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Identification of Couple of Novel Genes Probably have Relation with Virulence in *Yersinia Enterocolitica* O:8 using Subtractive Hybridisation

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Abstract - *Yersinia enterocolitica* is one of the important enteric pathogens which are gram-negative rods that are motile when isolated from environment but become nonmotile in mammalian host which cause human disease due to consumption of contaminated water and food and it has an invasivness ability to cross the gastrointestinal mucosa to infect the underlying tissue The pathogenic *Yersinia enterocolitica* always harbouring the important virulence factors, Such as the virulence 70Kbp plasmid which encodes the Yop virulon and the HPI which encodes the Yersiniabactin iron responsible genes, but still other virulence genes in *Yersinia enterocolitica* exists need to be identified and chracterized. Subtractive hybridisation is one of the most powerfull tools for the identification of virulence genes in wide range of bacterial pathogens, and in this study the hybrydization of high pathogenic *Yersinia enterocolitica* O:8 and low pathogenic *Yersinia enterocolitica* O:5 was successful to identify the two novel genes which are probably have relation to virulence. The prepilin peptidase which was proved by PCR in most pathogenic *Yersinia* species, and it is responsible for the faimbrial and pilli formation which has an adhesive and congugative functions which are important for the genetic materials transfere and the other gene is an invasine Inv homolog sequence which has an ORF of invasine Inv of pathogenic *Yersinia enterocolitica* which could be named as *Inv2*.

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I. Introduction

Yersinia spp are gram-negative rods that are motile when isolated from environment but become nonmotile in mammalian host. Three species of *Yersinia* cause disease in humans. *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Yersinia pestis* These species differ considerably in invasivness, *Y. enterocolitica and Y. Pseudotuberculosis* can ceoss the gastroentistenal mucosa to infect underlying tissue, but infections usually remain localized in the submucosal area. *Y. pestis* is injected into the body by an insect bite and thus does not have to penetrate a body surface on its own, but once inside the body, it spreads rapidly and causes a systemic infections (Heesemann *et al* 1984; Mingrone and Fantasia, 1988; Cornelis, 2002).

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Y. enterocolitica infections have been particularly common among children, and outbreaks have occurred in day-care centers and schools. Symptoms of *Y. enterocolitica* infections vary from a mild form of the disease, characterized by diarrea and abdominal pain, to a more severe from *Yersinia enterocolitica* strains which cause disease are serogrouped using an O1H type of E.coli (Fukushima *et al*, 2001; Denecker *et al*, 2002; Hayashidani *et al*, 2002; Juris *et al*, 2002).

Pathogenic *Yersinia spp* share a common tropism for lymphoid tissue and are markable ability to resist the non-specific immune response of the host their main strategy seems to consist of avoiding lysis by complement, avoiding phagocytosis by polymer-phonuclear leucocytes and macrophages and forming extracellular microcolonies in the infected tissues and they have common basic virulence functions (Tauxe *et al*, 1987, Simmonei *et al*, 1990).

The chromosome of *Yersinia enterocolitica* encodes many virulence factors, such as enterotoxin Yst, Myf fibrille and Inv (Cornelis, 1992, Iriarte *et al*, 1993; Gruizkan *et al*, 1990).

Which have a great influence on the pathogenicity of such micro-organisms and the concept of pathogenicity island explained that particular genomic regions of pathogens, such as *Y. enterocolitica*, carry virulence-associated genes together with loci whose presence strongly indicates horizontal gene transfer of these regions between different species or even genera (Dobrindt *et al*, 1998; Elliot *et al*, 1998; Al-Hasani *et al*, 2001).

The yersiniabactin gene cluster responsible for the manifestation of lethality for mice was named the high-pathogenicity islands (HPI). The functional core of the islands consists of 12 genes, at least six genes encoding iron-responsible proteins (irp1 to 5 and irp9) in *Y. enterocolitica* (YbtE and YbtT are Y. Pestis synonyms for irp4 and irp5, respectively) are involved in biosynthesis of the Yersiniabactin (Heesemann, 1987; Heeseman et al, 1993; Corniel et al, 1996; Pelludat et al, 1998; Rakin et al, 1999; Xu et al, 2000).

