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Plesiomonas shigelloides sipD mutant, generated by an efficient gene transfer system, is less invasive

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ABSTRACT

Plesiomonas shigelloides is widely associated with human diarrheal disease. Research on this pathogen has been hampered by the absence of an effective genetic manipulation system. In the present study, an efficient and precise conjugation transfer procedure, mediated by suicide vector pRE112 was used to overcome this limitation. The efficiency of generating double recombinants was average 74.3%, and the conjugation protocol may be applied to other *P. shigelloides* strains. We also identified that the SipD protein of *P. shigelloides* G5884 (serotype O45) is 65% similar to the SipD in *Salmonella* pathogenicity island 1 (SPI-1), which is a key element of the type III secretion system related to *Salmonella* invasion. A *P. shigelloides sipD* null mutant was generated via the conjugation system, using the suicide vector pRE112. The isogenic mutant strain lacking *sipD* showed a 50% reduction in its capacity to invade Caco-2 cells.

1. Introduction

Plesiomonas shigelloides is a rod-shaped gram-negative bacterium recognized as a causative agent of diarrhea and other diseases, including acute secretory gastroenteritis, an invasive shigellosis-like disease (Tsukamoto et al., 1978; McNeeley et al., 1984; Krovacek et al., 2000). In addition, certain extra-intestinal infections, such as meningitis, bacteremia (Billiet et al., 1989), and pseudo appendicitis (Brenden et al., 1988) are associated with *P. shigelloides*. Fresh and estuarine water are considered the natural environments of *P. shigelloides*, which is often isolated from fish and other seafood (Salerno et al., 2010).

A suicide vector containing homologous sequence to host chromosome is useful to integrate foreign DNA into a host chromosome. The positive clones that the suicide vector integrating into the chromosomal gene by homologous recombination are selected via sucrose sensitive gene *sacB*, the vector DNA is removed when the sucrose is added to the medium, resulting in possible replacement of the wild-type (wt) allele with a mutant one. This study aimed to improve an efficient genetic manipulation system for *P. shigelloides* based on the suicide vector pRE112 (with *sacB* for counter-selection) (Edwards et al., 1998) using the conjugative machinery from *Escherichia coli* S17–1 λ*pir*.

P. shigelloides strain G5884 (serotype O45) possesses the highest invasiveness among six serotypes (Table 1) tested. The strain contains a gene cluster of 34 genes that is similar to *Salmonella* pathogenicity island (SPI-1) for the type III secretion system (T3SS) (Burkinshaw and Strynadka, 2014; Fabrega and Vila, 2013). We selected the *sipD* gene, the product is 65% homologous with the SipD protein of SPI-1 and is a key element of T3SS related to *Salmonella* invasion, to test the genetic transfer system.

In this report, we created a Δ*sipD* mutant using the conjugation system, and compared its capacity to invade the eukaryotic cells (Caco-2 cells) with that of the wild type strain G5884.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used are listed in Table 1. *P. shigelloides* was cultured in trypticase soy broth (TSB) and on trypticase

Abbreviations: SPI-1, *Salmonella* pathogenicity island 1; T3SS, type III secretion system; Δ*sipD*-U, The upstream DNA sequences of the *sipD* gene; Δ*sipD*-D, The downstream DNA sequences of the *sipD* gene; Δ*sipD*-UD, The overlap fragment with *sipD*-U and *sipD*-D; *sipD*$^+$-UD, The overlap fragment with *sipD*-U, *sipD* and *sipD*-D; pRE112-*sipD*$^-$Ps, pRE112 containing the homologous arms of *sipD* gene; pRE112-*sipD*$^+$-Ps, pRE112 containing the homologous arms and the *sipD* gene

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Table 1
Bacterial strains and plasmids used in this study.

