行政院國家科學委員會三年期專題研究計畫之第三年成果報告

計畫名稱：***Salmonella choleraesuis* 之鐵調節基因與毒力表現(3/3)**

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*本計畫部份結果已投稿於**DNA Sequence**期刊並審查通過接受刊登。*

題目：Cloning and Characterization of an Iron Regulated Locus, iroA, in Salmonella entrica serovar Choleraesuis.

中文摘要

豬霍亂沙氏桿菌（*Salmonella choleraesuis*, SC）是豬隻常見細菌性病原，感染豬隻會出現腸炎、肺炎與敗血症等不同的臨床症狀。SC 亦可感染人類，爲人畜共通傳染病病原。SC 的分子致病機制目前並不清楚。由鼠傷寒沙氏桿菌（*Salmonella typhimurium*, ST）的研究顯示，許多存在染色體之毒力基因聚在一起，稱爲沙氏桿菌致病島（*Salmonella* pathogenicity island, SPI）。其中第 1 和第 2 ST 致病島（SPI-1, SPI-2）屬於第 3 型分泌系統（type III secretion system）。已知 SPI-1 與 ST 侵入上皮細胞有關，而 SPI-2 則與 ST 在巨噬細胞存活複製及全身性感染有關。利用聚合連鎖反應與南 SC 也具有驗 SPI-1 與 SPI-2。本實驗選殖與定序 SC 的 *hilA* 與 *spiCAB* 基因，這些基因與 ST 相似性很高。利用對偶基因交換技術（allelic exchange）建構 SC 的 *hilA* 與 *spiC* 突變株，此二突變株的 LD_{50} 與野外型相同； *hilA* 突變株對 HeLa 細胞的侵入能力較野外型弱，但 *spiC* 突變株的侵入能力則與野外型相同。由此實驗結果可以提供研發 SC 減毒活菌疫苗之依據。

關鍵詞：豬霍亂沙氏桿菌，沙氏桿菌致病島，*對偶基因交換技術*，致病機制

Construction, Genotypic and Phenotypic Characterization of mutant strains of type III Secretion systems of *Salmonella choleraesuis*

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ABSTRACT

Salmonella choleraesuis is a common swine bacterial pathogen. The infected pigs show septicemia, enterocolitis, and pneumonia. *S. choleraesuis* is also an important zoonotic pathogen. The molecular pathogenesis of *S. choleraesuis* is not clear. In *S. typhimurium*, many virulence genes, clustered together on chromosome, are *Salmonella* pathogenicity island (SPI). SPI-1 and SPI-2 encode structurally similar but functionally distinct type III secretion systems. The SPI-1 plays a role in invasion of epithelial cells. The SPI-2 is required for bacterial proliferation in macrophage and systemic infection. This study proved that *S. choleraesuis* had both of SPI-1 and SPI-2 by Southern hybridization and polymerase chain reaction. The PCR products of *hilA* and *spiCAB* genes of *S. choleraesuis* were cloned and sequenced. These genes had high homology with *S. typhimurium*. The *hilA* mutant and *spiC* mutant were constructed by allelic exchange. The LD_{50} of *hilA* mutant was similar to that of wild *S. choleraesuis*. The *hilA* mutant was less invasive for HeLa cell, however, *spiC* mutant had the same invasion ability with wild type. These data paved the way to develop live *S. choleraesuis* vaccine.

Key words: *Salmonella choleraesuis, Salmonella* pathogenicity island, allelic exchange, *pathogenesis*

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INTRODUCTION

Of the more than 2,400 species of *Salmonella*, all seem capable of causing disease in humans. Some strains (host-adapted) are especially adapted to a single host, such as *S. typhi* in humans, *S. choleraesuis* in pigs, and *S. pullorum* in poultry. *S. typhimurium* and *S. dublin* are two of the well-known strains that are universal serovars. Salmonellosis is one of the most significant infectious diseases of pigs. It is a serious worldwide problem causing swine disease estimated to cost $100 million annually in the United States. It is clinically characterized by one of three major symptoms: a peracute septicemia, acute enteritis, or a chronic enterocolitis. The septicemia is usually seen in young animals and is generally caused by *Salmonella choleraesuis*. The mortality rate may reach 100 %, with death frequently occurring within a few days. Salmonellas also are important human pathogens and are of great concern in food poisoning. Children are the most likely to get septic salmonellosis.

