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Original article

Mutations in the *Salmonella enterica* serovar Choleraesuis cAMP-receptor protein gene lead to functional defects in the SPI-1 Type III secretion system

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Abstract – *Salmonella enterica* serovar Choleraesuis (*Salmonella* Choleraesuis) causes a lethal systemic infection (salmonellosis) in swine. Live attenuated *Salmonella* Choleraesuis vaccines are effective in preventing the disease, and isolates of *Salmonella* Choleraesuis with mutations in the cAMP-receptor protein (CRP) gene (*Salmonella* Choleraesuis Δ*crp*) are the most widely used, although the basis of the attenuation remains unclear. The objective of this study was to determine if the attenuated phenotype of *Salmonella* Choleraesuis Δ*crp* was due to alterations in susceptibility to gastrointestinal factors such as pH and bile salts, ability to colonize or invade the intestine, or cytotoxicity for macrophages. Compared with the parental strain, the survival rate of *Salmonella* Choleraesuis Δ*crp* at low pH or in the presence of bile salts was higher, while the ability of the mutant to invade intestinal epithelia was significantly decreased. In examining the role of CRP on the secretory function of the *Salmonella* pathogenicity island 1 (SPI-1) encoded type III secretion system (T3SS), it was shown that *Salmonella* Choleraesuis Δ*crp* was unable to secrete the SPI-1 T3SS effector proteins, SopB and SipB, which play a role in *Salmonella* intestinal invasiveness and macrophage cytotoxicity, respectively. In addition, caspase-1 dependent cytotoxicity for macrophages was significantly reduced in *Salmonella* Choleraesuis Δ*crp*. Collectively, this study demonstrates that the CRP affects the secretory function of SPI-1 T3SS and the resulting ability to invade the host intestinal epithelium, which is a critical element in the pathogenesis of *Salmonella* Choleraesuis.

***Salmonella enterica* serovar Choleraesuis / cAMP-receptor protein / CRP / type III secretion system / attenuation**

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1. INTRODUCTION

Salmonella enterica serovar Choleraesuis (herein termed *Salmonella* Choleraesuis) is one of the prevalent serovars causing salmonellosis in swine. Clinically, pigs infected by *Salmonella* Choleraesuis develop not only gastroenteritis, but also lethal systemic manifestations including pneumonia, hepatitis, and meningitis [32]. Although *Salmonella* Choleraesuis is a host-adapted pathogen of pigs, studies have revealed this serovar may also cause systemic infections in humans [6, 7]; however, the relationship between isolates of *Salmonella* Choleraesuis from humans and swine remains to be elucidated [4].

Vaccines represent an effective strategy for the control of swine salmonellosis [32]. However, bacterins composed of inactivated cultures of *Salmonella* are less effective than live vaccines consisting of attenuated strains of *Salmonella* [15]. During the past decades, a variety of live attenuated *Salmonella* Choleraesuis mutants have been developed as vaccine candidates. These include strains having mutations in genes involved in purine synthesis (*pur*), thymidine synthesis (*thy*), the cAMP-receptor protein (*crp*), adenylate cyclase (*cya*), type III secretion system (T3SS) apparatus (*ssaC*, *ssaJ* and *ssaV*), Gifsy-1 prophage protein (*gifsy-1*), and D-galactonate transport protein (*dgoT*) [8, 19–21, 27]. Among these, *Salmonella* Choleraesuis strains with mutations in *crp* alone or in combination with other genes were shown to be greatly attenuated in virulence and to serve as effective vaccine candidates against swine salmonellosis [8,20,34].

In *Escherichia coli*, the cAMP-receptor protein (CRP) activates transcription of more than 100 genes and acts as a global gene regulator [22]. Upon coupling with the allosteric effector, cyclic AMP, dimeric CRP initiates transcription by binding to specific DNA sites and enhancing the binding of RNA polymerase holoenzyme to activate transcription [2]. In this manner, regulons or genes involved in the uptake and utilization of carbon source, flagellum synthesis, iron uptake, glycogen synthesis, outer membrane proteins etc. are regulated in *E. coli* and *Salmonella* Typhimurium [1, 22]. Moreover, insertional mutations in *crp* and *cya* have been reported to affect the production of proteins exported by the Ysc (*Yersinia* secretion), Ysa (*Yersinia* secretion apparatus), and flagellar T3SS in *Yersinia enterocolitica* [29]. Nevertheless, little was known regarding the role of CRP in *Salmonella* virulence until a recent study reported that *crp*/*cya* are required for expression of *sirA* gene which is an upstream regulator for *Salmonella* pathogenicity island 1 (SPI-1) gene clusters [37].

The SPI-1 T3SS is one of the virulence determinants of *Salmonella* that is important for intestinal colonization and invasion of epithelial cells as a prelude to systemic infection. By delivering more than 10 different effector proteins into the host cells via SPI-1 T3SS, *Salmonella* actively invades intestinal mucosa and causes enteropathy in the host [31, 42]. Moreover, the SPI-1 T3SS effector protein SipB induces caspase-1 dependent apoptosis in macrophages and plays an essential role in *Salmonella* pathogenesis [16, 25, 26]. Therefore, the aim of this study was to investigate the functional relationship between CRP and SPI-1 T3SS in *Salmonella* Choleraesuis using a *crp* mutant (Δ*crp*) and to better understand the basis of attenuation in the mutant strain.