Strains/plasmids	Relevant characteristics[a]	Reference
Plesiomonas shigelloides strains		
G5877	Serotype O2:H1a1c	CNCTC[b] Aer 33/89
G5996	Serotype O4:H3	CNCTC[b] Aer 53/89
G6001	Serotype O27:H3[b]	CNCTC[b] Aer 30/89
G5884	Wild type, serotype O45:H2[b]	CNCTC[b] Aer 44/89
G6006	Serotype O46:H3	CNCTC[b] Aer 46/89
G5270	Serotype O66:H3	Italy[c] H3
△*sipD*	*sipD* gene deletion mutant of G5884	This study
△*sipD*$^+$	*sipD* complementation strain	This study
E. coli strains		
DH5α λ*pir*	Transformation host	Lab collection
S17–1 λ*pir*	Tp^R Sm^R*recA*, *thi*, *pro*, *hsdR*-M$^+$RP4: 2-Tc:Mu: Km Tn7 λ*pir*, Km^r, Sm^r, Tp^r	Simon et al., 1983
Plasmid		
pRE112	Widely used gene knocked vector, with onT RP4, Cm^r	Edwards et al., 1998
pRE112-*sipD*$^-$-Ps	pRE112 containing the homologous arms of *sipD* gene of G5884, Cm^r	This study
pRE112-*sipD*$^+$-Ps	pRE112 containing the homologous arms and the *sipD* gene of G5884, Cm^r	This study

[a] r = resistant.
[b] CNCTC, Czech National Collection of Type Cultures, the Czech Republic.
[c] Italy, Department of Environmental Sciences, Parthenope University, Centro Direzionale, Isola C4, 80143 Naples, Italy.

soy agar (TSA) plate at 37 °C. *E. coli* was cultured in Luria-Bertani medium (LB) at 37 °C. Conjugation assays were performed in TSA medium. When needed, 10% sucrose and 25 μg/ml of chloramphenicol (Cm) were added to the medium.

2.2. Genomic DNA and plasmid DNA extraction

The genomic DNA was extracted by using a Rapid Bacterial Genomic DNA Isolation kit (Sangon Biotech Co., Ltd., Shanghai, China). Plasmid DNA was extracted using a SanPrep Column Plasmid Mini-Preps Kit (Sangon Biotech Co., Ltd.).

2.3. Sequencing and bioinformatic analysis

Whole-genome sequencing of G5884 was performed on the Solexa paired-end sequencing platform. Genomic DNA was prepared using the Illumina Sample Preparation Kit according to the manufacturer, protocols. Genomic libraries were constructed that 500 bp paired-end inserts, and sequencing was performed with Solexa sequencing technologies to produce ~100-fold coverage for the genome. Sequence reads were assembled using the de novo genome-assembly program Velvet to generate multi-contig draft genome. Bioinformatic analysis via clustalX was used to search similar genes or proteins.

2.4. Primer design

The two pairs of primers (Δ*sipD*-U-F/Δ*sipD*-U-R and Δ*sipD*-D-F/Δ*sipD*-D-R) for *sipD* mutant, used to amplify the upstream and downstream DNA sequences (named Δ*sipD*-U and Δ*sipD*-D) of the target *sipD* gene from G5884 genomic DNA, were designed using Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) (Table 2). Additionally, we added a cleavage site for the *Sac*I restriction enzyme at the 5′ end of Δ*sipD*-U-F, and added a cleavage site for the *Kpn*I restriction enzyme at the 5′ end of Δ*sipD*-D-R. To perform the overlap-extension PCR, 5′-CAATAGGTGTGTCATA-3′ was added at the 5′ ends of Δ*sipD*-U-R and Δ*sipD*-D-F. Similarly, the three pairs of primers of *sipD*$^+$-U-F/*sipD*$^+$-U-R, *sipD*-F/*sipD*-R and *sipD*$^+$-D-F/*sipD*$^+$-D-R for the complementation were designed as well. The restriction enzyme sites are indicted in bold underlined type in Table 2.

2.5. Development of the overlap-extension PCR method

The upstream and downstream DNA sequences (Δ*sipD*-U and Δ*sipD*-D) of the target *sipD* gene were amplified in a 20 μl reaction system containing 10 μl of 2 × Phusion mix, 5 μl of ddH_2O, 1 μl of forward primer, 1 μl of reverse primer, and 100 ng of G5884 genomic DNA as the template, and mixed by instantaneous centrifugation.

Table 2
Primers used in this study.