The molecular pathogenesis of *S. choleraesuis* is unclear. Recent works have shown that a significant proportion of *S. typhimurium* virulence genes are located in distinct chromosomal regions called *Salmonella* pathogenicity islands (SPI). *S. typhimurium* has five SPIs (SPI-1 to SPI-5). SPI-1 and SPI-2 are two pathogenicity islands, located at 63 and 30 centisomes, respectively, containing genes that encode two distinct type III secretion system. SPI-1 is required for the membrane ruffling and invasion of epithelial cells, induction of apoptosis in macrophages, and interleukin 8 productions. SPI-2 is necessary for *Salmonella* proliferation within macrophages and bacterial growth during systemic infection. In this study, we cloned and sequenced the partial genes of SPI-1 and SPI-2, constructed mutant strains by allelic exchange. Furthermore we analyzed virulence for the LD_{50} in mice and the invasiveness and intracellular survival of HeLa cell.

MATERIALS AND METHODS

Bacterial strains and growth conditions

All bacterial strains, and plasmids used in this study are described in Table 1. Bacterial strains were maintained at –70°C until used. All bacteria were cultured aerobically at 37°C in Luria-Bertani (LB) broth or on LB agar plates as required. The appropriate antibiotics were added to the medium in the following concentrations: ampicillin (Ap), 50 μg/mL; chloramphenicol (Cm), 35 μg/mL, kanamycin (Km), 50 μg/mL

Recombinant DNA techniques.

Chromosomal and plasmid DNA extractions, restriction endonuclease digestions, DNA ligations, plasmid transformations, and dot-blot hybridizations were performed according to standard methods. Cloning of DNA fragments was performed according to standard protocols (Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The PCR products of *hilA* and *spiCAB* genes were cloned into pCR vector (Invitrogen) and sequenced. The PCR primers used in this study were listed below. HILA-1 (5`-TCATCTAGATTTTCACC CTGTAAGAGA-3`), HILA-2 (5`-TACTCTAGAAACCAGATTACGATGATA-3'), SPICAB-1 (5`-ATCTCTAGATT TTACGGATGTGGTTGT-3'), and SPICAB-2 (5`-TACTCTAGAACCGGGTCAAAGTTGTCA-3`) were used for *hilA* and *spiCAB* genes cloning. HILA-1 and HILA-5 (5`-CTACCCGGGGTCGGGAGTTTGCTATTC -3`), HILA-6 (5`-GTACCCGGGCCGAACTATCTCAATCTG-3`) and HILA-2 were used to construct the *hilA* mutant. SPIC-5 (5`TTTGAATTCTTTTTGCGGGATGTAT GA-3`) and SPIC-6 (5`-CTTCCCGGGTCCCTCCTCAGACATAAA-3`), SPIC-7 (5`-

TTTCCCGGGCTT TATTCGGGTGGGGTA-3`) and SPIC-8 (5`-TTAGAGCTCAGC CACTTTTTCTCCCTG-3`) were used to construct the △*spiC* mutant.

Construction of mutants by allelic exchange△*hilA* SC

The △*hilA* gene fragments were amplified by PCR with HILA-1 and HILA-5, HILA-6 and HILA-2.The PCR products were digested with appropriate restriction enzymes, ligated with *aph*A-3 fragment, subcloned to the suicide vector pRE118 of *E. coli* cc118. This suicide vector was subcloned to *E. coli* S17-1(*pir*) to conjugate with *S. choleraesuis* wild type, and screened the conjugates by LB plates containing 10% sucrose or kanamycin.△*spiC* SC

The △*spiC* gene fragments were amplified by PCR with SPIC-5 and SPIC-6, SPIC-7 and SPIC-8. The PCR products were digested with appropriate restriction enzymes and subcloned to the suicide vector pRE118 of *E. coli* cc118. This suicide vector was subcloned to *E. coli* S17-1(l *pir*) to conjugate with *S. choleraesuis* wild type, and screened the conjugates by LB plates containing 10% sucrose or kanamycin.

