Y. pseudotuberculosis* and *Y. pestis* strains did not carry the *yts1* genes.

In order to assess the impact of the *yts1* gene cluster on pathogenicity, a *yts1E* mutant was constructed. Isogenic pairs (parental and mutant strains) were compared for mouse virulence by oral or intravenous challenge. The *yts1E* mutant was significantly attenuated, as demonstrated by lower bacterial burden in liver and spleen compared to the parental strain, when the oral route of inoculation was used. In contrast, no significant differences in virulence between mutant and parental strain were observed when the intravenous route of inoculation was used. Therefore the Yts1 secreton may be important in the dissemination of bacteria from Peyer's patches to liver and spleen.

The *yts1* secretion cluster is flanked downstream by a gene (*chiY*) that encodes a putative chitin binding protein, ChiY, a likely substrate of the Yts1 secreton. A 55-kDa polypeptide is predicted from the sequence of ChiY. Further analysis revealed two well defined chitin-binding domains located at both N and C termini of the protein. A GST-ChiY recombinant protein fusion demonstrated that the ChiY protein bound chitin *in vitro*.

Sequence analysis of the almost completed genome of *Y. enterocolitica* 8081 (biotype 1B high-pathogenicity strain) revealed a possible second type II secretion cluster, designated *yts2*

in this work. The *yts2* cluster was shown through high fidelity PCR reactions to be distributed among all tested *Y. enterocolitica* strains, pathogenic and non-pathogenic strains alike, but absent in *Y. pestis* and *Y. pseudotuberculosis* strains. By reverse transcription/PCR, we could demonstrate significant transcription of the *yts2* genes at 27 °C.

A novel insertion sequence, designated IS*1330* was also uncovered by the subtraction technique. Through Southern blot hybridizations, IS*1330* was shown to be present only in pathogenic *Y. enterocolitica* strains and could therefore be useful in the epidemiological typing of *Y. enterocolitica*.

This work represents a novel approach at elucidating differential intraspecific genomic sequences within *Y. enterocolitica* strains using subtractive hybridization, thus broadening our understanding of the diversity and genomic heterogeneity existing within this bacterial species. The *yts1* secretion cluster described here for the first time represents a novel locus that contributes to the enhanced virulence and mouse lethal phenotype of the high-pathogenicity *Y. enterocolitica* strains.

ZUSAMMENFASSUNG

In dieser Arbeit wurde die Methode der subtraktiven Hybridisierung angewandt, um neue Virulenzfaktoren zu finden, die spezifisch für hochpathogene *Yersinia enterocolitica* Stämme sind. Hierfür wurde die DNA eines nicht pathogenen „Treiber"-Stammes (*Y. enterocolitica* NF-O, Biotyp 1A) gegen die DNA eines hochpathogenen „Tester"-Stammes (*Y. enterocolitica* WA-314, Biotyp 1B) subtrahiert. Mit Hilfe der subtraktiven Hybridisierung konnten verschiedene Tester-spezifische Sequenzen ermittelt werden, die sowohl für bereits bekannte als auch neue potentielle Virulenzmarker kodieren. In dieser Arbeit konnte ein neues TypII-Sekretionscluster, genannt *yts1* (*Yersinia* TypII Sekretion 1), ermittelt werden. Das *yts1*-Gencluster umfasst ein 13 kb großes Operon-ähnliches Modul, welches die Gene *yts1C-S* enthält. Mittels reverser Transkription/PCR konnte eine bevorzugte Transkription bei 37 °C gezeigt werden. Southern Blot-Analysen sowie PCR haben gezeigt, dass das *yts1*-Gencluster nur in den hochpathogenen *Y. enterocolitica* Stämmen vorkommt. Dagegen sind *yts1*-Gene weder in schwachpathogenen sowie apathogenen *Y. enterocolitica* Stämmen noch in *Y. pseudotuberculosis*- und *Y. pestis*-Isolaten zu finden.

Durch Inaktivierung des *yts1E*-Gens in *Y. enterocolitica* wurde eine Mutante hergestellt und hinsichtlich Mauspathogenität mit dem Mutterstamm verglichen. Bei oraler Infektion der Mäuse erwies sich die *yts1E*-Mutante als attenuiert (geringere Keimzahlen) in Leber und Milz im Vergleich zum Mutterstamm. Im Gegensatz dazu konnte bei intravenöser Infektion der Mäuse kein Unterschied zwischen Mutante und Mutterstamm festgestellt werden. Dies könnte ein Hinweis darauf sein, dass das TypII-Sekretionssystem die Erregerdissemination von den Peyer-Plaques in Milz and Leber fördert.