Name	Sequence (5′–3′)	Annealing temperature (°C)
Δ*sipD*-U-F	AGA**GAGCTC**GCCAGCAAATTAAACCTGAA	68
Δ*sipD*-U-R	TATGACACACCTATTGACCACATCCGCACT	70
Δ*sipD*-D-F	CAATAGGTGTGTCATATAAGGCTCAGGAAG	66
Δ*sipD*-D-R	GAT**GGTACC**GCGAGGCTAATTGGTGTTC	69
sipD$^+$-U-F	AGA**GAGCTC**GCCAGCAAATTAAACCTGAA	68
sipD$^+$-U-R	TTATATCCATACCACATCCGCACT	65
sipD -F	CGGATGTGGTATGGATATAAATAATAGTACA	67
sipD -R	CTGAGCCTTATCAACCTTGCAGGAATGACT	66
sipD$^+$-D-F	GCAAGGTTGATAAGGCTCAGGAAG	64
sipD$^+$-D-R	GAT**GGTACC**GCGAGGCTAATTGGTGTTC	69

Restriction enzyme sites are indicted in bold underlined type.

The overlap-extension PCR of Δ*sipD*-UD was performed in 20 μl reaction system containing 10 μl of 2 × Phusion mix, 5 μl of ddH_2O, 1 μl of Δ*sipD*-U-F primer, 1 μl of Δ*sipD*-D-R primer, 100 ng of upstream DNA fragment and 100 ng of downstream DNA fragment as the templates.

The PCR reactions were conducted under the conditions of pre-denaturation at 98 °C for 30 s; 29 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 20 s, an extension at 72 °C for 30 s; with a final extension at 72 °C for 10 min. The PCR products were detected using electrophoresis in a 1% agarose gel and recovered. The PCR products were stored at −20 °C. And the PCR product of *sipD*$^+$-UD for Δ*sipD*$^+$ complementation were generated by using the same method with the corresponding pairs of primers.

2.6. Construction of the recombinant plasmid pRE112-*sipD*$^-$-Ps and pRE112-*sipD*$^+$-Ps

The Δ*sipD*-UD from the overlap-extension PCR was digested with *Sac*I and *Kpn*I, and ligated into the *Sac*I/*Kpn*I sites of pRE112. The recombinant plasmid pRE112-*sipD*$^-$-Ps was transformed into *E. coli* DH5α λ*pir*. The recombinant plasmid was extracted from positive colonies and confirmed by plasmid digestion. *E. coli* DH5α λ*pir* with pRE112-*sipD*$^-$-Ps was stored at −80 °C. The same method was used for the construction in pRE112-*sipD*$^+$-Ps. When needed, the recombinant

plasmids were extracted from *E. coli* DH5α λ*pir* and transformed into *E. coli* S17–1 λ*pir*.

2.7. Conjugation and screening the ΔsipD mutant and ΔsipD$^+$ complementation strains

Briefly, the recipient strain G5884 and donor *E. coli* S17–1 λ*pir* strain were grown at 37 °C with shaking overnight. The two strains were then diluted 1:100 into fresh medium and incubated at 37 °C with shaking until the OD_{600} reached approximately 0.6. The cells were harvested by centrifugation (2000 ×*g*, 4 °C for 5 min), washed, and resuspended with fresh TSB medium. The donor *E. coli* strain S17–1 λ*pir* and the recipient strain were mixed by brief vortexing at a ratio of 3:1 (v/v). The mixture was collected by centrifugation (2000 ×*g*, 4 °C for 30 s), resuspended in 100 µl of fresh TSB medium, spotted on a TSA plate, and incubated at 37 °C for 48 h.

After mating, the cells were collected, washed with TSB, and spread on TSA plate with 25 µg/ml of chloramphenicol to screen for clones that acquired chloramphenicol resistance via a single crossover event. The growing clones were then transferred into TSB medium with 25 µg/ml chloramphenicol at 37 °C overnight. The overnight culture was diluted and spread on TSA plates containing 10% sucrose and grown at 37 °C for 12–24 h. Positive colonies were transferred onto TSA plates and TSA plates containing 25 µg/ml chloramphenicol simultaneously, and colonies that were sensitive to chloramphenicol were collected and confirmed by PCR and DNA sequencing. A schematic diagram of the conjugation transfer procedure mediated by suicide vector pRE112 in *E. coli* S17–1 λpir is shown in Fig. 1.

In the TSA plates containing 10% sucrose, any 100 colonies were randomly selected and confirmed by PCR and DNA sequencing. The efficiency was calculated as the percentage of positive transconjugants among the 100 selected colonies.