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids and media

A wild type (WT) strain of *Salmonella enterica* serovar Choleraesuis (No. 13312) and an *E. coli* K-12 strain (No. 10798) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Bacteria were cultured at 37 °C in Luria-Bertani (LB) broth. To induce the expression of SPI-1 T3SS genes in the experiments described, bacteria were cultured at 37 °C in LB supplemented with 0.3 M NaCl under conditions of low aeration [14, 28, 36]. Where appropriate, ampicillin (100 μg/mL), kanamycin (50 μg/mL) or chloramphenicol (12.5 μg/mL) were added to the medium. Quantification of viable bacteria, expressed as colony forming units (CFU) per mL of culture or g of tissue, was determined for each experiment by preparation of serial dilutions of cultures or tissue homogenates in phosphate buffered saline (PBS) and plating on MacConkey or brilliant green (BG) agar plates

Table I. Bacterial strains used in this study.

Strain	Genotype	Relevant characteristics	Source
Salmonella enterica serovar Choleraesuis			
WT	Wild type		ATCC, No. 13312
CP327	*crp-327*::*km*	Derived from WT, Km^R	This study
CP359	*crp-359*::*loxP*	Derived from WT	This study
PQ518	*phoQ518*::*km*	Derived from WT, Km^R	This study
Escherichia coli			
K12	Wild type		ATCC, No. 10798

(Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

2.2. Construction of *crp* gene mutants

Mutants were generated by allelic exchange between suicide vectors and bacterial genomic DNA using a method described previously [40]. The mutant strains generated in this study are listed in Table I. Briefly, genomic DNA of *Salmonella* Choleraesuis was PCR amplified using the following primer pair: 5′- cat taa aat ctg gta ccg gag gag gac tct c -3′ and 5′- gat gaa ttc cca cca gca gaa gtt gcg tta cca -3′. The resulting 1 318 bp PCR product containing the full length *crp* (633 bp) flanked by partial sequences of the putative inner membrane protein genes, *yhfA* and *yhfK*, was digested with *Kpn*I and *Eco*RI, ligated into pGP704, and maintained in the permissive *E. coli* strain SY327λ*pir* [24]. The resulting plasmid was termed pGCRP. Thereafter, a *Hinc*II DNA fragment harboring a kanamycin-resistance determinant (*km*) was subsequently cloned and inserted into the *crp* gene of pGCRP. The resulting plasmid, pGCRPK, was a suicide vector for allelic exchange of *crp* by transformation into *Salmonella* Choleraesuis via electroporation (1.25 kV/cm, 600 Ω and 25 μF). The resulting bacterium CP327 strain exhibiting kanamycin-resistance and ampicillin-sensitivity was isolated and characterized by Southern hybridization using DNA probes specific to *crp* and *km*.

In order to construct a *crp* mutant free of the *km* resistance gene, similar cloning procedures were applied. Briefly, a *km* determinant flanked by two direct repeats of the *loxP* sequence (5′- ata act tcg tat aat gta tgc tat acg aag tta t -3′) was cloned into the *crp* gene of pGCRP. The resulting suicide vector was transformed and resulting mutants with kanamycin-resistance and ampicillin-sensitivity were selected and genotypically characterized. The selected strain was a *crp* mutant harboring a *loxP*-flanked-kanamycin determinant. To excise the *km* gene, bacteria were further transformed with a temperature sensitive p705-Cre plasmid that harbored a Cre recombinase gene (Gene Bridges GmbH, Dresden, Germany). Excision of the *km* determinant from genomic DNA of candidate strains via Cre-*loxP* recombination was achieved by shifting the incubation temperature from 30 °C to 42 °C [44]. The increased temperature induced expression of Cre recombinase and simultaneously inhibited replication of p705-Cre, leading to the elimination of the plasmid. The resulting bacterium CP359 strain lacking an antibiotic gene was further identified by Southern hybridization analysis (data not shown).

2.3. Construction of plasmids encoding SopB, SipB and CRP

To construct a plasmid that encoded the N-terminal Myc-tagged SopB, genomic DNA of *Salmonella* Choleraesuis was amplified by PCR using primer pair sopB P1(F) and sopB P2(R) as shown in Table II. The resulting *sopB* gene was restriction digested with *Bgl*II and *Hind*III, and ligated into a pUC19 vector (GenBank Accession L09137) that harbored a promoter region for the neomycin determinant of the Tn5 transposon (GenBank Accession U00004). The resulting pTSopB plasmid contains the *sopB-myc* gene that is transcriptionally regulated by the neomycin promoter *in cis*. Similar procedures were used in the construction of plasmids pTSipB and pTCRP expressing N-terminal Myc-tagged SipB and CRP, respectively, using the pUC19 vector as a backbone (Tab. II).

Additionally, a plasmid that harbored the pSC101 replication of origin and the *crp* gene was constructed. Briefly, a p705 M plasmid that served as the backbone was generated from modification of p705-Cre by *BamH*I digestion and self-ligation. To

Table II. Oligonucleotide primers used and plasmids constructed in this study.

Primer	Sequences (5′ → 3′)[a]	Plasmid
sopB P1(F)	CAGAGATCT**GATCAAGAGACAGGATGAGGATCGTTTCGC**ATGCAAATACAGAGCTTC	pTSopB
sopB P2(R)	CCGAAGCTT***TTA***CAGATCCTCTTCAGAGATGAGTTTCTGCTCAGATGTGATTAATGAAGA	
sipB P1(F)	CAGAGATCT**GATCAAGAGACAGGATGAGGATCGTTTCGC**ATGGTAAATGACGCAAGTAGCATTAG	pTSipB
sipB P2(R)	CCGCTGCAG***TTA***CAGATCCTCTTCAGAGATGAGTTTCTGCTCTGCGCGACTCTGGCGCAGAATAAAACG	
crp P1(F)	CAGAGATCT**GATCAAGAGACAGGATGAGGATCGTTTCGC**ATGGTGCTTGGCAAACC	pTCRP
crp P2(R)	CCGAAGCTT***TTA***CAGATCCTCTTCAGAGATGAGTTTCTGCTCACGGGTGCCGTAGACGA	
crp P3(F)	AATGCCGGATCCTGGACAGCAAGCGAACCGGAATTGCC	p705CRP
crp P3(R)	CGGGGATCCTCACACAGGAAACAGCTATGAC	

[a] The Myc-tag sequences are shown in italics. Restriction sites are underlined. Stop codons are shown in bold italics. Sequences of partial neomycin promoter of Tn5 transposon are shown in bold.

clone *crp* into p705 M, the *crp* gene was PCR amplified using primers crp P3(F) and crp P3(R) with pTCRP as the template. The PCR product was restriction digested with *BamH*I and ligated into p705 M. The plasmid was termed p705CRP.