Y. pestis, Y. pseudotuberculosis and all pathogenic *Y. enterocolitica* strains harbor a 70-Kb

plasmid that is devoted to virulence. In *Y. enterocolitica*, this plasmid is called pYV (for plasmid involved in Yrsinia virulence) followed by the identification of strain in *Y. pestis* and *Y. Pseudotuberculosis*, the archetypes are called PCD1 and PIB1 respectively. Most of this plasmid encodes the Yop virulon, a sophisticated virulence apparatus which is conserved in the three species and is considered an archetype of the so-called type III virulence systems encountered in several plant and animal pathogens. (Heesemann and Gruter, 1987; Neubauer et al, 2000; wecks et al, 2002).

II. Materials and Methods

a) Bacterial Strains and Growth Media

The bacterial strains used are *Yersinia enterocolitica* O:8 and O:5 in the subtractive hybridisation and *E.coli* for carrying plasmid with subtracted DNA fragments and *E.coli* DH5α in the transformation and the growth media was L.B media considering the incubation of *Yersinia* strains at 27° and *E.coli* at 37°.

b) Bacterial Plasmids

In subtractive Hybridization pMOSBlue with 2.8 Kbp to carry the subtracted DNA fragments in *E.coli* and pSB315 which carry the Kanamycin cassette which is 1Kbp.

c) Subtractive Hybridisation

Subtractive hybridisation is a powerful technique that has been applied to research in many different fields for studying the eukaryotic systems. The application of subtraction techniques using clontech PCR-select bacterial genome ubtraction Kit user Manual PT3170-1, typically focused on differential gene expression differences between two cDNA populations-rather than differences between genomes. This is because eukaryotic genomes are too complex for existing subtraction technologies. In contrast, bacterial genomes are considerably smaller, and are even less complex than many eukaryotic cDNA populations. Thus subtraction methods can be used to identify sequences that are present in one bacterial genome but are absent in another.

Although there are several different methods, the basic theory behind subtraction is simple. The genomic DNA sample that contains the sequences of interest is called “tester” and the reference sample is called “driver”. Tester and driver DNAs are hybridized, and the hybrid sequences are then removed. Consequently, the remaining unhybridized DNAs represent tester-specific sequences.

Traditional subtractive hybridization methods involve several rounds of hybridization and require large amounts of DNA. In contrast the clontech PCR-select bacterial genome subtraction Kit overcomes these and other technical limitations of traditional subtraction procedures. Like PCR-select cDNA subtraction, this kit is based on the suppression subtractive hybridization (SSH) method, the PCR- select procedure requires only 1.5-2 μg of genomic DNA, takes only 2-3 days. Suppression PCR prevents undesirable amplification while enrichment of target molecules proceeds.

d) Sequencing of the DNA Fragments

- PCR for the fragments (Subtracted fragments of *Y. enterocolitica* O:8 carried on the plasmid pMos blue) using the primers T7 and U19

240 μl	buffer (10x)
240 μl	dNTPs
24 μl	primer1
24 μl	primer 2
9.6 μl	tag polymerase
1822.4 μl	distilled water

90 μl for each tube (24) + 10 μl DNA and the running in the PCR set with 30-35 cycles.

- Purify the PCR product (DNA) using the PCR purification Kit.
- Run on the Gel.

e) Preparation of the DNA for Sequencing

- 3 μl PCR product (DNA)
- 1 μl primer (5pmol / μl)
- 4 μl Big dyes
- 12 μl distilled water
- Then run in the PCR set.
- Sequencing was done using ABI prism Big Dye terminator cycle sequencing ready reaction kits, in the diagnostic lab, Max von pettenkofer Institute University of Munich Germany.
- The data were analysed with computer using the NCBI bast x and TIGR blast program.

f) Isolation of Plasmids

The plasmids were isolated with nucleobond application Kit using the ready buffer solutions S1, S2, S3, N2, N3 and N5.