2.8. Invasion assays

The invasion assay was carried out as described previously (Schubert and Holz-Bremer, 1999), with some modifications. Briefly, approximately 5×10^7 bacterial cells were layered onto confluent monolayers of approximately 1×10^5 Caco-2 cells (suspended in phosphate-buffered saline (PBS)) per well in 24-well plates, and incubated at 37 °C in 5% CO_2 for 1 h. The monolayer was washed extensively with PBS, and fresh, pre-warmed Dulbecco's modified Eagle's medium (DMEM) containing gentamycin (100 µg/ml) was added to kill extracellular bacteria. After 1 h of incubation, the monolayer was washed with PBS twice, and the cells were lysed with 0.01% Triton X-100 for 30 min; the released intracellular bacteria were enumerated using the plate counting method. The invasive ability was expressed as the percentage of the inoculum that survived the gentamycin treatment.

2.9. Statistical analysis

Statistical analysis of the data was performed using ANOVA. A probability value (P) ≤ 0.05 was considered statistically significant (***p ≤ .001; **p ≤ .01; *p ≤ .05; ns indicates none significance).

2.10. Nucleotide sequence accession number

The gene cluster of 34 genes in *P. shigelloides* G5884 (serotype O45) that is similar to SPI-1 of *Salmonella* has been deposited in GenBank under the accession No. MK256934.

3. Results and discussion

3.1. The sipD gene in P. shigelloides G5884 is likely related to host invasion

Analysis of the genome of G5884 (serotype O45) revealed a cluster of 34 genes that is similar to SPI-1 of *Salmonella* and named as the putative *P. shigelloides* T3SS, (Fig. 2). Furthermore, the data revealed that SipD protein (1441 bp) is highly similar (65%) to the SipD of SPI-1 with the coverage of 99% and e-value at $1e^{-103}$, suggesting the *sipD* gene in G5884 would be related to *P. shigelloides* invasion, because *Salmonella sipD* is a key element of the T3SS that participates in host invasion.

The T3SS creates a contiguous channel through the bacterial and

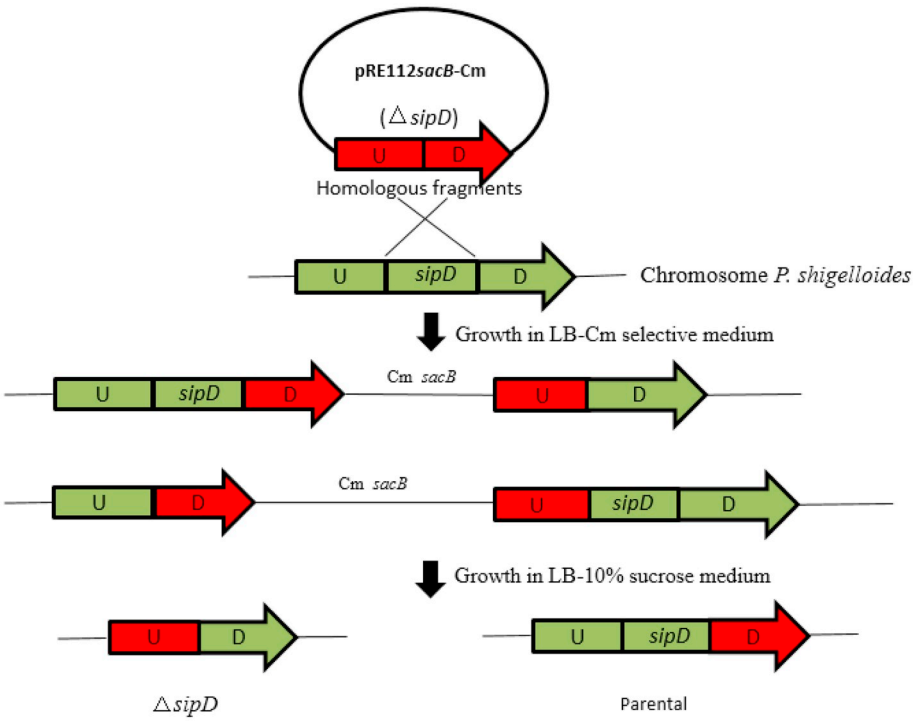

Fig. 1. Conjugation transfer procedure mediated by the suicide vector pRE112 in *Escherichia coli* S17–1 λpir.