2.4. Complementation experiments

Complementation experiments were performed by transformation of pTCRP or p705CRP plasmids into CP327 strain via electroporation (1.25 kV/cm, 600 Ω and 25 μF). In the experiments of in vitro survival and LDH assay, the pTCRP plasmid was used. The secretory function of the complemented *Salmonella* Choleraesuis Δ*crp* was investigated by co-transformation of pTSopB and p705CRP into CP327. These two plasmids are compatible in the CP327 strain.

2.5. In vitro survival assay

Stationary phase cultures of *Salmonella* in LB were washed twice with PBS, and resuspended in control media. The control medium was M9 (pH 7.0) for the acid killing assay and saline (0.9% NaCl, pH 7.0) for the bile salts killing assay. Approximately 1×10^8 CFU of bacteria were inoculated into the M9 medium (pH 3.0), 15% bile salts in saline (Becton, Dickinson and Company), 1% sodium deoxycholate in saline (Becton, Dickinson and Company), or control medium, followed by gentle mixing and incubation at 37 °C for 1 h. After incubation, viable bacterial counts were determined on MacConkey agar plates. The percentage of surviving bacteria was calculated as the number of viable bacteria before incubation/the number of viable bacteria after incubation.

2.6. Secretion assay and Western blot analysis

Salmonella strains harboring appropriate plasmid(s) were cultured into LB broth containing 0.3 M NaCl and grown at 37 °C overnight. The bacterial pellets were collected by centrifugation at 3 000 × *g* for 10 min, resuspended in the same medium to $\sim 1 \times 10^9$ CFU/mL and incubated at 37 °C for an additional 3 h. Thereafter, bacterial pellets were collected by centrifugation and culture supernatants were passed through a filter with a pore-size of 0.45 μm (Millipore, Billerica, MA, USA). All samples were normalized to the $OD_{595\ nm}$ values determined at harvest and subjected to Western blot analysis. The protein bands on immunoblots recognized by anti-Myc antibody (Abcam, Cambridge, UK) represented the quantity of recombinant proteins present within bacterial cells or that were secreted by bacteria into culture supernatants. Signals from immunoblots probed with an anti-DnaK antibody (Merck, Darmstadt, Germany) represented the quantity of bacteria in pelleted fractions or in the supernatants containing lysed cells. The intensity of the bands was quantified by analysis of the scanned

immunoblots using ImageJ software, version 1.37v (National Institutes of Health).

2.7. Animal experiments

The animals used in this study were cared for and handled according to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the National Chung Hsing University (permit number 94-67).

2.7.1. LD_{50} in mice

Healthy female BALB/c mice at 6 to 8 weeks of age were purchased from the National Laboratory Animal Center, Taiwan. The virulence of the *Salmonella* Choleraesuis strains was determined using both peroral (p.o.) and intraperitoneal (i.p.) routes of administration. For p.o. inoculations, mice were deprived of food and water for 4 h and orally given 30 μL of 10% sodium bicarbonate (Sigma, St. Louis, MO, USA) 5 min before administration of 40 μL of serially diluted *Salmonella* Choleraesuis in PBS. Food and water were resupplied 30 min after p.o. inoculation of bacteria. Intraperitoneal inoculation of mice was performed by injecting 100 μL of a bacterial suspension into the abdomen [9]. Mice that appeared severely ill were euthanized. Mortality was recorded at 21 days post inoculation and the LD_{50} was calculated by the method of Reed and Muench [30].

2.7.2. Porcine ligated ileal loop

Male Landrace × Yorkshire (LY) pigs at 8 weeks of age were obtained from a local commercial supplier. Pigs were confirmed to be negative for *Salmonella* prior to experiments by overnight enrichment of rectal swabs in Rappaport broth (Becton, Dickinson and Company) at 37 °C, followed by culture on BG agar plates overnight. For surgical manipulations, pigs were sedated by intramuscular administration of 4 mg/kg Stresnil (Janssen Animal Health, High Wycombe, UK), followed by induction of anesthesia by intravenous administration of 8 mg/kg thiamylal sodium (Shinlin Sinseng Pharmaceutical, Taiwan, R.O.C.), and maintenance of anesthesia with 2% isoflurane (Halocarbon Laboratories, River Edge, NJ, USA) in oxygen via an endotracheal tube. When a deep stage of anesthesia was reached, a midline incision was made through the abdominal wall, the ileocecal junction was revealed, and sequential 6-cm loops with 1-cm spacers were constructed with surgical silk. Twelve ileal loops were constructed in each pig and randomly assigned for bacterial inoculation. Bacteria grown under conditions to induce SPI-1 T3SS were injected into assigned loops at a concentration of 1×10^9 CFU/5 mL per loop. At least triplicate loops were injected for each bacterial strain. The abdomen was then closed with surgical thread and anesthesia was maintained for 3 h to allow intestinal invasion of bacteria. Thereafter, animals were euthanized and the ileal loops accompanied with the associated mesenteric lymph nodes were harvested.