Experimental animals Six to eight–week-old female BALB/c mice were obtained from Laboratory Animal Center, National Taiwan University College of Medicine.

Determination of LD50 Mice were inoculated oral or intraperitoneally (I.p.) with 0.1 ml volume of serial-diluted bacterial culture (dose from 1 to 10^8 CFU/ml) or LB. Animals were observed regularly over 3 weeks following inoculation. The 50% lethal dose was measured according to the method of Reed and Muench (1938).

Gentamicin protection assay HeLa cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum in 24-well plates to reach 80% confluency. The culture was removed and the wells were washed three times with phosphate-buffered saline. Overnight bacterial cultures were diluted 1:50 into 10 ml fresh LB and grown at 37℃ , 100 rpm to midlog phase. Bacteria were added to HeLa cells with DMEM containing no antibiotics at a multiplicity of infection (MOI)

of 100 and incubated at 37℃ for 2 hours in 5% CO2. The cells were washed three times with PBS and incubated in a mixture of DMEM, 10% FBS, 100 g/ml gentamycin at 37℃ for 2 hours in 5% CO2. Cells were washed three times with PBS and incubated with DMEM containing 10% FBS and antibiotics for 1 hour or 24 hours. Cells were lysed in 0.1% Triton X-100, and the number of intracellular bacteria (CFU) was determined by plating on LB plate.

RESULTS

Cloning and analysis of *hilA* and *spiCAB* genesThe PCR products of HILA-1 and HILA-2, and SPICAB-1 and SPICAB-2 were cloned to pCR vector by ZeroBlunt PCR Cloning kit (Invitrogen). The subcloned plasmids were isolated and digested with *Eco*RI (Fig. 1). The PCR product of HILA-1 and HILA-2 is 1.6 kb. The nucleotide sequence of PCR product has high identity (98%) with *hilA* of *S. typhimurium* and *S. typhi*. The PCR product of SPICAB-1 and SPICAB-2 is 2.9 kb (Fig. 1). The nucleotide sequence of PCR product has high identity (95%) with *spiCAB* of *S. typhimurium* and *S. typhi*.

Confirmation of mutants

△*hilA* SC constructed by allelic exchange was confirmed by PCR. The primer HILA-11 (5`-CGGT GACCATTACGAAGAACT-3`) and HILA-12 (5`-ATGGGCGA AAGTAAGTTAGCT-3`) was designed to distinguish the *hil*A gene deletion and *aph*A-3 fragment insertion (Fig. 2). △*hilA* SC was confirmed by Southern hybridization using *aph*A-3 fragment for probe. The genomic DNA was digested by *Sac*I and *Bam*HI. (Fig. 3) △*spi*C SC constructed by allelic exchange was confirmed

by PCR. The SPIC-5 and SPIC-8 primers were used to distinguish the *spi*C gene deletion (Fig. 4).

Virulence assay To determine whether SPI-1 and SPI-2 have a role in the virulence of *S. enterica* serovar Choleraesuis in mice, the LD_{50} assays of the wild type, *hilA,* and *spi*C mutant were performed via oral and intraperitoneal inoculation. The LD_{50} of both the wild type and *hilA* and *spi*C mutant were below 10^1 CFU by intraperitoneal route. The LD_{50} of both the wild type and *hilA* mutant were above 10^8 CFU by oral route

Growth curves of mutants The growth curve of SC and mutants were monitoring by absorbance of 600 nm wavelengths for eight-hour culture (Fig. 5). The growth curves of *hilA* and *spi*C mutants were similar with the wild type. The results show that △ *hilA* SC and △*spi*C SC have no growth defect in vitro.

The invasion and intracellular survival ability The CFU of HeLa cells infected by *hilA* mutant for 2 hours and incubated for 1 hour was less than that of SC wild type (Fig. 6). The CFU of HeLa cells infected by *spi*C mutant for 2 hours and incubated for 1 hour was similar with that of SC w.t. (Fig. 7a). The results showed that the *hilA* mutant was less invasive than SC wild type. The CFU of HeLa cells infected by *spi*C mutant for 2 hours and incubated for 24 hourn was larger than that of wild type (Fig. 7b).