The bacterial culture is harvested by centrifugation at 300 to 5000 rpm for 5-10 min at 4°C.

g) Electroporation

With ice cold environment 40 μl from competent cells plus 3.5 μl DNA (plasmid was diluted 1:10). Then electroporation was done in electroporation cuvetts with gene pulser. L.B. medium was added (1 ml) and incubated for 1hr in 37°C, then 100 μl was platted in L.B. medium with antibiotics and incubated overnight. Some colonies were selected and platted on L.B medium with antibiotics overnight and then screened for the plasmids.

h) Isolation of the plasmid pMos blue from E.coli

The plasmid pMos blue of the subtracted clone No 75, which carries the invasine homology was isolated from *E. coli*, and it was 2.8 Kbp and then cut with the restriction enzyme EcoRI

i) Isolation of the kanamycin kassette from the plasmid pBs 315

The pBs 315 plasmid was isolated from *E. coli*, then cut with the restriction enzyme Hinc II to give the kanamycine kassette band which is 1000 bp, and was cut from the gel and purified

j) Mutagenesis using linear transformation ET recombination

The principle of this method is the design of the required sequence (Invasin) with about 50 nucleotides which forms homology arms to the selection marker (kanamycin).

- Then PCR amplification and purification of the PCR product (linear DNA).
- Digestion with restriction enzyme DpnI.
- Direct transformation of the linear DNA into competent cells of the Nalidixin mutant of *Y. enterocolitica* to produce point mutation (homologous recombination) into the chromosomal DNA.
- Plat the mutants on the nalidixin, kanamycin L.B. medium plates
- Prove the insertion of the desired sequence with PCR (Wagner and Koszinowski, 2002).

III. Results

a) Subtractive hybridization and Sequencing of the Y. enterocolitica subtracted DNA fragments

96 subtracted clones of *Y.enterocolitica* O:8 which carried on the plasmid pMos blue were detected with PCR, and revealed different sizes (Fig. 1). Then purified and sequenced to show different important homology to translated proteins, which include some virulence factors (Table. 1), such as prepilin peptidase (Clone NO.3) and invasin (Clone NO.75).

b) Prepilin peptidase detection in different Yersinia strains

Prepilin peptidase was detected in different *Yersinia* strains such as *Yersinia enterocolitica, Y. pseudotuberculosis* and *Y. pestis* by PCR, using primers designed from the subtracted fragment DNA of the clone No 3 which have homology to prepilin peptidase and PCR revealed specificity of this protein to all strains of *Y. enterocolitica* (Fig. 2).

c) Detection of invasine homolog in Yersinia enterocolitica

PCR was applied on the subtracted DNA fragment No.75 which has homology to invasine using the proper primers T7 and U19, which is carried on the plasmid pMos blue, the DNA fragment was about 900 bp (Fig.3,4). and from the Data base of the genomic sequencing of the *Yersinia enterocolitica*, using blast matching it was found that the sequence has an ORF of *inv* gene (Fig.4)

