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Deletion of a prophage-like element causes attenuation of *Salmonella enterica* serovar Enteritidis and promotes protective immunity

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ABSTRACT

Salmonella enterica serovar Enteritidis (*S.* Enteritidis) is a wide host range serovar belonging to the *S. enterica* genus. Worldwide, it is one of the most frequent causes of food borne disease. Similar to *S.* Typhimurium, some virulence genes of *S.* Enteritidis are located in pathogenicity islands and prophages. In this study we have generated a mutant strain of *S.* Enteritidis lacking a prophage-like element, denominated ϕSE12. The resulting mutant strain was attenuated and promoted protective immunity in infected mice. Although *S.* Enteritidis strains lacking the complete prophage ϕSE12 remained capable of surviving inside phagocytic cells, they showed a significantly reduced capacity to colonize internal organs and failed to cause lethal disease in mice. Consistent with these data, infection with *S.* Enteritidis strains lacking prophage ϕSE12 promoted the production of anti-*Salmonella* IgG antibodies and led to protection against a challenge with virulent strains of *S.* Enteritidis. These results suggest that strains lacking this prophage can induce a protective immunity in mice and be considered as potential attenuated vaccines against *S.* Enteritidis.

1. Introduction

Some members of *Salmonella enterica* genus are pathogenic bacteria that cause an array of diseases in warm-blooded animals [1]. Among them, *S. enterica* serovar Enteritidis (*S.* Enteritidis herein) is a wide host range serovar that can cause diseases in more than one host [2]. Similarly to *S.* Typhimurium (another wide host range serovar), *S.* Enteritidis causes gastroenteritis in humans and a systemic and lethal infection in mice [3,4]. This pathogenic bacterium is an important public health problem in several countries as a frequent cause of gastroenteritis and zoonotic infection [5,6]. In addition, this pathogen can cause an asymptomatic infection in poultry, which results in egg contamination and transmission to humans upon consumption of raw or undercooked eggs and its derivatives [3].

The complete genome of *S.* Enteritidis PT4 was recently sequenced and made available to the scientific community [7]. Comparative analyses of the genomic sequences have revealed the presence of several DNA regions in *S.* Enteritidis, which are absent from the chromosome of *S.* Typhimurium [7]. Most of these sequences encode for prophages and genomic islands, which represent 6.4% of the *S.* Enteritidis PT4 genome [7]. It is thought that these regions were probably acquired by means of lateral gene transfer events and have been denominated "regions of difference" (ROD) [7].

Prophage ϕSE12 is one of the RODs found in the genome of *S.* Enteritidis PT4. This is a 19.6 kb incomplete lambda-related prophage and it contains 26 annotated genes [7]. Sequence analyses have shown that this prophage possesses two genes, *sodC* and *sopE*, which were previously related to virulence in other *Salmonella* serovars. For instance, the *sodC* gene in prophage ϕSE12 of *S.* Enteritidis is 100% identical to the *sodC* gene found in the prophage Gifsy-2 of *S.* Typhimurium [8,9]. This genetic element encodes for a Fe–Cu Superoxide dismutase precursor, which is specifically required to protect *S.* Typhimurium from reactive oxygen species (ROS) within phagocytic cells [8,9]. SopE, on the other hand, is a guanine exchange factor that promotes the stabilization of actin filaments, ruffle formation and phagocytosis of *Salmonella* by non-phagocytic cells, such as intestinal epithelia [10–12]. Most of the other putative genes included in the prophage ϕSE12 encode for phage related proteins or proteins of unassigned functions [7].

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Here we have evaluated the contribution of prophage ϕSE12 to *S.* Enteritidis virulence in the mouse. We observed that deletion of the complete prophage led to a severe attenuation of *S.* Enteritidis virulence in mice after oral infection. In addition, mice infected with the mutant strain produced high titers of anti-*S.* Enteritidis IgG antibodies and were protected against a lethal challenge with virulent *S.* Enteritidis. We also observed that mutant strains complemented with either the *sodC* or *sopE* gene recovered the capacity to cause a lethal disease in the mouse. Interestingly, deletion of complete prophage ϕSE12 did not affect the capacity of *S.* Enteritidis to survive inside phagocytic cells, such as macrophages and dendritic cells. In summary, our results suggest that genes in prophage ϕSE12 are fundamental for *S.* Enteritidis virulence in mice. Furthermore, strains lacking this prophage could be considered as potential vaccine strains to protect against virulent *S.* Enteritidis, or as an immunogenic vector for delivering heterologous antigens.