CONCLUSIONS

In these experiments, we found that the nucleotide sequences of *hilA* and *spi*CAB genes of *S. choleraesuis* have high identities with *S. typhimurium*. The

mutant strains constructed by allelic exchange were confirmed by PCR and Southern hybridization and both of them had no growth defect in vitro compared with wild type. The virulence of *hilA* and *spi*C mutants were similar with wild type, but only *hilA* mutant was less invasive in HeLa cell infection. The *spi*C mutant could invade into HeLa cell as well as wild type SC, but it seemed to survive inside HeLa cell better than the wild type. This preliminary data can provide a method to construct and evaluate the mutant for pathogenesis.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristic(s)[a]	Reference or source
E. coli strains		
DH5α	F⁻ *endA1hsdR*17(*rk-mk-*) *supE*44*t hi*-1 *recA*1 *gyrA relA*1 Δ(*lacZYA-argF*) U169 *deoR* {ϕ80 *dlac* Δ(*lacZ*)M15} λ⁻; cloning host	Gibco BRL
S17-1 λpir	*Prp thi recA hsdR*; chromosomal PR4-2 (*Tn1*::*ISR1 tet*::*Mu Km*::*Tn7*); λ*pir*	Simon *et al.*, 1993
cc118	PUT::flh (long promoter)-*lux*AB-km (Amp^r, Km^r) ; SphIligationPUT::LuxAB-Km(SphI)	de Lorenzo and Timmis, 1994
S. enterica serovar Choleraesuis strains		
SCWT	Wild type, hog isolated	This study
△*hilA* SC	SCWT △*hilA* ::*aph*A3	This study
△*spiC* SC	*spiC*⁻ *derivative* of SCWT	
Plasmids		
pBluescript SK+	3.0-k cloning vector; Ap^r	Laboratory stock
pGP704	3.7 kb *oriT oriV* Ap^r	Pettis, *et al.*,1988
pRE118	*oriT oriV sacB aphA*	Edwards *et al.*,1998

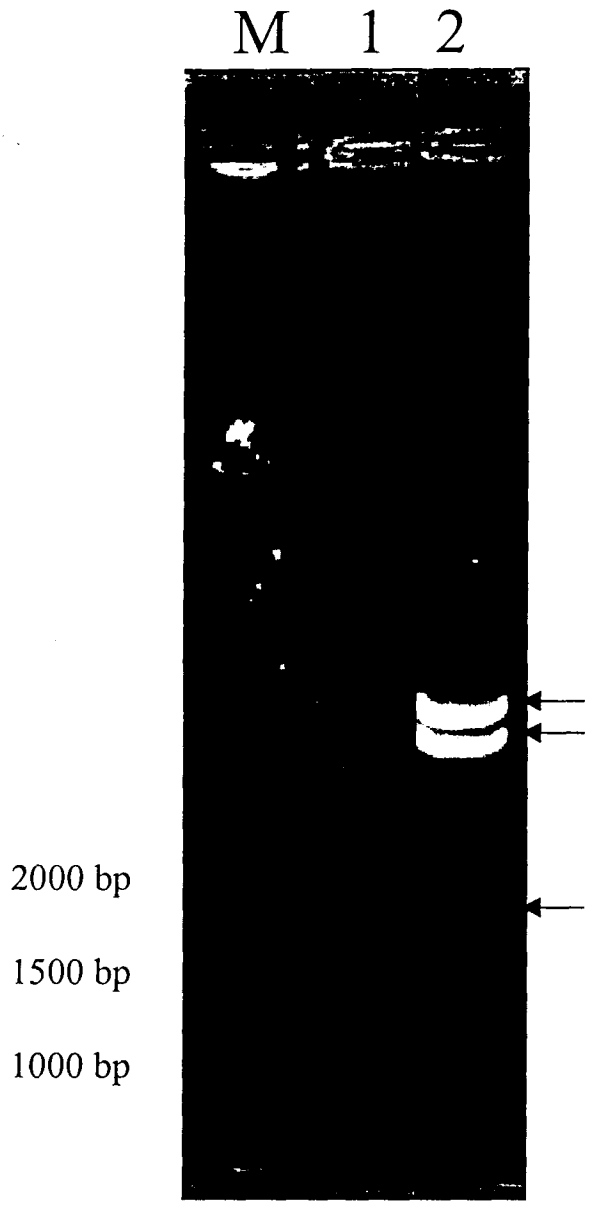

Fig. 1. Restriction enzyme digestion of subcloned pCR vectors.
Lane 1 is *hilA*/pCR. The insert fragment is 1.6 kb. Lane 2 is *spiCAB*/pCR. The insert fragment is 2.9 kb. pCR vector is 3.5 kb. Marker is 100 bp ladders.

