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以標籤式突變法分析 Salmonella choleraesuis 活體內毒力因子(2/3)

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計畫名稱：以標籤式突變法分析 *Salmonella choleraesuis* 活體內毒力因子(II): *Salmonella choleraesuis* 活體內表現之基因篩檢(2/3)

計畫編號：NSC 92-2313-B-002-136

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中文摘要

以標籤式突變法 (STM) 鑑定豬霍亂沙門氏菌在豬隻活體內存活有關基因。 將獨特 DNA 序列標籤 (tags) 築入具抗康黴素基因標誌 (marker) 之自殺性質體載體-pUTminiTn5Km2, 以接合作用將此載體送入對康黴素有感受性之豬霍亂沙門氏菌體內，構築 STM 基因庫。 帶有獨特 DNA 序列標籤之自殺性載體藉跳躍子突變 (transposon mutation) 插入抗康黴素豬霍亂沙門氏菌體內形成突變株。 960 個突變株組合為 20 群 (pool), 每群含 48 個突變株。 每群突變株接種 2 頭豬隻, 篩檢活體內存活有關基因。 經活體篩檢後, 有 34 株不能由腸系膜淋巴結回收，此 34 株可被認定為毒力弱化之突變株。 利用逆向 PCR 增幅嵌入康黴素基因兩端區域并定序之。 定序結果顯示，此 34 株突變區域各異，可歸為八類基因：(1) 細胞封套，(2) 第三型分泌系統，(3) 毒力質體，(4) 運送，(5) Gifsy-1 前噬菌體蛋白，(6) 調控基因，(7) 代謝，及(8) 未知功能。

關鍵詞：豬霍亂沙門氏菌，標籤式突變法，毒力因子， 活體內表現， 突變種

Genetic analysis of the *in vivo* virulence factors by signature-tagged mutagenesis (II): Screening the *in vivo* expressed genes in *Salmonella* Choleraesuis (2/3)

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ABSTRACT

A signature-tagged mutagenesis (STM) system was adapted to identify the genes required for in vivo survival of *Salmonella enterica* serovar Choleraesuis (*Sal.* Choleraesuis) in pigs. An STM library was constructed in *Sal* Choleraesuis (kanamycin sensitive) by conjugation with a suicide plasmid vector (pUTminiTn5Km2) which contains unique DNA sequence tags and a kanamycin resistance marker. *Sal* Choleraesuis (kanamycin-resistant) mutants were obtained by transposon mutation, resulting in gene inactivation by insertion of the suicide vector carrying a unique tag. 960 mutants of *Sal* Choleraesuis were pooled together (48 mutants/pool) and screened in a pig model. Out of 960 mutants, 34 of them were not recovered from the mesenteric lymph node. These 34 mutants were considered as virulence-attenuated mutants. In order to identify the virulence genes, the regions flanking the kanamycin gene of the attenuated mutants were amplified by inverse PCR and sequenced. The sequence analysis showed that the 34 mutants had insertions in 34 different genes that could be grouped in eight classes: (i) genes encoding cell envelope; (ii) type III secretion system; (iii) gene encoded in a virulence plasmid; (iv) genes involved in transport; (v) genes coding for Gifsy-1 prophage protein; (vi) genes encoding proteins involved in regulatory; (vii) genes involved in metabolic pathways; and (viii) DNA sequences that showed similarity to hypothetical genes with unknown function.

Keywords: *Samonella* Choleraesuis, signature-tagged mutagenesis, virulence factor, in vivo expression, mutant

INTRODUCTION

Salmonella enterica serovar Choleraesuis (*Sal.* Choleraesuis) is host-adapted pathogen caused swine paratyphoid. It was the common cause of salmonellosis in swine and occasionally in people. This gram-negative bacterium is a facultative intracellular pathogen and is usually manifested as septicemia. To prevent the infection of this bacterium in swine and/or human is needed. Understanding of the virulence factors encoded by this pathogen will not only lead to the development of new vaccines and/or treatments but also facilitate to unravel the pathogenicity of this pathogen.
Signature-tagged mutagenesis (STM) is one of several recently developed techniques, which will lead to the identification of genes required for in vivo survival or infection [Hensel *et al.* 1995]. STM allows the large-scale screening of mutants for reduced survival in vivo, colonization and adhesion defects, and decreased invasive ability [Chiang and Mekalanos, 1998].

