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Penetration of HEp-2 and Chinese Hamster Ovary Epithelial Cells by *Escherichia coli* Harbouring the Invasion-Conferring Genomic Region from *Salmonella typhimurium*

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Pathogenic *Salmonella typhimurium* can invade the intestinal epithelium and cause a wide range of diseases including gastroenteritis and bacteremia in human and animals. To identify the genes involved in the infection, the invasion determinant was obtained from *S. typhimurium* 82/6915 and was subcloned into pGEM-7Z. A subclone DH1 (pSV6235) invaded HEp-2 and Chinese hamster ovary epithelial cells and contained a 4.4 kb fragment of *S. typhimurium* genomic region. Compared with the host strain *E. coli* DH1, the subclone DH1 (pSV6235) invaded cultured HEp-2 and Chinese hamster ovary cells at least 75- and 68-fold higher, respectively. The invasion rate of *E. coli* DH1 for the cells significantly increased by harbouring the genomic region derived from pathogenic *S. typhimurium* 82/6915.

Key words: cosmid, Chinese hamster ovary, HEp-2, invasion, *Salmonella typhimurium*

Pathogenic *Salmonella* spp. is the etiological agent of salmonellosis from systemic disease including typhoid fever to limited diseases such as gastroenteritis and bacteremia in humans (7, 23). *S. typhimurium* is of particular interest because the pathogen is the most common species of *Salmonella* involved in the pathogenesis of gastroenteritis in humans, and it also causes diseases in domestic animals, wild mammals, birds, and reptiles (6).

Giannella *et al.* (18) proposed the use of cultured cells as a model for *Salmonella* invasion. In the study, only *Salmonella* strains which were able to invade rabbit mucosae, had the ability to invade HeLa cells. Since then, much evidence has been reported that *Salmonella* spp. could invade the host intestinal epithelium and cause the disease (12). The invasion of *S. typhimurium* induces membrane ruffling on the surface of infected cells (13, 14). In addition, invading *Salmonella* strains can cause the rearrangement of host actin filaments and cytoskeleton-associated proteins such as α-actinin and tropomyosin (13). The phosphorylation of the protein tyrosine is also involved in the internalization of the bacteria (25). In general, the entry and invasion of the mammalian cells by *S. typhimurium* transduces the rearrangement of the cytoskeletal elements, leading to ruffling and internalization of invading bacteria. The internalized bacteria can survive from the host immune system including phagocytosis and spread to other tissue organs including the liver, spleen, lymph nodes, and reticuloendothelial systems (4, 12). Therefore, the invasion of eukaryotic cells by *S. typhimurium* is considered the primary step contributing to *Salmonella* infection. To understand invasion determinants of *S. typhimurium* 82/6915 for HEp-2 and Chinese hamster ovary (CHO) epithelial cells, a determinant was introduced into non-invasive *E. coli* DH1.

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Materials and Methods

Bacterial strains, cosmid, plasmids, and culture conditions

The bacterial strains, cosmid, and plasmids used in this study are shown in Table 1. *S. typhimurium* 82/6915 virulent to mice was cultured in Luria-Bertani (LB) medium consisting of 0.5% (w/v) sodium chloride, 1% (w/v) bacto-tryptone and 0.5% (w/v) bacto-yeast extract. *E. coli* DH1 strains harbouring a cosmid vector pLA2917 (2) or plasmid vector pGEM-7Z were cultured in LB medium containing 20 μg/ml tetracycline and 100 μg/ml ampicillin, respectively.

Purification of cosmid DNA and S. typhimurium genomic DNA

Cosmid DNA was purified using the Megaprep™ DNA

Table 1. Bacterial strains, cosmid, and plasmids used in this study

	Description	Source
Bacterial strains		
S. typhimurium 82/6915	Wild type, Inv^+	(1)
E. coli DH1	*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17(R_k^-,M_k^+), *sup*E44, *rel*A1	(21)
Cosmid		
pLA2917	Km^rTc^r (21 kb) cosmid cloning derivative of pLA2901	(2)
Plasmids		
pGEM-7Zf(+)	Ap^r*lacZ'* (3 kb)	Promega, USA
pSI623	pLA2917 carrying a 27 kb genomic region from *S. typhimurium* 82/6915, Inv^+	This study
pSV6235	pGEM-7Z carrying a 4.4 kb genomic region from pSI623, Inv^+	This study

purification column (Promega, USA) according to the instructions of the supplier. Genomic DNA from *S. typhimurium* was purified as follows. A plate of an overnight culture was suspended in 4 ml of sucrose-Tris buffer [25% (w/v) sucrose in 50 mM Tris, pH 8.3] and transferred into a 50 ml centrifuge tube. 0.05 M EDTA (pH 8.0) and 1% (w/v) SDS were added to a final concentration, mixed, and incubated for 30 min at 37°C. The cell suspension was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) by inversion and spun at 13,000 rpm for 15 min. RNase (40 μg/ml) and pronase (20 μg/ml) were added and incubated for 30 min at 37°C. The DNA solution was again extracted with an equal volume of phenol/chloroform/isoamylalcohol (25 : 24 : 1). The DNA was precipitated with 0.3 volume of 7.5 M ammonium acetate (pH 4.6) and 2 volumes of absolute ethanol, and resuspended in sterilized Milli-Q water.