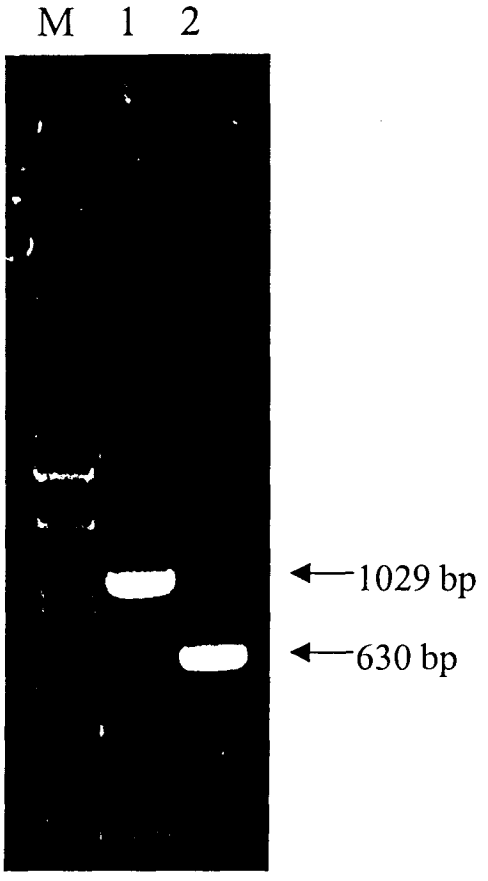

Fig 2. PCR result of △*hilA* SC.

M is 100 bp markers. Lane 1 is △*hilA* SC. Lane 2 is SC wild type.

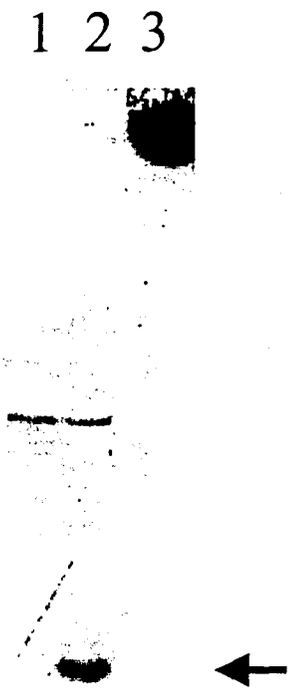

Fig. 3. Southern hybridization of △*hilA* SC.
Lane 1 is SC wt. Lane 2 is △*hilA* SC. Lane 3 is the dot of positive control. The arrow is *aph*A-3 fragment.

Fig 4. PCR result of △*spi*C SC.

M is 100 bp markers. Lane 1 is △ *spi*C mutant, the size of PCR product is 2,673 bp.

Lane 2 is SC wild type. The size of PCR product is 2,276 bp.