Das *yts1*-Sekretionscluster grenzt stromabwärts an ein Gen, welches für ein potentielles Chitin-Bindungsprotein (ChiY) kodiert. ChiY ist ein mögliches Substrat des Yts1-Sekretons. Sequenzanalysen sagen voraus, dass ChiY ein 55-kDa Protein mit zwei definierten Chitin-Bindungsdomänen ist, von denen sich die eine Domäne am N- und die andere am C-Terminus des Proteins befindet. Es konnte gezeigt werden, dass rekombinantes ChiY Chitin bindet.

Sequenzanalysen des zugänglichen fast kompletten Genoms von *Y. enterocolitica* 8081 (Biotyp 1B) führten zum Nachweis eines möglichen zweiten TypII-Sekretionscluster, das in

dieser Arbeit als *yts2* bezeichnet wird. Wie mittels PCR gezeigt werden konnte, kommt *yts2* - im Gegensatz zu *Y. pseudotuberculosis* und *Y. pestis* - in allen getesteten pathogenen und apathogenen *Y. enterocolitica*-Stämmen vor. Reverse Transkriptionsanalysen/PCR zeigten, dass die *yts2* - Gene bevorzugt bei 27 °C abgelesen werden.

Mittels subtraktiver Technik konnte auch ein neues Insertionselement (IS*1330*) charakterisiert werden. Durch Southern Blot-Analysen konnte gezeigt werden, dass IS*1330* nur in pathogenen *Y. enterocolitica* Serotypen vorkommt und somit für die epidemiologische Typisierung dieser Spezies eingesetzt werden kann.

Diese Arbeit repräsentiert einen neuen Ansatz zur Aufklärung von unterschiedlichen intraspezifischen Genomsequenzen von *Y. enterocolitica* mit Hilfe der subtraktiven Hybridisierung, um unser Verständnis der genetischen Vielfalt und Heterogenität dieser bakteriellen Spezies zu erweitern. Das zum ersten Mal hier beschriebene *yts1*-Cluster repräsentiert einen neuen Lokus, der eine wichtige Rolle für die Pathogenese der hochpathogenen *Y. enterocolitica* Stämme spielt.

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ABBREVIATIONS

A	Adenine
A. dest	distilled water
Amp	Ampicillin
AP	Alkaline Phosphatase
APS	Ammonium persulphate
ATP	Adenosine Triphosphate
au	arbitrary units
BCIP	5-Bromo-6-chloro-3-indolylphosphate
BG	Biogroup
BHI	Brain-Heart-Infusion
bp	base pair
BSA	Bovine serum albumen
CFU	Colony forming unit
CIAP	Calf intestinal alkaline phosphatase
Cm	Chloramphenicol
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP)
ds DNA	double stranded DNA
EDTA	Ethylenediamine triacetic acid
e.g.	for example
EtOH	Ethanol
$EtOH_{abs}$	absolute ethanol
FACS	Fluorescence activated cell sorter
Fig.	Figure
GFP	Green fluorescent protein
hr	hour
Hepes	N-2-hydroxyethyl piperazine- N'-2-ethane sulfonic acid
HMWP	High molecular weight protein
HPI	High pathogenicity island
i.p.	intraperitoneal
IPTG	Isopropyl β-D-thiogalactopyranoside
kb	kilobase
Kan	Kanamycin
kDa	kilo Dalton
LB	Luria Bertani
LD_{50}	Median Lethal dose (Lethal dose 50%)
µ	micro
m	milli
M	molar
MCS	multiple cloning site
min	minute
MU	4-Methylumbelliferyl
n	nano
Nal	Nalidixic acid
NBT	Nitroblue tetrazolium
OD	optical density
o/n	overnight

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PMSF	Phenylmethane sulfonyl fluoride
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	revolutions per minute
RT	Room temperature
RT	Reverse Transcriptase / Reverse transcription
SDS	Sodium dodecyl sulphate
sec	seconds
ss DNA	single stranded DNA
Sm	Streptomycin
Tab.	Table
TAE	Tris-Acetate-EDTA
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
Tet	Tetracycline
TRIS	Tris-(hydroxymethyl)-ammonium methane
Yen	*Yersinia enterocolitica*
U	Units
O/N	overnight
UV	Ultraviolet
V	Volt
Vol.	Volume
v/v	volume by volume
wt	wild type
w/v	weight by volume
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

Iwobi, A. N., Rakin, A., Garcia, E. and J. Heesemann. 2002. Representational difference analysis uncovers a novel IS*10*-like insertion element unique to pathogenic strains of *Y. enterocolitica*. *FEMS Microbiology letters* 210: 251-255.

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