2. Materials and methods

2.1. Bacterial strains

S. Enteritidis wild type (WT) used in this study is a clinical strain, phagotype 1 (PT1), which was kindly provided by Mrs. Alda Fernandez from the Public Health Institute of Chile (ISP). This *S.* Enteritidis strain was previously characterized as a virulent strain in mice [13]. The presence of prophage ϕSE12 was detected in PT1 strains and in a collection of other 30 clinical strains of *S.* Enteritidis, which were provided by Dr. Patricia García (Facultad de Medicina, Pontificia Universidad Católica de Chile). To test the presence of the prophage ϕSE12 in these strains, we prepared genomic DNA, as described previously [14]. The right and left boundaries of the prophage, along with *sodC* and *sopE* genes, were amplified by PCR using primer pairs described in the supplementary Table 1. Routine PCR amplifications were performed in a BioRad MJ Personal Mini Thermal Cycler (BioRad), using the following program: 1 cycle of 5 min at 95 °C, 30 cycles of 40 s at 95 °C, 40 s at 55 °C and 1–5 min at 72 °C, and a final cycle at 72° for 10 min. PCR products were resolved by electrophoresis in 1% agarose gels containing 1X SYBR® Safe (Catalog number S33102, Invitrogen), and visualized under UV light transilluminator (UVP, Inc.).

S. Enteritidis ΔϕSE12::Cm was generated by Lambda Red-mediated recombination, as described by Datsenko and Wanner [15]. Briefly, a PCR product encoding a chloramphenicol-resistance gene was generated by PCR amplification from the plasmid pKD3 [15]. The primers used were SEN_phage_12_(H1 + P1) (5′ CACCGATCATACCGGGCAGTCGTCAAACCTCCAGGATCGCgtgtaggctggagctgct tc 3′) and primer SEN_phage_12_(H2 + P2) (5′ AGATTTACTGGCCTTACGGTAGGTGAGGTTCCTCGGAAGGcatatgaatatcctcctta 3′). The first 40 bp of these primers (capital) align with bp 1,245,492–1,245,453 and 1,227,485–1,227,524 of the *S.* Enteritidis chromosome. The last 20 bp of these primers align with plasmid pKD3 (underscore). The PCR product obtained was electrotransformed to competent *S.* Enteritidis that harboured the thermosensitive plasmid pKD46 [15]. Previously to the transformation, bacteria were grown for 2 h in liquid Luria Bertani (LB) medium (Bacto Tryptone, 10 g/l; Bacto Yeast Extract, 5 g/l; NaCl, 5 g/l, DIFCO) and supplemented with 0.4% (w/v) Arabinose (Sigma–Aldrich). After electrotransformation, bacteria were incubated for 1 h at 37 °C with aeration in 1 ml of LB medium, and then seeded in solid LB medium supplemented with 20 μg/ml chloramphenicol. To test the correct replacement of prophage ϕSE12 by the chloramphenicol-resistance gene, PCR amplification was performed using the genomic DNA of the mutant strains and the primers SEN_1158_Fw (5′ GCCAGAAAAATTTCAGACGTT 3′) and SEN_phage_12_(H2 + P2). This PCR reaction generated a PCR product of 1909 bp only if the correct replacement of prophage ϕSE12 by the chloramphenicol-resistance gene occurred. To generate a chloramphenicol-sensitive *S.* Enteritidis ΔϕSE12, mutant strains were electrotransformed with the thermosensitive plasmid pCP20 (which encodes for the recombinase required to excise the chloramphenicol-resistance gene) and treated as previously described [15]. To corroborate the deletion of this gene in the resulting chloramphenicol-sensitive strains, a PCR amplification was performed using primers SEN_1158_(integrase)_Fw and SEN_phage_12_(H2 + P2), which generate a PCR product of 969 bp when the gene was correctly removed.

The ΔaroA::Kn strain of *S.* Enteritidis was generated using the Lambda Red-mediated recombination system described above. In this case, a kanamicin gene was amplified by PCR using the plasmid pKD4 as template [15] and primers aroA(H1 + P1) (5′ ATGGAATCCCTGACGTTACAACCCATCGCGCGGGTCGATGgtgtaggctggagctgcttc 3′) and aroA(H2 + P2) (5′ TTAGGCAGGCGTACTCATTCGCGCCAGTTGTTCGAAATAAcatatgaatatcctcctta 3′). The correct replacement of *aroA* gene by the kanamicin resistant gene was tested using primers aroA_Fw (5′ ATGGAATCCCTGACGTTACA 3′) and aroA_rev (5′ TTAGGCAGGCGTACTCATTC 3′). Amplification with these primers generated a PCR product of 1284 bp in the WT strain and a 1,557 bp product in the ΔaroA::Kn strain.