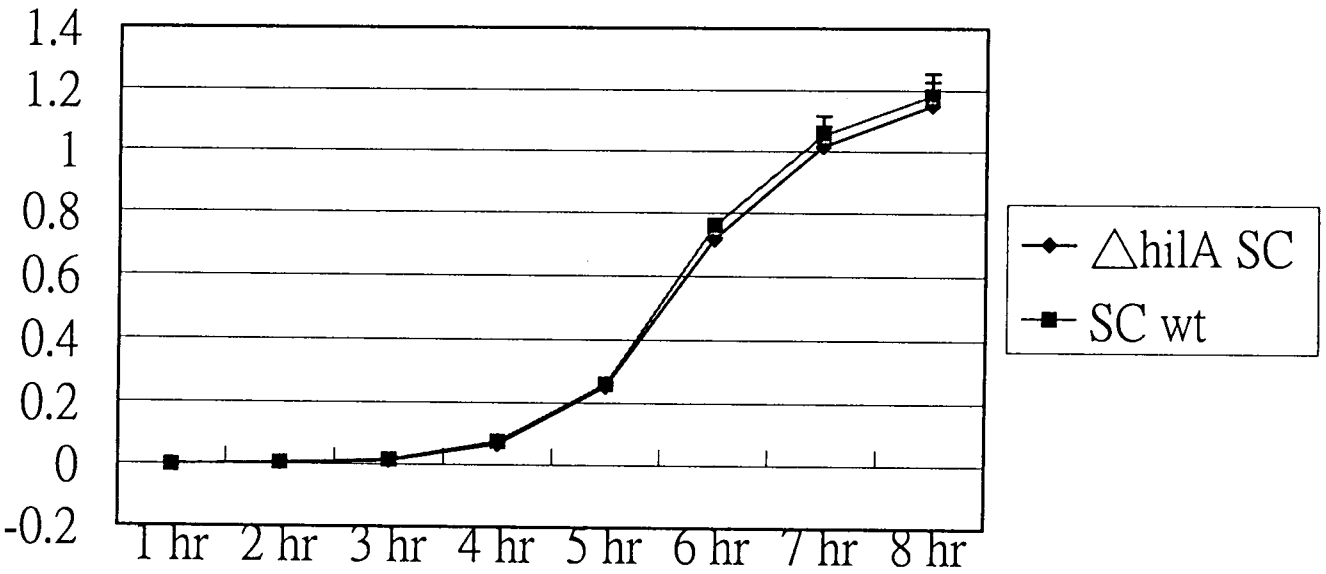

Fig. 5a. The growth curves of *hilA* mutant and SC wild type.

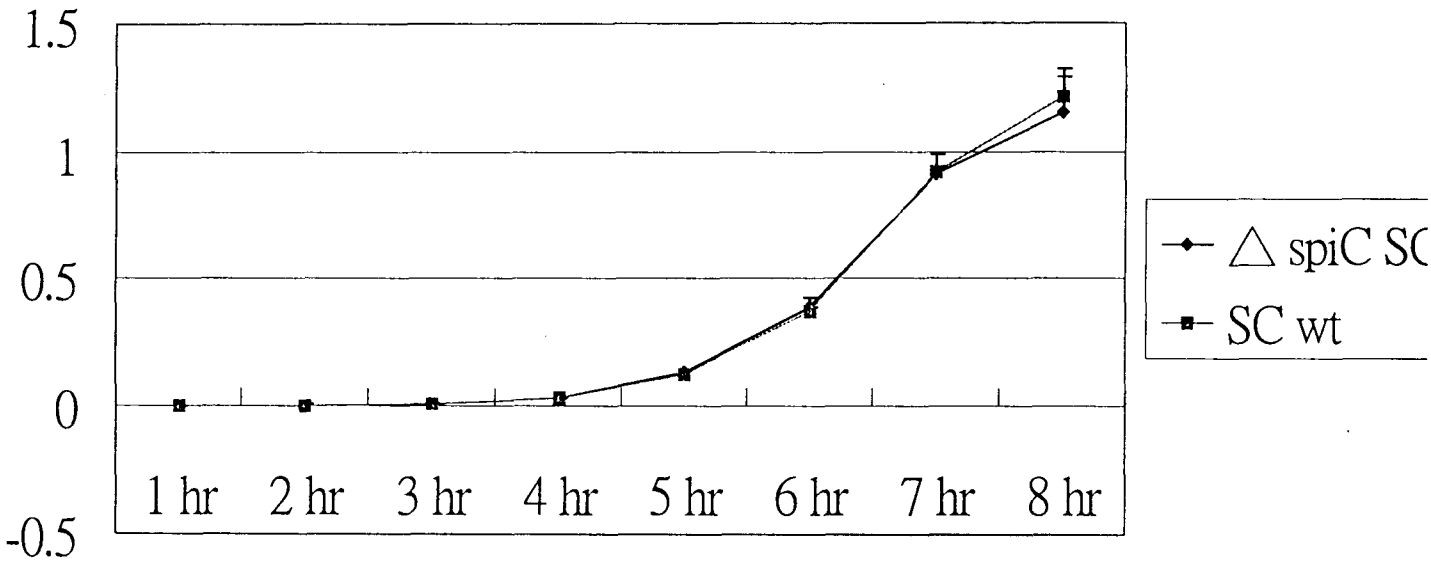

Fig. 5b. The growth curves of *spi*C mutant and SC wild type.