To quantify the invasion of *Salmonella*, the ileal loops were dissected and gently washed with 250 mL of ice-cold saline to remove mucus and excess bacteria in the lumen. The intestinal walls with Peyer patches were harvested and were homogenized in PBS containing 0.5% bovine serum albumin (Sigma) and 0.5% Tergitol TM10 (Fluka, Buchs, Switzerland). Serial dilutions of homogenates of the intestine or mesenteric lymph nodes were plated on BG agar with appropriate antibiotic and incubated overnight at 37 °C. Bacterial counts in each preparation were performed as described previously.

2.8. Invasion and intracellular survival assays

Porcine alveolar macrophages (PAM) were obtained from 3- to 6-week old healthy piglets through lavage and stored in liquid nitrogen as previously described [5, 18]. For experiments, PAM were thawed, resuspended and cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. Invasion and survival assays were performed as described previously with minor modifications [10, 12]. Briefly, PAM were plated in 96-well tissue culture plates at a density of 2×10^5 cells per well and incubated at 37 °C in 5% CO_2 overnight. Fifty microliters of SPI-1 T3SS induced bacteria in culture medium (RPMI-1640 supplemented with 10% FBS) were added to PAM in each well to provide a final multiplicity of infection (MOI) of ~ 20. The plates were then centrifuged at $167 \times g$ for 5 min and incubated at 37 °C in 5% CO_2 to allow invasion. Two hours post inoculation (hpi), the cells were washed five times with culture medium and incubated in 200 μL of culture medium supplemented with 100 μg/mL gentamicin (Gibco Labs, Grand Island, NY, USA) for an additional 2 h in order to kill extracellular bacteria. Subsequently, the medium was replaced with culture medium containing 10 μg/mL gentamicin. At 4, 8 and 16 hpi, the cells were washed once with culture medium and lysed in 200 μL of 0.1% Triton X-100 in PBS for 5 min. Cell lysates were diluted serially in

PBS and plated onto BG agar plates to determine the number of bacteria surviving intracellularly. The invasiveness of each strain was calculated as follows: CFU at 4 hpi/total bacterial counts at 2 hpi, and the relative invasion rate was expressed after normalization to WT. The survival rate of each strain at 8 or 16 hpi was calculated as (CFU at 8 or 16 hpi/CFU at 4 hpi) × 100%.

2.9. LDH assay

Murine RAW 264.7 macrophage cells were maintained in RPMI-1640 supplemented with 10% FBS. Bacteria grown under conditions that induce SPI-1 T3SS were used [28]. To determine the cytotoxic effect of *Salmonella*, RAW 264.7 macrophage cells were seeded onto 96-well cell culture plates at a density of 5×10^4 cells/well and incubated at 37 °C overnight. The cells were washed with RPMI-1640 and incubated for 1 h in RPMI-1640 containing 50 μM of the caspase-1 inhibitor Ac-YVAD-CMK (Calbiochem, Darmstadt, Germany) or the vehicle (0.5% dimethylsulfoxide, DMSO) used to dissolve the caspase-1 inhibitor. Bacterial suspensions representing an MOI of ∼ 50 were added to the cell cultures and incubated for 1 h at 37 °C. Thereafter, low serum media with gentamicin (RPMI-1640 containing 1% FBS, 100 μg/mL gentamicin, and 50 μM Ac-YVAD-CMK or 0.5% DMSO) was added to kill extracellular bacteria and the cultures were incubated for an additional 6 h. Cytotoxicity was quantified using a kit (Roche, Basel, Switzerland) that detects LDH released from the cytoplasm. Percent cytotoxicity was calculated as 100 × ((experimental release − spontaneous release)/(total release − spontaneous release)), in which the spontaneous release was the amount of LDH activity in the supernatant of uninfected cells and the total release of LDH was measured from cells lysed with 1% Triton X-100 (Sigma).

3. RESULTS

3.1. The *Salmonella* Choleraesuis Δ*crp* was attenuated in BALB/c mice

To examine the role of the *crp* gene in the virulence of *Salmonella* Choleraesuis, BALB/c mice were infected by p.o. or i.p. administration of the WT strain of *Salmonella* Choleraesuis or an isogenic *crp* gene mutant strain CP327. The results showed that the WT strain was highly virulent with an LD_{50} of 2.4×10^1 CFU and 1.2×10^6 CFU via delivery by i.p. and p.o. routes, respectively. Mice infected with the WT isolate developed acute systemic symptoms and death was observed within 10 days post inoculation (Fig. 1). By contrast, CP327 was greatly attenuated with LD_{50} doses of 3.5×10^4 CFU (i.p.) and $> 2.1 \times 10^{10}$ CFU (p.o.), that were approximately 10^3- and 10^4-fold greater than those for the WT, respectively (Fig. 1). Mice inoculated with CP327 showed a high tolerance for infection by the mutant strain and survived longer compared with those infected with WT, i.e. 40% of mice survived at 2 weeks after i.p. injection with 1×10^5 CFU of Δ*crp*. Notably, all mice challenged with 2.1×10^{10} CFU of CP327 delivered p.o. survived throughout the experimental period, indicating that CP327 was highly attenuated when administered via this route.

3.2. Attenuation of Δ*crp* was not due to greater susceptibility to low pH or bile salts

The fecal-oral route is the primary pathway for transmission of *Salmonella* infections among swine [11]. Before intestinal colonization, *Salmonella* needs to overcome the bactericidal effects of gastric acid and bile salts present in the upper alimentary tract. To determine if the attenuation of the Δ*crp* mutant was due to increased susceptibility to these bactericidal factors, survival of bacteria under conditions mimicking gastric acid and bile were investigated. The results show that both WT and Δ*crp* (CP327 and CP359) were highly sensitive to low pH (pH 3.0) with nearly 50% of bacteria being killed after 1 h incubation (Fig. 2A). However, the Δ*crp* strains showed ∼ 10% higher resistance than WT ($p < 0.05$) indicating that Δ*crp* strains were less susceptible to acidic environments. Furthermore, incubation of WT in the presence of 15% bile salts or 1% sodium deoxycholate (an active component of bile) resulted in the inactivation of nearly 30% and 60% of the bacteria, respectively (Fig. 2B). By contrast, Δ*crp* strains were significantly more resistant to both reagents than the WT and the sensitivity of the CP327 was restored by complementation *in trans* with a plasmid encoding CRP (Figs. 2A and 2B).