In this study, we report the screening of an STM bank of *Sal.* Choleraesuis mutants in a pig model to identify potential host-specific virulence factors.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

The mutant bank was constructed during the first year project. Bacteria were grown on Luria-Bertani (LB) medium with additional antibiotics where appropriate at the following concentrations: nalidixic acid (nal), 20μg mL^{-1}; kanamycin (kan), 50μg mL^{-1}.

Animals, husbandry, and housing

White crossbred piglets (*n* = 40), 5-9 weeks of age, were used in the present study. These animals were in overall good health, free of clinical signs of enteric diseases and negative for *Salmonella* species by microbiological culture and serology. Pigs were housed in separate rooms in a biosafety. Animals were acclimated to the diet and facilities for 5 days prior to initiation of the study. Pigs were fed a non-medicated diet, and feed and water were provided *ad libitum* except where noted

Infection studies

Frozen plates of pooled *Sal.* Choleraesuis transposon mutants were removed from −80°C storage and subcultured by transferring 20 μL from each well to a new 96 well plate (Corning Costar) containing 180μL of LB (nalidixic acid 20 μg/mL + kanamycin 50 μg/mL). Plates were incubated with shaking (50 rpm) overnight at 37°C. Each plate was pooled to form the ‘‘input pool’’. One mL of the input pool (48 mutants) containing $2x10^9$ mutant cells in PBS were given to pig by intranasal route and rest of them was used for the preparation of the input pool chromosomal DNA. The inoculum was verified by viable counts after plating serial dilutions of the bacterial suspension on selective LB agar to determine the colony forming units (cfu). Each mutant pool was used to infect two animals. At approximately 5 days [Lawson and Dow, 1965] post infection, surviving animals were humanely killed and organs were removed aseptically [Gray *et al.*, 1995] and homogenized in deionized water. Aliquots of the bacterial suspension were plated on selective media and following growth overnight at 37°C, approximately 10,000 colonies were pooled (the "output pool") and total chromosomal DNA was prepared.

DNA manipulations and hybridization.

Chromosomal DNA from *Sal.* Choleraesuis was extracted by the CTAB method. For dot blots, duplicate hybridization membranes were prepared from the master plate of *E. coli* containing 48 plasmid tags. Chromosomal DNA from input pool and recovery pool were used as template for the preparation of radiolabeled tags. Constant regions were removed by digestion with *Hin*dIII and the tags were used to probe duplicate hybridization membranes. Hybridizations were performed according to standard molecular biology techniques [Sambrook et al., 2001].

Characterization of transposon insertion sites

To determine whether 34 mutants harbored unique mini-Tn5 insertions, we carried out Southern hybridization analysis. Transposon flanking sites were amplified by inverse PCR as previously described [Hensel *et al.*, 1995] and subcloned by TA cloning kit (Invitrogen, CA, USA). Alternatively, DNA from mutants was digested with *Eco*RV, ligated into *Sma*I restricted enzyme cut cloning vector pUC19 and followed by transformation into *E. coli* CC118. The transformants (Km^r) were subjected to DNA sequencing. Sequence similarity searches were carried out by using Blast (NCBI, National Center for Biotechnology Information).

RESULTS and DISCUSSION

STM screen of *Sal.* Choleraesuis insertional mutants bank in pigs.