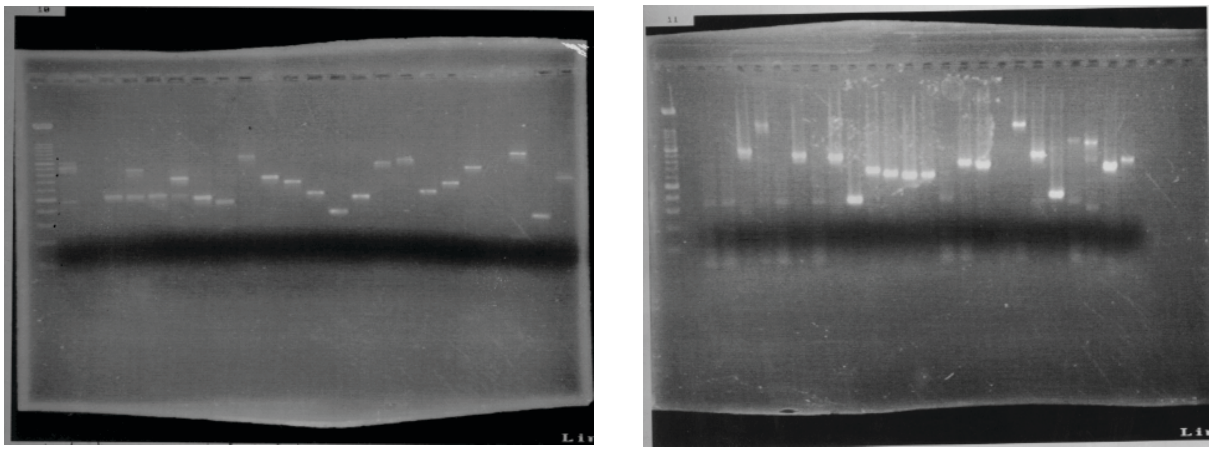
d) Transformation of the pMos blue plasmid which carries the kanamycine kassette into E.coli DH5α

The kanamycine kassette was ligated with the plasmid pMos blue 75 which carries the invasine homology, and then transformed into *E. coli* DH5α, and the transformants were grown well on the L.B. medium plates containing the antibiotic Kanamycine, then the ligated plasmid was isolated again from *E. coli* (Fig.5)

e) Mutagenesis of Y. enterocolitica using E.T recombination

The proper primers were designed from the kanamycine kassette sequence and the sequence of invasine homology which is carried on the plasmid pMos blue 75 to make 50 nucleotides homology arms to the kanamycine kassette. With the application of PCR, the DNA fragment 1000 bp was obtained from the DNA obtained from the cooked cells of *E.coli* DH5α and the resulted DNA was electroporated into the competent cells of the Nalidixin mutant *Y. enterocolitica* WA314, and the transformation was positive where the mutants were grown on L.B. medium containing the antibiotics Nalidixin and kanamycine.

(A) (B)



Mr ABCD EF GH I J K LM NO PQ RS TUVW Mr ABCDEFGHIJKLMNOPQRSTUVWX

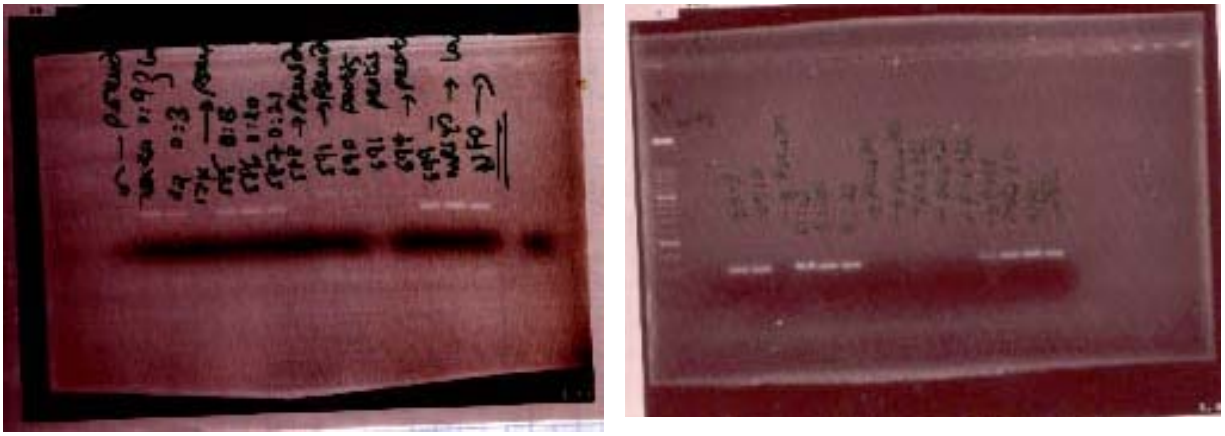
Fig. 1 : Detection of the subtracted fragments of DNA of *Y. enterocolitica* O:8 using PCR. Mr = 100 bP DNA marker. (A) A to W = the subtracted fragments from 1 to 23. (B)A to X = the subtracted fragments DNA from 73 to 96