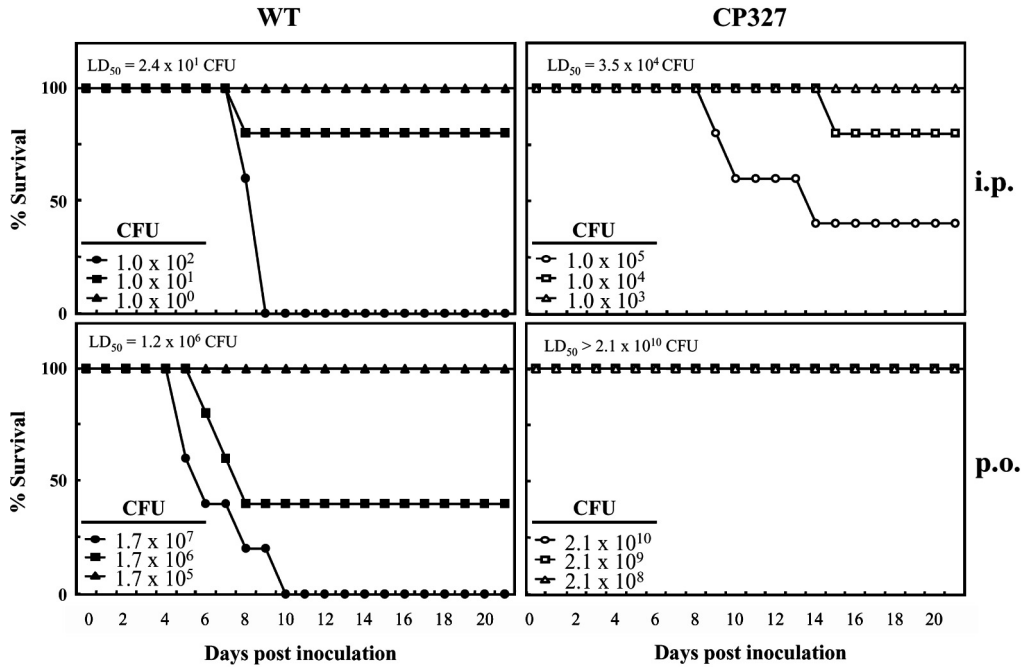

Figure 1. *Salmonella* Choleraesuis Δ*crp* is attenuated in BALB/c mice. Survival of mice was recorded up to 3 weeks after peroral (p.o.) or intraperitoneal (i.p.) administration of varying doses of *Salmonella* Choleraesuis (WT) or the CP327 strain. Data are representative of two independent experiments (n = 5 in each group).

3.3. Intestinal invasion was impaired in Δ*crp*

During infection, *Salmonella* Choleraesuis must penetrate the intestinal epithelial barrier prior to establishing systemic infection. To examine the role of the *crp* gene in intestinal invasiveness, an in vivo experiment was performed using porcine ligated ileal loops. The result shows that, 3 h after incubation, fewer colonies of CP327 were recovered from the ileal wall and associated Peyer patches as compared to WT ($p < 0.05$). Further examination of the *Salmonella* strains that had penetrated the intestinal epithelial barrier revealed that significantly fewer CP327 were recovered from the mesenteric lymph nodes as compared to WT ($p < 0.001$) (Fig. 3A) suggesting that the mutation in the *crp* gene was associated with reduced intestinal colonization and/or invasion of *Salmonella* Choleraesuis.

Salmonella is able to invade and survive within macrophages that in turn serve as vehicles for bacteria to spread systemically. To determine the basis for our observation that fewer numbers of CP327 were present in mesenteric lymph nodes in ligated ileal loop experiments, we performed in vitro assays of bacterial invasion and survival in PAM. The results show that, compared to the WT, mutations in *crp* resulted in a 10% reduction in invasiveness of CP327 (Fig. 3B). These results can be compared with those from co-cultivation of PAM with a non-invasive strain of *E. coli* (K12) where only 4% of the viable bacteria were recovered, representing a background level of bacterial phagocytosis by PAM. In comparing the bacterial survival rate within PAM, it was noted that both *Salmonella* strains, but not *E. coli* K12, were able to survive within PAM. However, there was no significant