A total of 960 clones (20 pools each containing 48 mutants) were screened for loss of virulence in a pig model. Approximately 2 x 10^9 cfu of each pool were intranasally inoculated into two pigs per pool. A post-infection time period of 4 to 5 days was based on the results of a previous study in recovery of *Sal.* Choleraesuis from swine (Lawson and Dow, 1965). Five days post infection, two pigs were sacrificed and the bacterial load in the spleens and mesentery lymph nodes were evaluated (Gray *et al.*,

1995). More than 1 x 10^4 cfu were recovered from the mesentery lymph nodes (approximate 2 g). Recovery of bacteria from the spleen was variable. Therefore, the mesentery lymph nodes were chosen to recover the bacteria. For each pig, 10^4 colonies were harvested (recovered pool) and used to amplify the tags. These tags were labeled with [^{32}P] (dCTP, NEN Life Science Products, Inc.) and used as probes to screen a membrane onto which the 48 different tags had been transferred. Probe tags giving a strong hybridization signal from the input pool and weak signals from the two recovered pools allowed to identify the attenuated mutant candidates (Fig. 1). A total of 34 potential attenuated mutants were selected for further study.

Identification of disrupted genes

Southern blot analysis of all attenuated mutants confirmed the presence of single insertion (Fig. 2). The nucleotide sequence of the DNA flanking the site of transposon insertion obtained for each of the 34 attenuated mutants was used to search the GenBank databases for homologous genes. The putative identities listed in Table 1 were assigned by comparison with bacterial databases and were organized into eight classes: cell envelope, type III secretion system, virulence plasmid, transport, Gifsy-1 prophage protein, regulation, metabolism and unknown gene function.

The first category encodes proteins involved in cell envelope. Mutants 9C5 and 19C11 were disrupted in sequences related to *manC* and *wzzE*, respectively, which are involved in lipopolysaccaride biosynthesis. Additionally, a transposon insertion was identified in *steB* (strain 3A5) that encodes a fimbrial usher protein. This protein involved in biogenesis of gram-negative bacterial pili.

The second category encodes proteins involved in type III secretion system. Sequence

analysis of seven attenuated strains (7B9, 10A9, 9C11, 10C11, 15C6, 8D12, and 19B5) showed that the transposon was inserted in genes involved in the *Salmonella* pathogenicity island (SPI-1) (*invH*, *hilA*, *sipC*, *invA*, *spaP*, *sprB*, and *hilD*). The products of the *inv/spa* gene cluster are proteins that form a type III secretion system required for the assembly of surface appendages mediating entry into epithelial cells. The *sipC* encodes cell invasion protein, SspC that involved in translocation of secreted proteins into host cell. High osmolarity, low aeration, and slightly basic pH enhanced transcription of *hilA*, one of the central regulatory proteins. HilD is one of the important environmental sensors. HilD and HilC activate HilA and all 3 activate *InvA-F* genes. Sequence analysis of five attenuated strains (8D2, 10B1, 6F7, 12B12, and 10C2) showed that the transposon was inserted in genes involved in the SPI-2 (*spiR, spiA, spiB, ssaJ, and ssaV*). Mutant 8D2 contains transposon insertion in *spiR* (*ssrA*), which encodes a putative component that regulates the expression of the type III secretion system of SPI-2 [Ochman *et al.*, 1996].

The third category of gene is associated with a 50-kilobase virulence plasmid. Mutant 14A4 contains an insertion in *tlpA* that encodes a possible virulence-associated regulatory protein, TlpA, with a function as a thermometer by regulating its own transcription according to temperature [Haneda et al., 2001].