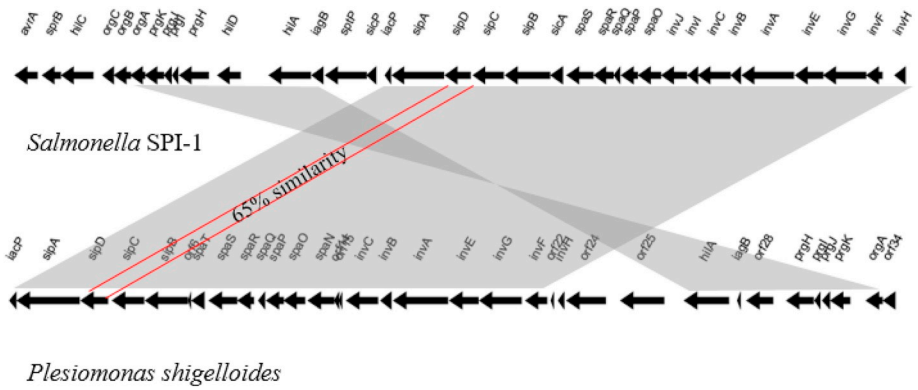

Fig. 2. Schematic representation of the gene cluster of *Salmonella* pathogenicity island 1 (SPI-1) and the predicted *Plesiomonas shigelloides* type III secretion system. And the similarity was blast by using the clustalX.

host membranes, allowing injection of specialized bacterial effector proteins directly into the host cell. The T3SS encoded by the *inv/spa* invasion locus resides on a pathogenicity island of *Salmonella* and is named as pathogenicity island 1 (SPI-1) (Shea et al., 1996). Among the 34 genes of the SPI-1 cluster, *sipD* encodes a needle tip protein, similar to IpaD of *Shigella* (Meghraoui et al., 2014), EspA of enteropathogenic *E. coli* (An et al., 2000), LcrV of *Yersinia* spp. (Espina et al., 2007), and PcrV of *Pseudomonas aeruginosa* (Sato et al., 2011). We found that the SipD protein of *P. shigelloides* G5884 (serotype O45) is 65% similar to the SipD in *Salmonella* SPI-1, which is a key element of T3SS related to *Salmonella* invasion (Wee and Hughes, 2015).

3.2. Construction of the recombinant plasmid pRE112-sipD$^-$-Ps and pRE112-sipD$^+$-Ps

The schematic illustration of the overlap-extension PCR method used is shown in Fig. 3A. The fragments of Δ*sipD*-U (689 bp), Δ*sipD*-D (752 bp) and Δ*sipD*-UD (1441 bp) were detected by electrophoresis and showed in Fig. 3B. The recombinant plasmid pRE112-*sipD*$^-$-Ps was transformed into *E. coli* DH5α λ*pir*. The recombinant plasmids were extracted from positive colonies and digested with *Sac*I and *Kpn*I, and the products were detected by electrophoresis (Fig. 3C). Positive *E. coli* DH5α λ*pir* with the recombinant plasmid pRE112-*sipD*$^-$-Ps was stored at −80 °C. And recombinant plasmid pRE112-*sipD*$^+$-Ps was constructed accordingly.

3.3. Intergeneric conjugation between E. coli and P. shigelloides

Electroporation is a standard molecular biological technique that is widely applied for gene transfer in bacteria. However, the published protocols are strain-dependent, requiring optimization for individual strains (Dominguez and O'Sullivan, 2013). Previously, conjugative transfer of transposition element miniTn*5*Km-1 was used for gene knockout in *P. shigelloides* (Aquilini et al., 2013). However, Mutants 302-A and 302-B were selected among 1200 mutants. Conjugation based on the vector pRE112 is a better alternative as it is suitable for a wide range of hosts.

When the recipient and donor strains were spotted on TSA plate after being mixed by brief vortexing, the suicide plasmid pRE112-*sipD*$^-$-Ps was mobilized from *E. coli* S17–1 λ*pir* into *P. shigelloides* G5884 by intergeneric conjugation. After mating, cells were spread on TSA plate with chloramphenicol to screen for clones in which the suicide plasmid pRE112-*sipD*$^-$-Ps had integrated into the G5884 genome via a single crossover event. To select mutants in which the second recombination had occurred, the overnight culture was diluted and spread on TSA plates containing sucrose and grown at 37 °C for 12–24 h. Sucrose resistant colonies were transferred onto TSA plates and TSA plates containing chloramphenicol simultaneously, and colonies sensitive to chloramphenicol were collected and confirmed by PCR using primers Δ*sipD*-U-F and Δ*sipD*-D-R (Fig. 3D), followed by DNA sequencing. And the complementation was confirmed by PCR using primers *sipD*$^+$-U-F and *sipD*$^+$-D-R (Fig. 3E), followed by DNA sequencing.