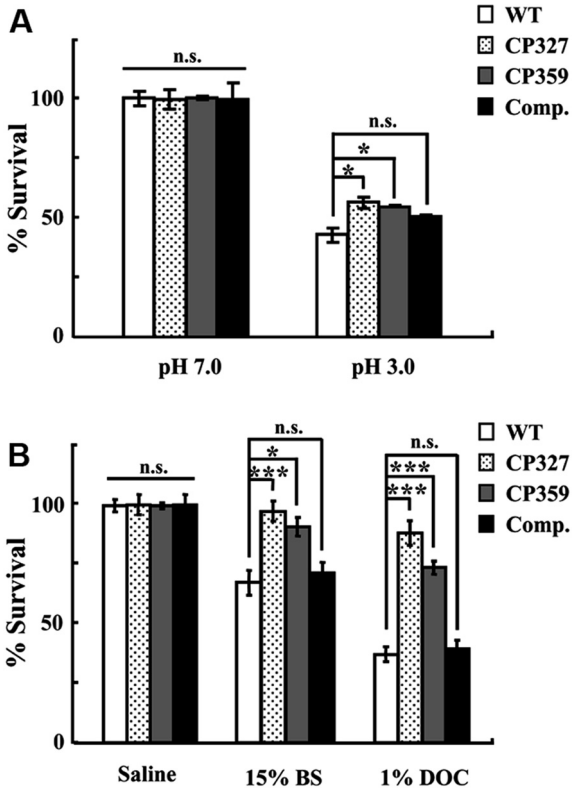

Figure 2. *Salmonella* Choleraesuis Δ*crp* is more resistant to low pH or bile salts. Bacteria grown to stationary phase in LB broth were incubated for 1 h in M9 medium at pH 3.0 or 7.0 (A) or in saline, 15% bile salts, or 1% sodium deoxycholate (B). Survival of bacteria before and after incubation was determined on MacConkey agar plates. Percent survival was normalized to the initial inoculum. WT, wild type *Salmonella* Choleraesuis; CP327 and CP359, *crp* gene mutants; Comp., CP327 complemented with pTCRP plasmid; BS, bile salts; DOC, sodium deoxycholate. The results are from three independent experiments of at least triplicate determinations. Error bars are SEM. $*p < 0.05$; $***p < 0.001$; n.s.: no significant difference from each other (ANOVA).

difference between WT and CP327. At 16 hpi, the percent of viable *Salmonella* strains decreased to approximately 25% (Fig. 3C).

3.4. Secretion of SPI-1 effector proteins SopB and SipB was impaired in Δ*crp*

The SPI-1 encoded T3SS delivers a set of effector proteins which facilitates bacterial entry into intestinal epithelial cells [45]. To understand whether a mutation in the *crp* gene affected SPI-1 T3SS and contributed to the impaired intestinal invasiveness of *Salmonella* Choleraesuis, the secretory function of SPI-1 T3SS was investigated by ectopic expression of the Myc-tagged recombinant effector proteins SopB and SipB in WT and mutant strains. It was observed that, under conditions that induced SPI-1 T3SS, WT bacteria successfully expressed the recombinant protein SopB and secreted it into the supernatant (Fig. 4A). To investigate whether the presence of SopB in culture supernatants was attributed to spontaneous bacterial lysis, the leakage of

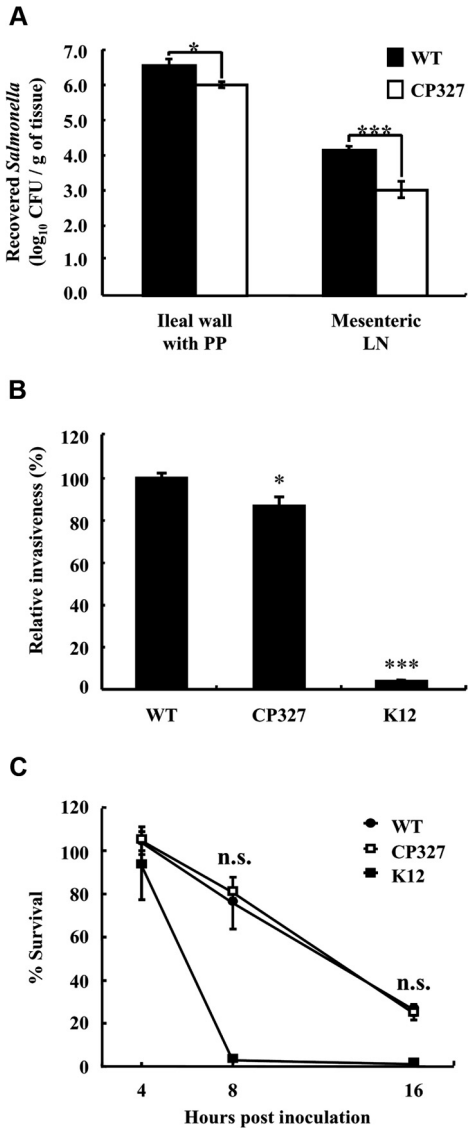

Figure 3. *Salmonella* Choleraesuis Δ*crp* was less invasive in porcine ligated ileal loops and in macrophages. The degree of intestinal invasion by wild type *Salmonella* Choleraesuis (WT) and CP327 was determined at 3 h after inoculation of the loops (A). To recover bacteria, portions of the ileal wall with Peyer patches (PP) and associated mesenteric lymph nodes (LN) were harvested, homogenized, and plated onto BG agar. The results are from at least triplicate determinations. For in vitro invasion (B) and intracellular survival assays (C), porcine alveolar macrophages were co-cultured with WT and CP327 of *Salmonella* or *E. coli* (K12) strains at an MOI of ∼ 20 for 2 h and intracellular survived bacteria were assessed. Relative invasion was determined at 4 hpi while intracellular survival rates were at 8 and 16 hpi. The results are representatives of three independent experiments of at least triplicate determinations. Error bars are SEM. $*p < 0.05$ and $***p < 0.001$ compared to WT; n.s.: no significant difference from each other (ANOVA).