2.2. Complementation assays

To incorporate *sodC* and *sopE* genes back to *S.* Enteritidis ΔϕSE12, these genes were amplified by PCR using genomic DNA from WT *S.* Enteritidis (as template) and primers sopE-phage12-NcoI-Fw (5′ GGCCATGGGAGTGACAAAAATAACTTTAT 3′) and sopE-phage12-HindIII-Rev (5′ GGAAGCTTTCAGGGAGTGTTTTGGATA 3′) to amplify the *sopE* gene; and primers sodCI-phage12-NcoI-Fw (5′ GGCCATGGGAATGAAATACACAATATTGT 3′) and sodCI-phage12-HindIII-Rev (5′ GGAAGCTTTTATTTCTCAATGACACCA 3′) to amplify the *sodC* gene. PCR products were cloned in the pCR®-Blunt II-TOPO® vector (Invitrogen) according to the manufacturer instructions. Next, plasmids containing *sopE* and *sodC* genes and the expression vector pKK233.2 (Clontech) were digested with enzymes *Nco*I and *Hind*III (New England Biolabs) for 4 h at 37 °C. DNA fragments were purified and ligated using T4 DNA ligase, as indicated by the manufacturer (New England Biolabs). The resulting plasmids were used to electrotransform *S.* Enteritidis ΔϕSE12 (chloramphenicol sensitive) and the complemented strains were tested in survival assays. As controls for these experiments, WT or ΔϕSE12 were electrotransformed with the plasmid pKK233.2-OVA [13], which encodes for an irrelevant protein.

2.3. Mouse infection assays

Groups of 4–6 male C57BL/6 mice (5–6 week age) were used in this study to evaluate the virulence of strain *S.* Enteritidis ΔϕSE12::Cm. Infections with WT, ΔaroA::Kn or ΔϕSE12::Cm strains were performed by growing these bacteria in LB medium, with aeration provided by a shaker at 37 °C until OD_{600} equal to 0.6. The volume of bacterial culture containing 1×10^6 CFUs was centrifuged in a refrigerated microcentrifuge (CT15RE Hitachi) at $10{,}000 \times g$ for 5 min. Bacteria pellets were resuspended in 20 μl of PBS and used to orally infect mice. After infection, survival rate was recorded on a daily basis. When the infections were performed with ampicillin resistant strains, mice received a daily intraperitoneal injection of ampicillin (50 μl of a 20 μg/ml solution in PBS). After 9 days of infection, a group of mice infected with either WT *S.* Enteritidis or *S.* Enteritidis ΔϕSE12::Cm were euthanized to obtain livers and spleens. Organs were homogenized using two sterile slides in PBS and cells were counted using a haemocytometer. The volume containing 100,000 cells was incubated for 15 min

in 1 ml of PBS/Triton X-100 0.25%, at room temperature, to release intracellular bacteria. One hundred μl of lysed cells were seeded in LB-Agar plates and incubated 12 h at 37 °C, in order to quantify bacterial colonies per cell. Similar experiments were performed to detect organ colonization by *S.* Enteritidis ΔϕSE12::Cm strain after 30 days of infection. To perform re-infection experiments, mice previously challenged with either *S.* Enteritidis ΔϕSE12::Cm or ΔaroA::Kn, and mice previously challenged with PBS, were orally infected with 10^6 CFU of virulent *S.* Enteritidis. The survival rate was recorded daily for each group of mice. As a control, a group of naïve mice were orally challenged with 20 μl of PBS. Mice were maintained at the facilities of the Pontificia Universidad Católica de Chile animal centre and all animal work was performed according to institutional guidelines and supervised by a veterinarian.

2.4. Detection of anti-Salmonella antibodies

Before and after 28 days of re-infection with WT *S.* Enteritidis, serum samples were obtained from each mouse and stored at −80 °C. Anti-*Salmonella* IgG antibodies were detected as follows: 10^8 CFU of *S.* Enteritidis were resuspended in 1 ml of sterile 1X PBS and heat inactivated at 65 °C for 30 min. 50 μl of the bacterial suspension were placed in each well of a 96-well plate and incubated for 12 h at 4 °C. Then, bacterial suspension was discarded and wells were blocked with 3% BSA in PBS for 2 h at room temperature. Serial dilutions of mice sera were prepared in PBS–BSA 1%, and 50 μl of each dilution were placed per well. After 1 h of incubation at room temperature, three washes with PBS–Tween 0.02% were performed. Then, 50 μl of anti-mouse IgG-HRP (1/2000 dilution, BD Pharmingen) were added per well and incubated for 1 h, at room temperature. Three washes with PBS–Tween 0.02% were performed and then, 50 μl of TMB solution were added to each well and incubated at room temperature until colour development was evident (5–15 min) (TMB solution: 3-3′-5-5′-tetramethyl-benzidine solution in DMSO, final concentration 100 μg/ml, Sigma–Aldrich). To stop the reaction, 50 μl of H_2SO_4 2 M were added to each well, and the absorbance at 450 nm was determined in an ELISA plate reader (TM-36 Thermo).