Table 1 : Results of sequencing of the subtracted fragments DNA of the pathogenic *Y. enterocolitica* O:8

Clone No.	Size bP	Significant similarity homologous protein
3	483	Prepilin peptidase
5	610	Putative transposase for Ins
10	623	Putative transposase / yersinia
30	589	Phospho-N-acetymuranoyl-pen
31	725	Cell wall surface anchor
32	590	Orf hyoth
34	590	DNA-Damag-inducible protein
37	637	Hemin transport protein HMV
38	783	Cytochrome C oxidases
39	789	Hypothetical protein
40	999	(M74027) mucin (Homo sapiens)
41	999	(M74027)mucin (Homo sapiens)
42	644	Protein Y HHw > gi 125 180891
43	600	Hypothetical protein PA5101
44	624	(AP002552)deoxyribopyrimidin
45	516	(AE005566-1glycogen ph
49	757	(AE006974) PE- PGRS family protein
51	653	(AF068066) cytochrome c
54	646	(AB005245) ATP- dependent RNA
55	649	(AP002839) hypothetical protein
59	692	Extension homolog F28A2180
62	672	(K03503) threonine dehydratase
64	451	(Y14835) beta- galactosidase
65	660	(Aj231116)257r (*Vibrio cholerae*)
66	745	(Ac006585)hypothetical
70	626	Beta-galactosidase-complent-protein
73	645	Orf, hypothetical protein
75	632	Putative invasin
76	654	Ribonuclease G
77	688	Orf. Hypothetical protein

78	676	Conserved hypothetical protein
79	784	(M7 40279 mucin (homo sapiens)
81	643	NADP-alcohol dehydrogenase
83	624	(AF 285784) Clp protease
84	629	(Aj 414143) UDP-N-actylmuramoyl
86	644	(L37382) beta- galactosidase-comple..
88	801	(M7 4027) mucin (Homo sapiens)
90	628	Transcriptional regulator (lys..
92	401	(AJ 414153) putative long-chain..
93	641	(AJ 414146) putative surface ant..
94	629	KIAA0579 protein (Homo sapiens)
95	573	(Y08949) tipA (synthetic construct)
96	628	(AJ 414156) conserved hypothetic..

(A) (B)



Mr AB CD EFG HIJ KLMNO Mr AB CDE FGHI

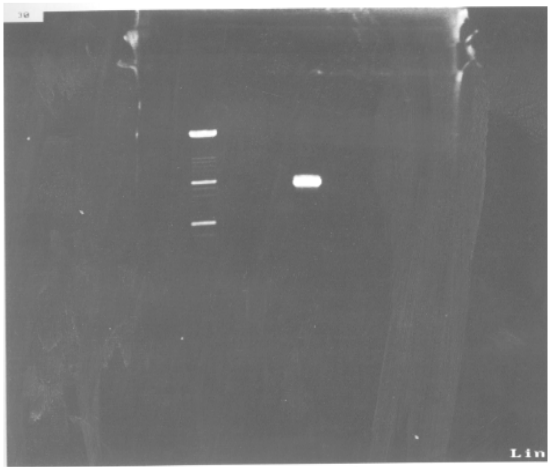
Fig. 2 : Detection of prepilin peptidase gene in different *Yersinia* strains using PCR

(A)

Mr = 100 bP DNA marker
A = *Yersinia pseudotuberculosis*
B = *Y. enterocolitica O:9*
C = *Y. enterocolitica O:3*
D = *Y. pseudotuberculosis*
E = *Y. enterocolitica O:13*
F = *Y. enterocolitica O:20*
G = *Y. enterocolitica O:21*
H = *Y. pseudotuberculosis*
I = *Y. pseudotuberculosis*
J = *Y. pestis*
K = *Y. pestis*
L = *Y. pestis*
M = *Y. enterocolitica*
N = *Y. enterocolitica*
O = *Y. enterocolitica*