The suicide vector pRE112 had been applied to other hosts other than *Plesiomonas shigelloide*. For example, Xu et al. constructed the Δ*lacZ*, Δ*slmA* and Δ*metJ* mutants in *Vibrio cholera*e (Xu et al., 2018). Panina et al. constructed the Δ*bteA* and Δ*btcA* mutants in *Bordetella bronchiseptica* (Panina et al., 2005). Lan et al. contstructed the Δ*esrB* mutant, which encodes a regulator protein of the type III secretion system in *Edwardsiella tarda* (Lan et al., 2007). Yang et al. constructed the Δ*lamB* mutant in *Aeromonas veronii* (Yang et al., 2019).

3.4. The efficiency of generating double recombinants

On the TSA plates containing 10% sucrose, three batches of 100 random colonies selected and screened with 65, 73 and 85 colonies were confirmed as positive colonies by PCR and DNA sequencing, indicating a high efficiency of 74.3% ± 8.22%. Meanwhile, higher conjugation efficiencies were obtained when the ratio of *E. coli* to *P. shigelloides* was 3:1 (v/v) after incubation at 37 °C with shaking to an OD_{600} of approximately 0.6, showing a slight improvement in the conjugation efficiency (5-fold) when compared with the 1:1 ratio that is typically suggested in conjugation protocols.

3.5. Invasion of Caco-2 cells

Compared with the *P. shigelloides* wild-type strain G5884, the isogenic mutant lacking *sipD* showed a 50% reduction in its capacity to invade eukaryotic cells, and the Δ*sipD*$^+$ complementation strain could restore the invasive ability to wild type strain (Fig. 4). The assay was repeated three times. The difference in invasion capabilities between the wild-type and Δ*sipD* was statistically significant (p = .0148). The data demonstrated that SipD is an important invasion protein, similar to its counterpart in *Salmonella.*

4. Conclusion

Epidemiological evidence has shown that *P. shigelloides* is a causative organism of diarrheal disease (Tsukamoto et al., 1978). An efficient genetic manipulation system is required to study *P. shigelloides* in vivo. We improved an efficient genetic manipulation system, based on the suicide vector pRE112 and the conjugative machinery from *E. coli* S17–1 λ*pir*, to overcome the current limitations of genetic manipulation in *P. shigelloides*. And we demonstrated that *sipD* is related to invasion in