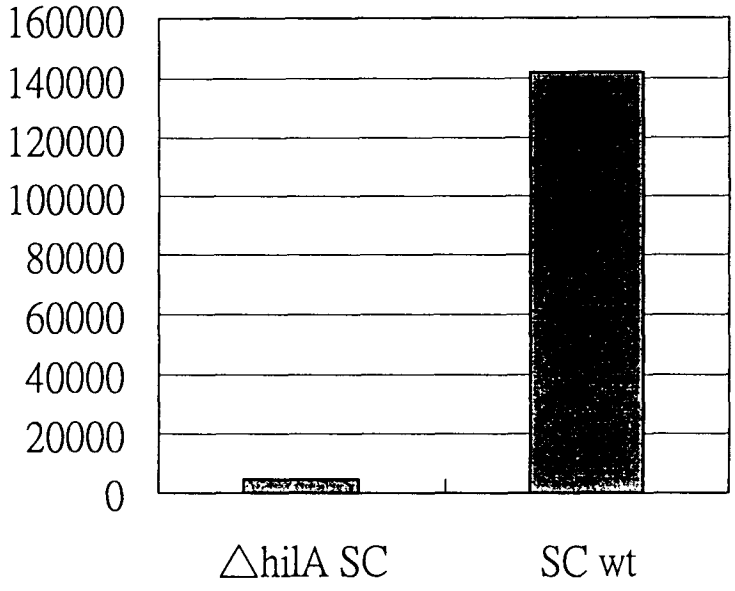

Fig. 6. The intracellular bacterial counts of *hilA* mutant and SC wild type.

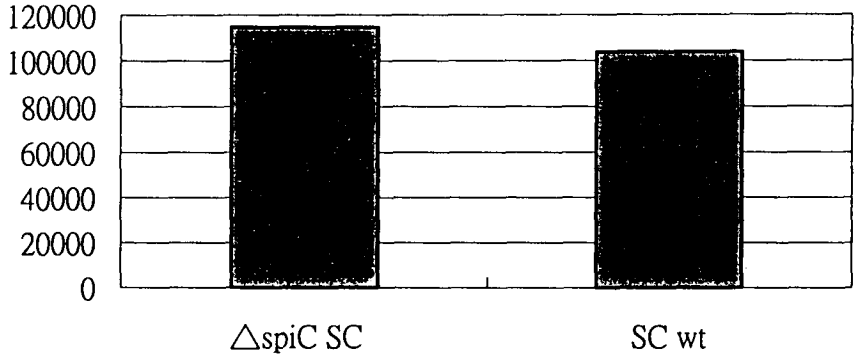

Fig. 7a The intracellular bacterial counts of *spi*C mutant and SC wild type.

Culture for 1 hour.

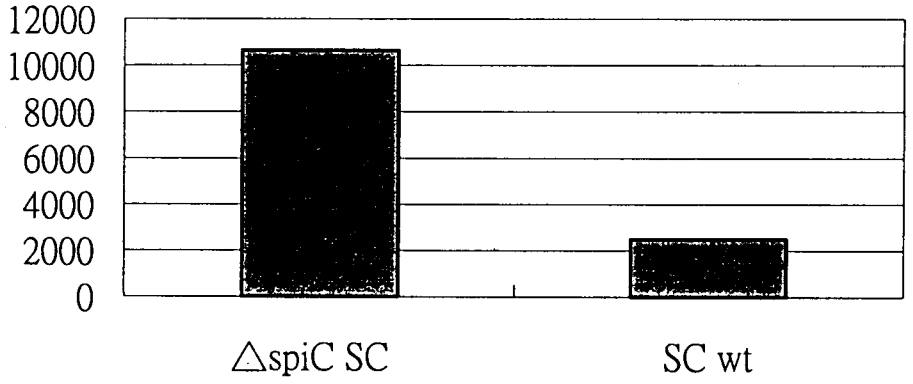

Fig. 7b. The intracellular bacterial counts of *spi*C mutant and SC wild type. Culture for 24 hours.