(B)

Mr = 100 bP DNA marker
A = *Y. enterocolitica O:9*
B = *Y. enterocolitica O:3*
C = *Y. enterocolitica O: 13*
D = *Y. enterocolitica O:20*
E= *Y. enterocoliticaO:21*
F = *Y. enterocolitica*
G = *Y. enterocolitica*
H = *Y. enterocolitica*
I = *Y. enterocolitica*



M A

Fig. 3 : detection of the subtracted fragment DNA (75) which is invasine homolog and carried on pMos blue plasmid using PCR. M = 100 bP DNA marker. A = The subtracted fragment DNA (75)

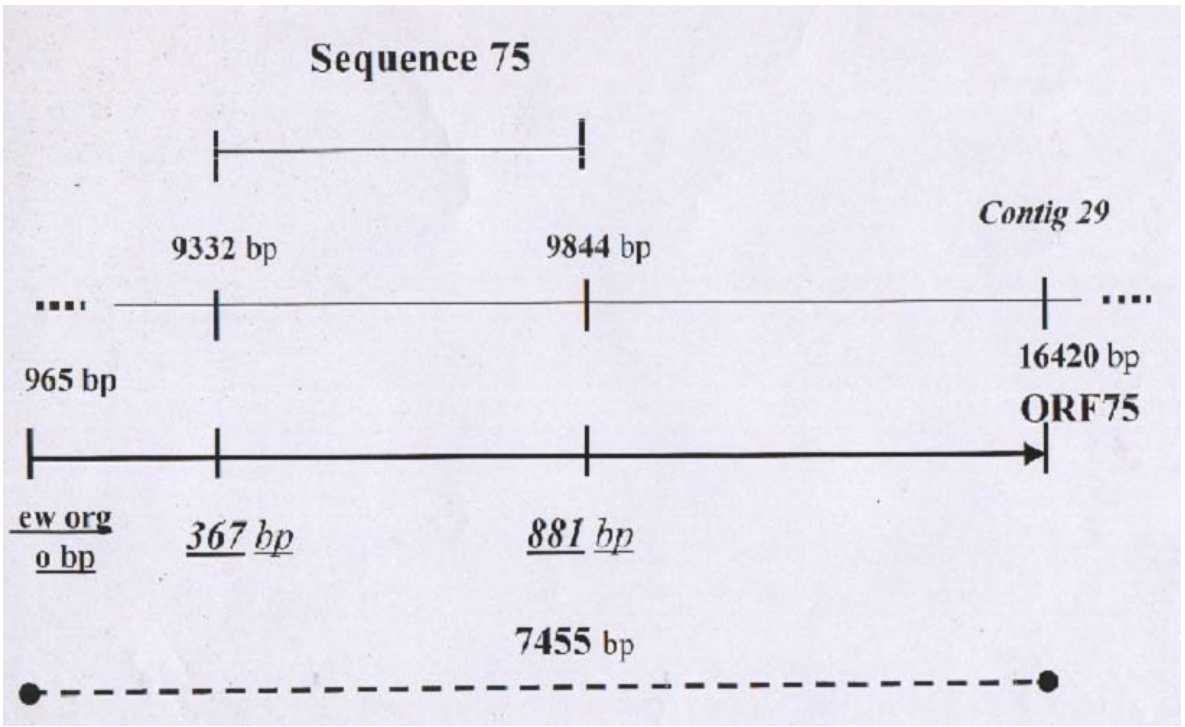
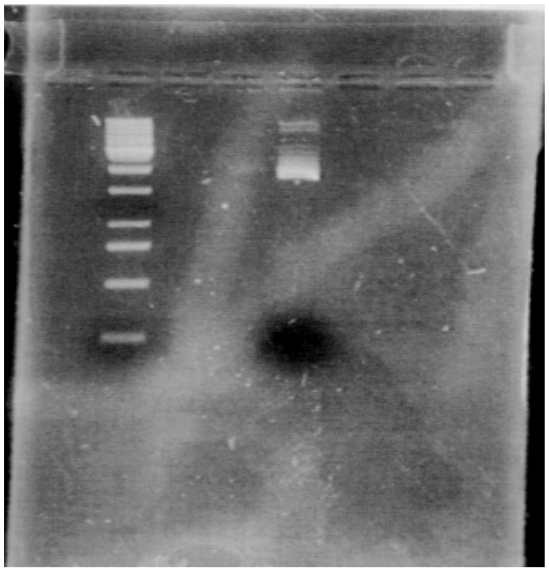