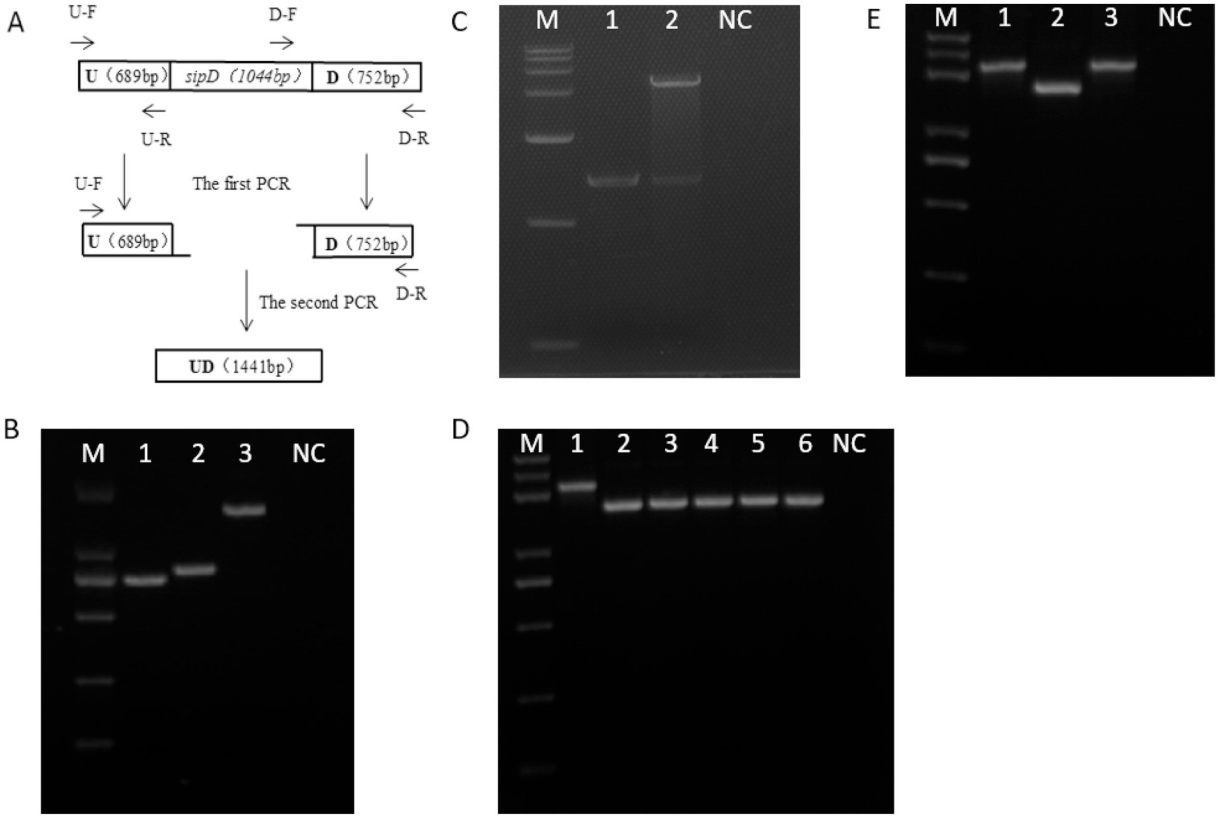

Fig. 3. Confirmation of the deletion of *sipD* in G5884. A. Overlap-extension PCR. B. PCR detection of the upstream and downstream DNA sequences of *sipD*, and the product of the overlap-extension. M, DL2000 DNA marker. 1, *sipD*-U. 2, *sipD*-D. 3, *sipD*-UD. NC indicates negative control ddH_2O. C. The recombinant plasmid pRE112-*sipD*$^-$-Ps extracted from the positive clone was confirmed by double digestion. M, DL15000 DNA maker. 1, *sipD*-UD acquired from the overlap-extension and digested with *Sac*I and *Kpn*I indicates the positive control. 2, pRE112-*sipD*$^-$-Ps extracted from positive clone was digested with *Sac*I and *Kpn*I. NC indicates negative control (ddH_2O). D. PCR confirmation of the Δ*sipD* mutant by using *sipD*-U-F and *sipD*-D-R primers. M, DL5000 DNA maker. 1, G5884 wild-type strain. 2–6, five independent colonies. NC indicates negative control ddH_2O. E. PCR confirmation of the Δ*sipD*$^+$ complementation strain by using *sipD*$^+$-U-F and *sipD*$^+$-D-R primers. M, DL5000 DNA maker. 1, G5884 wild-type strain as positive control. 2, Δ*sipD* strain as negative control. 3, Δ*sipD*$^+$ complementation strain. NC indicates negative control ddH_2O.

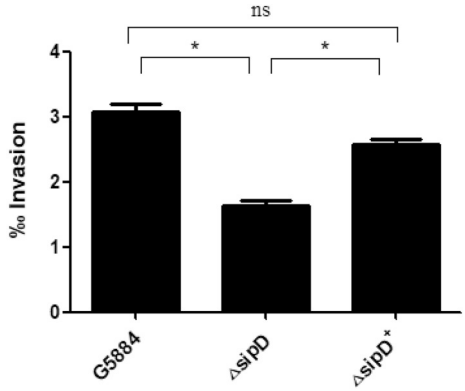

Fig. 4. Effect of the invasion capability of the wild type G5884, the mutant Δ*sipD* and the Δ*sipD*$^+$ complementation strain into Caco-2 cells for 1 h. Results are performed using ANOVA of three independent assays.

P. shigelloides G5884 similar to *Salmonella* by using this genetic manipulation system. The genetic manipulation system will be useful to study the pathogenicity of *P. shigelloides*.

Conflict of interest

The authors declare that there are no financial or commercial conflicts of interest.

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