Transposon insertion was also found in genes with transport function. Strain 6F9 contains transposon insertions in *dgoT* encoding D-galactonate transport. The *dgoT* (*yidT*) gene is a member of the major facilitator superfamily (MFS) of transporters (Pao et al., 1998). It is likely that DgoT functions as a galactonate/proton symporter. Imported galactonate is modified and cleaved by the enzymes derived from the *dgo* operon to yield glyceraldehyde 3-phosphate and pyruvate. Strain 14D7 contains

transposon insertion in *ydiN* that encodes an amine transport protein. The YdiN protein is an uncharacterized member of the major facilitator superfamily (MFS) of transporters [Pao et al., 1998]. Based on sequence similarity, it may be function as a proton-driven metabolite uptake system. Strain 8A1 contains transposon insertion in *ybaE* that encodes a putative ABC transporter periplasmic binding protein. Strain 16F6 contain transposon insertions in *ydeD* that encodes a probable cysteine metabolite efflux pump [Dassler et al. 2000].

A fifth category represents genes are encoding component of Gifsy prophage protein. Strain 14A4 contains transposon insertion in STM2626 that encodes a Gifsy prophage protein. Several lines of evidence suggested that the Gifsy prophage protein contributes to *Salmonella* virulence [Figueroa-Bossi and Bossi, 1999; Figueroa-Bossi .et al., 2001]. In *Salmonella* Typhimurium, Gifsy-1 bacteriophage protein is important for the survival in the Peyer's patches of BALB/c mice [Stanley et al., 2000].

The sixth category of gene is associated with regulatory mechanism. Strain 15D12 contains transposon insertion in *clpB* that encodes an ATP-dependent protease. The ClpB protein is a heat shock protein for intracellular protein degradation and is important for virulence of *Salmonella* [Turner *et al.*, 1998]. Strain 13A4 contains transposon insertion in *minC* that encodes a cell division inhibitor; which inhibits FtsZ ring formation when activated [Hu et al., 2003]. Therefore, the evidence indicates that MinC from *Neisseria gonorrhoeae* is essential for maintaining proper division [Ramirez-Arcos et al., 2001].

The seventh category of genes is associated with metabolism. Strain 2C3 contains

transposon insertion in *idnD* that encodes a L-idonate 5-dehydrogenase. L-idonate 5-dehydrogenase catalyzes the reversible oxidation of L-idonate to 5-ketogluconate. The enzyme specifically oxidizes L-idonate using NAD and catalyzes the specific reduction of 5-ketogluconate using NADH or NADPH [Bausch et al., 1998; Peekhaus and Conway. 1998]. Strain 10B3 contains transposon insertion in STM1002 that encodes a diaminopropionate ammonia lyase.

Analysis of DNA sequences for 9 STM mutants revealed unknown function.

The STM technique has been used successfully to identify attenuated transposon insertion mutants of *Salmonella* spp [Hensel et al., 1995; Shea *et al.*, 1996; Tsolis et al., 1999; Bispham et al., 2001; Lichtensteiger and Vimr, 2003]. Application of STM to *Salmonella* led to the identification of a number of virulence genes and discovered the second type III secretion system that plays an important role in salmonella pathogenesis.

Genetic analysis of *Sal.* Choleraesuis has identified a number of genes that are important for virulence, including virulence plasmid such as *tlpA* [Rotger and Casadesus, 1999; Haneda et al., 2001], type III secretion system such as *hilA* [Maddox et al., 1997] and *inv* [Altmeyer et al., 1993]. Surface protein mutants decreased to pass through epithelial barrier [Finlay et al., 1988]. In *Salmonella*, reduced amounts of LPS affect both stress tolerance and virulence [Thomsen et al., 2003]. The *spi* within *Salmonella* encodes a regulator and a type III secretion system essential for virulence in mice and survival in macrophages [Ochman et al., 1996].

This study described the first global research of genes important for the pathogenicity of *Sal.* Choleraesuis. It allowed the identification of some different loci that were not previously associated with virulence in *Salmonella*. In the third year, we will continue to perform a competitive index (C.I.) test. The C.I. is a sensitive measure of the relative degree of virulence attenuation of a particular mutant in mixed infection with the wild-type strain. Certain mutants will be chosen for safety and vaccine efficacy test in swine.