Fig. 4 : Sequence of subtracted fragment DNA-sequence No. 75 which is homologous to a part of ORF of inv-gene from the data-base of *Y. enterocolitica*



Mr A

Fig. 5 : Detection and isolation of pMos blue plasmid (75) from E.coli DH5α after ligation with Km cassette. M = 100 bP DNA marker. A = pMos blue plasmid (75) after ligation with Km cassette

IV. Discussion

For most bacterial pathogens virulence is a multifactorial process requiring two general classes of determinants. The first encompasses genes that participate in physiological processes necessary for survival in host and non-host environments and these genes are generally found in both pathogenic and non-pathogenic organisms. The second class of virulence genes specifies traits that are unique to pathogens, and not surprisingly, these genes are rarely detected in non-pathogenic organisms, based on the initial characterization of plasmids from *Yersinia* and *Shigella*. Such sequences were originally thought to be confined to extrachromosomal elements but recently, several virulence cassettes have been mapped to the chromosome of pathogenic organisms. These segments of the chromosome, termed pathogenicity islands (Groisman and Ochman., 1996; Thoerner *et al*, 2003).

Subtractive hybridization of *Y. enterocolitica* O:8 and O:5 and sequencing of the subtractive fragments after cloning, revealed many homology. The most important fragments which gives indication of the homology to virulence genes is prepilin peptidase for clone No 3. The bifunctional enzyme pripilin peptidase (pilD), is the key determinant in both type-IV pilus biogenesis and extracellular protein secretion. Pripline peptidase cleave, among other substrates, the leader sequence from pripline-like proteins that are required for type II protein secretion in Gram-negative bacteria type 4 pili, that are surface organelles required for diverse activities that contribute to board attributes, such as genetic transfer, virulence and environmental persistence of a wide array of Gram-negative bacterial pathogens (Lory and Strom, 1997; Lapointe and Taylor, 1999; Liles *et al.*, 1999).

Sequencing of the subtractive fragment clone DNA No 75 revealed homology to the Orf of Invasin gene (Inv), where the three pathogenic species of the genus *Yersinia*, invade host tissues and cause disease syndromes, ranging from gastroenteritis *(Y. enterocolitica* and *Y. pseudotuberculosis*) to plague (*Y. pestis*).

Early studies showed that pathogenic strains of *Y. enterocolitica* could invade eukaryotic cells in vitro whereas nonpathogenic strains could not. A molecular analysis of cellular penetration led to the cloning of the chromosomally encoded invasion gene (Inv) of *Y. pseudotuberculosis* Invasin, the Inv gene product, initiates the entry process directly by attaching to mammalian cell receptors, which include multiple members of the integrin superfamily subsequently, the homologous Inv gene from *Y. enterocolitica*. (Pepe and Miller, 1993; Dersch and Isbery, 1999; Grassl *et al*, 2003).

In this study, the homologous of Invasin of *Y. enterocolitica* O:8, which is not found in the other low pathogenic strains could be another Invasine, which could be called (Inv2) and this possible for the first time to be another unknown virulence gene of *Yersinia* spp., and could be the first step to discover another gene cluster which is related to virulence and that needs more work in the light of pathogenicity island concept. Particularly using the Et recombination to block the original Invasine with the kanamycine (Km) cassette and production of mutant lacking one of the most important virulence determinants, reduce the virulence of *Y. enterocolitica*. Such mutants produced by point mutation of *Y. enterocolitica* could be useful in the production of mutants applied as vaccines for pathogenic *Yersinia* infections.

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