Subcloning of the invasion determinant

S. typhimurium genomic DNA was partially cleaved with *Sau3*AI (Promega, USA) to generate fragments in the range of 17 to 31 kb, layered directly onto a 10 to 40% (w/v) sucrose gradient in 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 50 mM NaCl. The DNA-sucrose gradient was centrifuged for 18 hrs at 23,000 rpm in a SW 27 rotor (Beckman Instrument) at 25°C. A genomic library of *S. typhimurium* was then ligated to pLA2917 and introduced into *E. coli* DH1 using the Packagene® Lambda DNA Packaging Extract (Promega, USA), leading to the construction of a *S. typhimurium* cosmid library in non-invasive *E. coli* DH1. To define the invasive properties, the genomic region carrying the putative invasion genes derived from *S. typhimurium* 82/6915 was cleaved with *Bgl*II and eluted by the Geneclean II kit (Bresatec, Australia). The *Bgl*II digests were introduced into *E. coli* DH1 by subcloning into the *Bam*HI site of pGEM-7Z by standard methods (26).

Tissue-culture invasion assay

HEp-2 and Chinese hamster ovary (CHO) cells (CSIRO, Australia) were grown in Dulbeccos modification of Eagle's medium (DMEM) (Biosciences, Australia) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), penicillin (100 IU/ml) and streptomycin (100 μg/ml) in 24-well tissue culture plates. The cells were incubated at 37°C in a CO_2 incubator to the confluent monolayers. The recombinants, DH1 (pSI623) and DH1 (pSV6235), carrying the invasion-conferring genes were cultured overnight in LB medium containing 20 μg /ml tetracycline and 100 μg/ml ampicillin, respectively, washed twice with phosphate buffered saline (PBS) and resuspended in DMEM. The composition of PBS was 0.8% NaCl, 0.02% KCl, and 0.1% Na_2HPO_4. The HEp-2 and CHO cells were washed three times with PBS to remove any antibiotics. For invasion, aliquots of 1.5×10^6 CFU were added to the HEp-2 and CHO monolayers (2×10^4 cells in 18-mm tissue culture plates) and incubated in a CO_2 incubator for 1.5 hrs at 37°C. For the invasion assay, extracellular bacteria were killed by treating 150 μg/ml gentamicin (Sigma, USA) for 1.5 hrs at 37°C in a CO_2 incubator and the intracellular bacteria which invaded the cultured HEp-2 and CHO cells were released by the treatment of 0.5% (w/v) sodium deoxycholate for 10 min at room temperature, serially diluted, and plated on LB medium.

Results

Construction of a S. typhimurium cosmid library in E. coli

200 μg of purified genomic DNA from *S. typhimurium* 82/6915 was partially cleaved with 35 units of *Sau*3AI for 1 h at 37°C, fractionated by the gradient of 10 to 40% (w/v) sucrose, and pooled out according to the molecular weight of genomic DNA. A genomic library representing 15 to 30 kb fragments in length was cloned into the *Bgl*II site of pLA2917, leading to a *S. typhimurium* cosmid library in non-invasive *E. coli* DH1.

Subcloning of the invasion determinant

The genomic region of pSI623, which conferred the invasion of cultured HEp-2 and CHO cells by *S. typhimurium* 82/6915, was completely digested by *Bgl*II. The resulting 7.4-, 6.3-, 5.5-, and 4.4 kb of the genomic regions from pSI623 were successfully ligated to the *Bam*HI site of the

plasmid vector, pGEM-7Z, producing the subclones carrying the genomic fragments from pSI623 involved in the invasion of the cells (Fig. 1).