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Table 1. Classes of genes identified by STM screens in pigs

No. of Mutant	Classification	Homology	Hypothesized functional		
Mutants					3
Cell envelope		19C11	wzzE	LPS biosynthesis	
			9C5	manC	mannose-1-phosphate guanyltransferase
			3A5	steB	outer membrane usher protein
12	Type III secretion system				
	pathogenicity island (SPI-1)	7B9	invH	cell adherance/invasion protein	
			10A9	hilD~hilA	
			8D12	sprB	Transcriptional regulator
			9C11	sipC	cell invasion protein
			10C11	invA	invasion protein
			15C6	spaP	surface presentation of antigens
			19B5	hilD	araC family
	pathogenicity island (SPI-2)	10B1	spiA (ssaC)	putative outer membrane secretory protein	
			6F7	spiB (ssaD)	virulence protein
			8D2	spiR (ssrA)	secretion system regulator: Sensor component
			12B12	ssaJ	secretion system apparatus protein
			2D2	ssaV	secretion system apparatus protein
1	Virulence plasmid	14A4	tlpA	TlpA	
4	Transport	1B4	ybaE	ABC transporter family	
			6F9	dgoT	MFS family transport
			14D7	ydiN	MFS family transport
			16F6	ydeD	Cysteine metabolite efflux pump
1	Gifsy-1 prophages protein	4A9	STM2626	Replication protein	
2	Regulatory	15D12	clpB	Clp family of oligomeric ATPases.	
			13A4	minC	Cell division inhibitor
2	Metabolism		2C3	idnD	L-idonate 5-dehydrogenase
			10B3	STM1002	putatiave diaminopropionate ammonia lyase 9
Unknown function		7B3	STM1459	oxidoreductase protein	
			14B6	STM3125	putative cytoplasmic protein
			7D11	ygiF	putative cytoplasmic protein
			1B3	STM2800	putative inner membrane protein
			4C11	STM0342~STM0343	
			8C2	orf~ssrB	
			9D11		putative bacteriophage tail protein
			14D11		hypothetical protein
			19D11		hypothetical protein