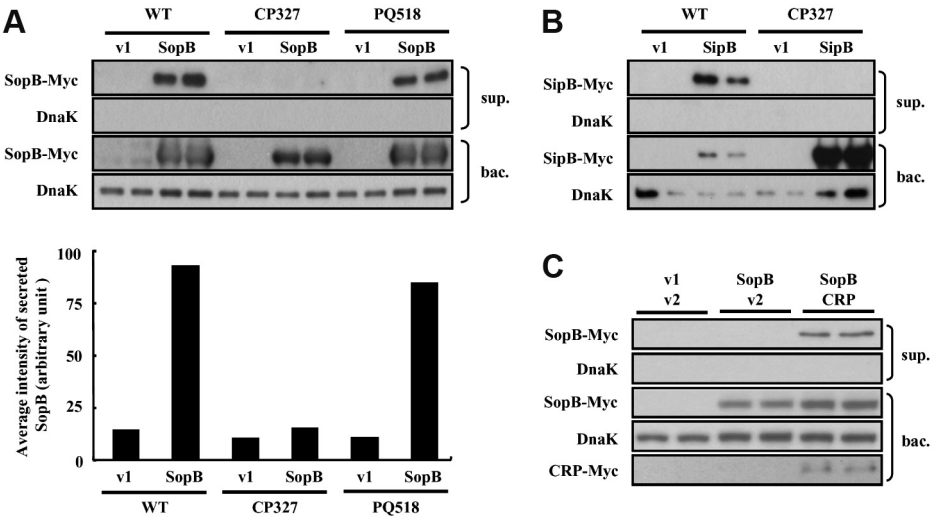

Figure 4. Secretion of effector proteins SopB and SipB via SPI-1 T3SS was impaired in Δ*crp*. Wild type (WT), CP327 or PQ518 strains transformed with pUC19 (v1), pUC19 encoding *sopB-myc* (SopB), *sipB-myc* (SipB), p705 M (v2) or p705 M encoding *crp-myc* (CRP) were grown under conditions that induced SPI-1 T3SS function. Bacterial culture supernatants (sup.) and pellets (bac.) were harvested, normalized and subjected to Western blot analysis with an antibody to Myc-tag. The results of the SopB secretion assay in WT, CP327 and PQ518 strains (A), SipB secretion assay in WT and CP327 (B) and SopB secretion assay in CP327 complemented with p705CRP (C). Immunoblots were also probed with an anti-DnaK antibody as a loading control and to detect signals resulting from bacterial lysis. The average intensity of secreted proteins was quantified and normalized to the intensity of DnaK for WT, CP327 and PQ518 strains (A, lower panel).

DnaK, a cytoplasmic chaperon protein, was examined by probing the same immunoblot with a DnaK-specific antibody. The lack of a DnaK signal in the bacterial culture supernatant confirmed that SopB was indeed secreted and not released due to bacterial lysis. By contrast, recombinant SopB was ectopically expressed in CP327, however, secretion of this protein was not detectable in the culture supernatant (Fig. 4A). To exclude the possibility that the altered function of SPI-1 T3SS was due to genetic manipulations used during the construction of CP327, an isogenic *phoQ* gene mutant PQ518 strain, constructed using a similar strategy, was tested for SopB secretion. The results showed that the secretory phenotype of PQ518 was comparable to that of the WT strain (Fig. 4A) suggesting that the genomic manipulation procedures used in this study did not affect SPI-1 T3SS.

To further demonstrate that the impairment of SPI-1 T3SS was a general effect and not restricted to SopB, secretion of another effector protein SipB was also assessed (Fig. 4B). Recombinant SipB was secreted efficiently in WT bacteria. However, there was no detectable signal in the supernatant of CP327 cultures even though a large amount of SipB was expressed within the CP327 cells (Fig. 4B). Furthermore, complementation of CP327 with co-expressed recombinant CRP restored the ability of the mutant to secrete SopB extracellularly, while complementation with a control plasmid remained non-secretory (Fig. 4C).

3.5. Caspase-1-dependent cytotoxicity in RAW 264.7 macrophage cells was reduced in Δ*crp*

Salmonella is known to activate caspase-1-dependent cytotoxicity in macrophages by

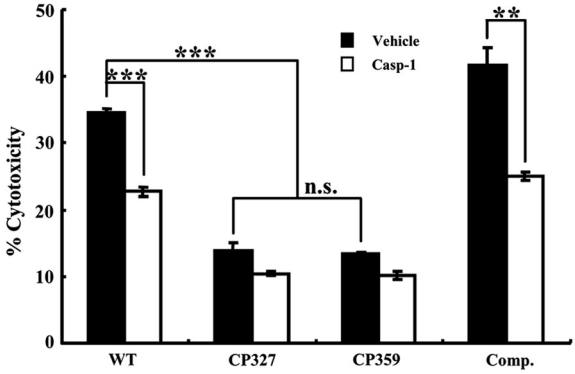

Figure 5. *Salmonella* Choleraesuis induced caspase-1 dependent cytotoxicity in RAW 264.7 cells was impaired in Δ*crp* mutants. Bacteria grown under conditions that induce SPI-1 T3SS were co-cultured at a MOI of ~ 50 with RAW 264.7 cells treated with 0.5% DMSO carrier (Vehicle) or with 50 μM caspase-1 inhibitor Ac-YVAD-CMK (Casp-1). Cell death was assessed at 6 hpi by measuring the release of lactate dehydrogenase (LDH). WT, wild type *Salmonella* Choleraesuis; CP327 and CP359, *crp* gene mutants; Comp., CP327 complemented with pTCRP plasmid. The results are from three independent experiments of at least triplicate determinations. Error bars are SEM. $^{**}p < 0.01$; $^{***}p < 0.001$; n.s.: no significant difference from each other (ANOVA).

delivering the SPI-1 T3SS effector protein SipB to the cell membrane [25, 38]. To determine whether there was impaired secretion of SipB by Δ*crp* affected macrophage cytotoxicity, RAW 264.7 cells were co-cultured with *Salmonella* at an MOI of ~ 50 for 6 h in the presence of the caspase-1-specific inhibitor Ac-YVAD-CMK or DMSO and subjected to cytotoxicity analysis using an LDH release assay. The results showed that WT *Salmonella* Choleraesuis induced approximately 35% cytotoxicity in RAW 264.7 cells and the addition of Ac-YVAD-CMK significantly reduced cytotoxicity to 23% (Fig. 5). By contrast, cytotoxicity caused by CP327 or CP359 strains was only ~ 14%, indicating that Δ*crp* mutants were less cytotoxic to macrophages (Fig. 5). To further confirm that the reduction in cytotoxicity was attributed to the mutation of the *crp* gene, the effect of CRP complementation was investigated. It was noted that when complemented with a plasmid expressing CRP, the CP327 strain regained the ability to induce cytotoxicity in macrophages and the cytotoxicity was reduced significantly by a caspase-1 inhibitor Ac-YVAD-CMK ($p < 0.01$) (Fig. 5).