Tissue culture invasion assay

Invasion of cultured HEp-2 and CHO cells was estimated by infecting the *S. typhimurium* cosmid library constructed in non-invasive *E. coli* DH1. Out of approx. 1,000 clones a *S. typhimurium* cosmid, DH1 (pSI623), invaded the cultured cells (Table 2). Compared to *E. coli* DH1 carrying pLA2917, the invasion efficiency of DH1 (pSI623) increased at least 26 times. The 27 kb fragment of the genomic region conferring the invasive capacity to non-invasive *E. coli* DH1 was completely digested by *Bgl*II and subcloned into pGEM-7Z (Fig. 2). Cultured HEp-2 and CHO cells were infected by the subclones. The subclone DH1(pSV6235), which carried a 4.4 kb fragment of the genomic region from pSI623 was easily able to invade the cells (Table 2). Other subclones, DH1 (pSV6232), DH1 (pSV6233) and DH1 (pSV6234), did not significantly invade cultured HEp-2 and CHO cells. In comparison with *E. coli* DH1 carrying pGEM-7Z, the invasion efficiency of DH1(pSV6235) increased more than 68 fold. The invasion efficiency of non-invasive *E. coli* DH1 was significantly improved by harbouring the invasion determinant.

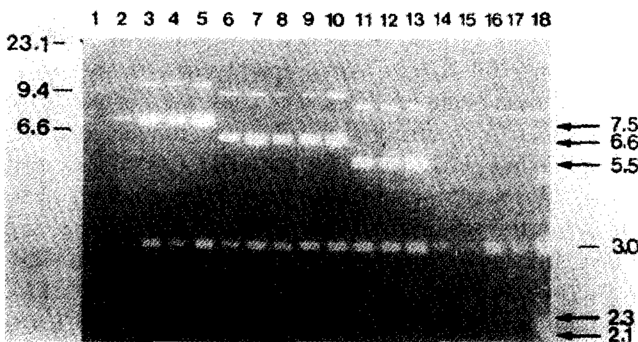

Fig. 1. Restriction cleavage of the subclones carrying the potential invasion genes from pSI623 conferring the invasion of HEp-2 and CHO epithelial cells. Lane 1, λ / *Hin*dIII; lanes 2 to 5 (pSV6232) carried 7.5 kb fragments from pSI623; lanes 6 to 10 (pSV6233), 6.3 kb fragments from pSI623; lanes 11 to 13 (pSV6234), 5.5 kb fragments from pSI623, lanes 14 to 17, 0.8 kb fragments from pSI623 (not shown in the gel); lane 18, 4.4 kb fragments from pSI623. DH1(pSV6235) was able to invade cultured HEp-2 and CHO cells. The numbers on the right and left are molecular mass markers in kb. The arrow indicates the inserts from pSI623.

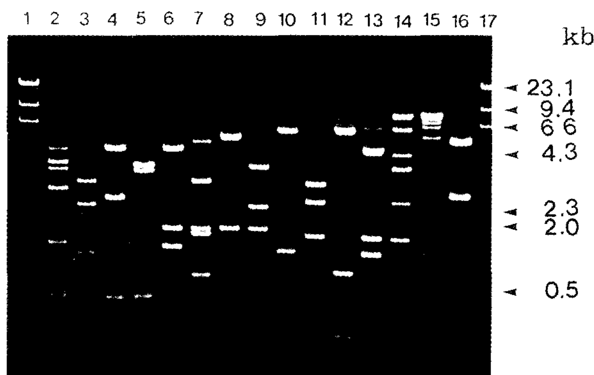

Fig. 2. Restriction digestion of pSV6235 carrying the 4.4 kb genomic region involved in the invasion. Lanes 1 and 17: λ / *Hin*dIII. Lane 2: pSV6235 / *Eco*RI + *Hin*dIII. Lane 3: pSV6235 / *Eco*RI + *Sac*I. Lane 4: pSV6235 / *Eco*RI + *Kpn*I. Lane 5: pSV6235 / *Eco*RI + *Eco*RV. Lane 6: pSV6235 / *Eco*RI + *Sal*I. Lane 7: pSV6235 / *Hin*dIII + *Sac*I. Lane 8: pSV6235 / *Hin*dIII + *Kpn*I. Lane 9: pSV6235 / *Hin*dIII + *Eco*RV. Lane 10: pSV6235 / *Hin*dIII + *Sal*I. Lane 11: pSV6235 / *Sac*I + *Kpn*I. Lane 12: pSV6235 / *Sac*I + *Eco*RV. Lane 13: pSV6235 / *Sac*I + *Sac*I. Lane 14: pSV6235 / *Kpn*I + *Eco*RV. Lane 15: pSV6235 / *Kpn*I + *Sal*I. Lane 16: pSV6235 / *Eco*RV + *Sal*I.

Discussion

Pathogenic enteric bacteria including *Salmonella* strains invade mammalian epithelial cells and cause a wide spectrum of diseases including enteric fever, gastroenteritis, bacteremia, and focal infections in humans as well as animals (2). The pathogenesis of the *Salmonella* infections is attributed to a series of functions; 1) entry through adherence and invasion of the bacteria into the mammalian cells, 2) selection of a unique niche within the mammalian cells and evasion of the host immune response, and 3) multiplication (11).