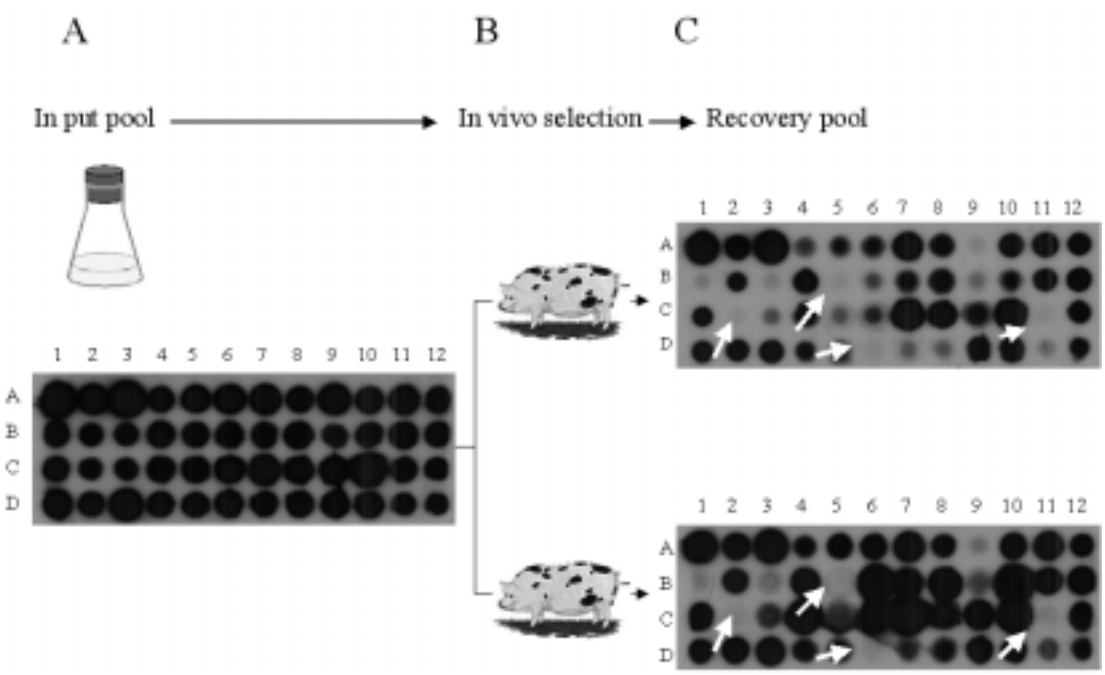

Fig 1. Negative selection of *Salmonella* Choleraesuis mutants. Dot blot hybridization results of STM generated *Sal.* Choleraesuis mutants that were unable to survive in pig. Tags were amplified from a mixed mutant inoculum and probed against a DNA dot blot of the 48 different tags on filters (A); two 5-7 weeks old pig were given exactly the same inoculum of $2x10^9$ cfu of mutants (B); surviving mutants were recovered from the mesentery lymph node 5 days post-infection, and tags were amplified from recovered pools and probed again (C). The mutant in well B5, C2, C11 and D6 are attenuated mutants (white arrow).

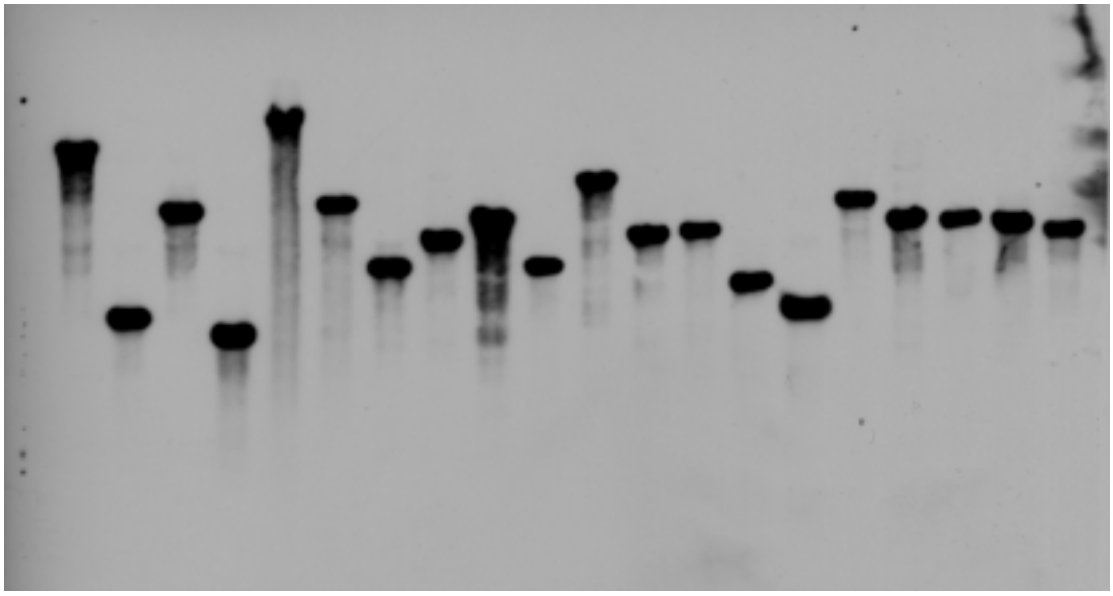

Fig. 2. Southern blot hybridization analysis of 20 of 34 putative attenuated *Sal.* Choleraesuis mutants. Chromosomal DNA was digested with *EcoR*V. All showed a single insertion in different sites. A labeled *kan* cassette from mini-Tn5 was used as probe for this study.