4. DISCUSSION

The application of live *Salmonella* vaccines as a preventive measure has been effective in the control of swine salmonellosis [32]. By eliciting effective humoral and cellular immune responses in the vaccinated animals, live *Salmonella* Choleraesuis vaccines harboring a mutation in *crp* have been shown to induce protective immunity in mice and pigs [8, 20]. One of the characteristics of such mutants is the attenuation in virulence due to unknown mechanism(s). In this study, we showed that, at the early stage of infection, the attenuation in Δ*crp* was not attributed to increased susceptibility to low pH or bile salts, but to a reduction in invasiveness associated with an impairment of the secretory function of SPI-1 T3SS and lower invasion rate and cytotoxicity to macrophages. This study is the first to demonstrate that a mutation in the *crp* gene leads to a functional defect in the SPI-1 T3SS and to delineate the role of CRP in *Salmonella* Choleraesuis virulence.

Genes targeted for the development of live *Salmonella* vaccines fall into two major categories, metabolic and virulence-associated

genes, of which, the former were found to be more immunogenic [23]. Nevertheless, not all mutants deficient in a metabolic-gene are attenuated or effective in inducing immunity. For example, in contrast to the findings in *Salmonella* Typhimurium [17], Nnalue et al. (1987) reported that *Salmonella* Choleraesuis strains with mutations in the *aroA* (aromatic biosynthesis) or *galE* (UDP-glucose-4-epimerase) genes were attenuated, but did not induce a protective response in BALB/c mice [27]. Distinctively, mutations in the *crp* gene of *Salmonella enterica* serovars Typhimurium, Choleraesuis, or Typhi lead to the attenuation of yielding strains that may serve as effective vaccine candidates in mice, pigs, horses or human beings [8, 20, 33, 35, 43]. However, it is not clear how *crp* mutants display reduced virulence yet manage to elicit a protective host immune response.

Using ligated ileal loops of pigs, we found that intestinal invasion of *Salmonella* Choleraesuis Δ*crp* was significantly impaired as compared to WT. Nevertheless, there were still approximately 10^6 CFU (per gram of tissue) of Δ*crp* attached to or invaded into the ileum wall, and 10^3 CFU (per gram of tissue) of Δ*crp* were able to reach mesenteric lymph nodes, suggesting that the ability of Δ*crp* to invade intestinal tissues was not fully abolished and that a pathway independent of SPI-1 T3SS might have allowed *Salmonella*-infected phagocytes to gain access and spread to mesenteric lymph nodes, where the immune response occurs. This hypothesis is supported by other investigations showing that *Salmonella* Typhimurium deficient in SPI-1 also gained access to the spleen from the intestine [13] and that CD18-expressing phagocytes are responsible for the extra-intestinal dissemination of *Salmonella* [39]. Therefore, it is plausible that *Salmonella* Choleraesuis *crp* mutants might also invade and subsequently induce immune responses in the spleen and/or lymph nodes via a similar pathway. Moreover, because SPI-1 T3SS activity was impaired in *Salmonella* Choleraesuis Δ*crp*, we speculate that the immunogenicity of *Salmonella* Choleraesuis does not rely on a functional SPI-1 T3SS. This hypothesis is supported by a recent study that demonstrated that protection against *Salmonella* Typhimurium in pigs is independent of SPI-1 T3SS secreted proteins [3].

Survival within macrophages is an essential feature of *Salmonella* pathogenesis in vivo [12]. In this study, we found that the Δ*crp* mutant was less invasive for PAM as compared to WT, but the survival rates were similar. Therefore, a reduction in the invasiveness of the Δ*crp* mutant probably accounts for the reduced number of Δ*crp* cells observed in mesenteric lymph nodes of ligated ileal loops. Other investigators have demonstrated decreased colonization of Peyer patches and spleens of mice infected by *Salmonella* Choleraesuis strains having mutations in the *crp*/*cya* genes. Moreover, adhesion/invasion in CHO cells and bacterial transcytosis in polarized Madin–Darby canine kidney cells was significantly reduced as compared to WT [19]. Taken together, these findings strengthen our results that suggest these phenotypes are correlated to impaired SPI-1 T3SS function in *Salmonella* Choleraesuis Δ*crp*.

However, CRP is pleiotropic and mutations in the *crp* gene have been shown to alter diverse characteristics of *Salmonella* including carbon source utilization, synthesis of flagella, reduced growth rate, and attenuation in virulence [9, 43]. Due to the versatile role of CRP, we cannot exclude the possibility that virulence determinants other than SPI-1 T3SS may also be affected in *Salmonella* Choleraesuis Δ*crp*. For example, a recent report indicated that there is a significant decrease of SPI-2 gene expression in the *crp* mutant of *Salmonella* Typhimurium [41]. Also, our results show that a mutation in the *crp* gene increased the resistance of *Salmonella* Choleraesuis to low pH or bile salts, and Δ*crp* was more resistant to gentamicin as comparing to the parental strain (data not shown). These data led us to hypothesize that a mutation in *crp* might have effects on the composition or structure of the cell wall resulting in decreased permeability to bile salts or gentamicin.

In conclusion, this study explored the gastrointestinal factors that affect *Salmonella* Choleraesuis Δ*crp* colonization/invasion and provides evidence to delineate one of the attenuation mechanisms of *Salmonella* Choleraesuis Δ*crp* that is widely approached as a candidate of live *Salmonella* vaccine.

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