The mechanisms for *Salmonella* infection have been explained using cultured epithelial cells (5, 10, 18, 24). A genetic locus *inv* region, which conferred the ability to invade tissue culture cells to a non-invasive *E. coli* strain, was identified from genomic DNA of *S. typhimurium* (15). Groisman and Ochman (20) isolated a gene cluster involved in the invasion of eukaryotic cells by *S. typhimurium* and *S. flexneri*, which is similar to the gene cluster mediating the presentation of surface antigens (*spa*) on the plasmid involved in the *Shigella* infection. The monolayers of HEp-2 epithelial cells were used to identify the

Table 2. Invasion of HEp-2 and CHO epithelial cells by bacterial cells

Strains	Invasion efficiency[a]	
	HEp-2	CHO
S. typhimurium 82/6915	0.418 ± 0.042	0.351 ± 0.041
E. coli DH1	<0.001	<0.001
DH1 (pLA2917)	<0.001	<0.001
DH1 (pGEM-7Z)	<0.001	<0.001
DH1 (pSI623)	0.035 ± 0.004	0.027 ± 0.003
DH1 (pSV6235)	0.081 ± 0.006	0.073 ± 0.005

[a]Invasion efficiency [%] = 100 × (number of bacteria resistant to intracellular killing by gentamicin/total number of bacteria added). The values shown are the averages of at least three independent experiments done in duplicate (mean ± SD).

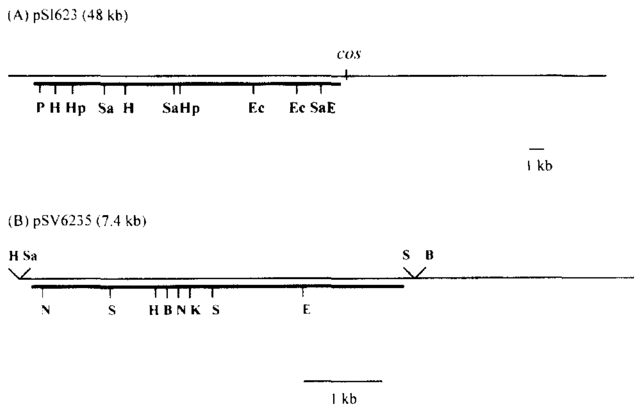

Fig. 3. The plasmid constructs of the invasion-conferring recombinants. (A) The plasmid construct carrying a 27 kb genomic region from *S. typhimurium* 82/6915. (B) The plasmid construct carrying a 4.4 kb genomic region from pSI623. The size of the constructs is indicated next to the recombinants. The thick line represents the genomic region of *S. typhimurium* 82/6915 carrying the invasion determinants. B, *Bst*XI; E, *Eco*RI; Ec, *Eco*91I; H, *Hind*III; Hp, *Hpa*I; K, *Kpn*I; N, *Nsi*I; P, *Pst*I; S, *Sac*I; Sa, *Sal*I.

gene cluster of *S. typhimurium*, *spa* region. The *invABC* (9, 16, 17), *invD* (16), *invE* (19), *invFG* (22), *invH* (3), and *invIJ* (8) were subsequently defined in genomic DNA of *S. typhimurium*.

The invasion of the monolayers of HEp-2 and CHO cells by the clone DH1 (pSI623) and a series of subclones, which carried 7.4-, 6.3-, 5.5-, and 4.4 kb fragments of the genomic regions from pSI623, was measured to derive the invasion characters for the cells. The invasion efficiency of HEp-2 and CHO monolayers by DH1(pSV6235) were 75- and 68 times higher than that of *E. coli* DH1 strains harbouring pLA2917 and pGEM-7Z, respectively (Table 2). In comparison to *S. typhimurium* 82/6915, the lower invasion efficiency of DH1(pSI623) and DH1(pSV6235) were presumably due to the intracellular replication of *S. typhimurium* within the cultured cells during a tissue-culture invasion assay. In addition, both DH1(pSI623) and DH1(pSV6235) may not have produced unique transcription and translation products of wild type *S. typhimurium* 82/6915.

Restriction enzyme analysis showed that the 4.4 kb fragment of the genomic region from pSI623 was different from the *inv* and *spa* gene clusters. The genomic region of the invasion-conferring clone, pYA2219 carried two *Bgl*II sites on the *invABC* operon (16). The 6.4 kb of the *spa* gene cluster carried a single *Bgl*II site. However, there was no *Bgl*II site in the 4.4 kb fragment of the genomic region of pSV6235 because of cohesive end ligation between the linearized *Bgl*II sites of pSI623 and *Bam*HI sites of pGEM-7Z (Fig. 3). The identification of the genes contributing to the invasive properties of *S. typhimurium* could lead to a clearer understanding of the host-pathogen interaction.

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