2.5. Phagocytic cells preparation

The monocyte/macrophage J774.3 cell line used in this study was kindly provided by Dr. María Inés Becker (Biosonda S.A., Chile). J774.3 cells were routinely grown in T75 bottles containing high glucose DMEM medium (GIBCO®, Invitrogen), which was supplemented with 10% fetal bovine serum (HyClone) and 1 mM HEPES (GIBCO®, Invitrogen). Cells were incubated at 37 °C and 5% CO_2 until 95% confluency was achieved. Before infection assays, cells were treated with 0.1 mg/ml trypsine (HyClone) for 5 min, recovered in 50 ml polypropylene tubes and centrifuged at $1800 \times g$ for 5 min at room temperature. After three washes with supplemented DMEM medium, cell number and viability was determined in a haemocytometer, using trypan blue staining (1 mg/ml, Invitrogen). 5×10^5 cells/ml were seeded in 24-well plates and incubated overnight at 37 °C and 5% CO_2. DCs were prepared from bone marrow precursors of C57BL/6 mice. Cells were incubated in complete RPMI 1640 medium, which was supplemented with 5% FCS (Hyclone), 2 mM glutamine, 1 mM non-essential amino acids, 1 mM pyruvate, 1 mM HEPES and 10 ng/ml of recombinant murine GM-CSF (Peprotech). All cell culture media and supplements were acquired from GIBCO® (Invitrogen). Culture media was replaced every 2 days and the phenotype of DCs was analyzed at day 6 of culture by flow cytometry for the expression of the surface markers CD11c, I-A^b, H-$2K^b$, CD80, CD86 and CD40. Routinely, over 70% of cells expressed the DC marker CD11c and showed an immature phenotype. Previous to the infection assays, DCs were washed three times with PBS and then culture media was replaced with complete RPMI medium without antibiotics.

2.6. Gentamicin protection assays

J774.3 or DCs were infected with either WT *S.* Enteritidis or ΔϕSE12::Cm at a multiplicity of infection (MOI) equal to 25 bacteria per one cell. The infection period extended for 1 h, and then cells were washed with PBS 1X and treated with 50 μg/ml gentamicin to kill extracellular bacteria. After 2 and 24 h of infection, cells were washed with 1 ml PBS 1X and 100,000 cells were permeabilized for 15 min with 0.1% Triton X-100 in PBS, at room temperature. One hundred μl of the cell lysates were seeded on LB-agar plates and incubated at 37 °C for 12 h, in order to count the amount of CFUs per cells.

2.7. Detection of T cell response against S. Enteritidis

C57BL/6 mice were orally infected with either *S.* Enteritidis ΔϕSE12::Cm or ΔaroA::Kn as described above and, after 28 days of infection, were challenged with WT *S.* Enteritidis. 30 days after infection, spleens were recovered, homogenised and perfunded with PBS. Cells were treated with 10 ml of ACK solution (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.15 mM EDTA), washed three times with 10 ml of PBS and resuspended in complete culture media at a final concentration of 1×10^6 cells/ml. Six-day DCs were pulsed with heat-killed (HK) WT *S.* Enteritidis (MOI 50) and incubated for 24 h at 37 °C. Then, decreasing amounts of DCs were co-cultured with 1×10^5 splenocytes for 24 h at 37 °C. After 24 h of incubation, 100 μl of cell supernatants were collected to detect IL-2 and IFN-γ secretion by sandwich ELISA, as described previously [13].

3. Results

3.1. Characterization of prophage ϕSE12 in the chromosome of S. Enteritidis

Prophage ϕSE12 is an exclusive *S.* Enteritidis defective prophage, which is located between coordinates 1,226,088 and 1,264,440 in the *S.* Enteritidis PT4 NCTC13349 genome. It is 19,638 bp long and has 26 annotated genes. This prophage contains two genes that have been associated to virulence in other *Salmonella* serovars: *sopE* and *sodC*. In addition, on its right boundary, there are genes encoding for an excisionase (SEN1132) and an integrase (SEN1131) (Fig. 1A).

The prophage ϕSE12 is located upstream Centisome (CS) 40 island, which is another degenerate lambdoid prophage of 16,649 bp that was previously described in *S.* Typhimurium LT2 [16]. As shown in Fig. 1A, the CS40 island is conserved between the genomes of *S.* Enteritidis and *S.* Typhimurium. Sequence analyses on the CS40 island allowed us to define two "modules" of DNA in these *Salmonella* serovars (modules 1 and 2, from right to left in Fig. 1A). These two DNA modules were probably acquired through independent lateral gene transfer events and are bounded by direct repeated sequences (DRS). As shown in Fig. 1B, these DRS consist of two 26 and 37 bp-long DNA segments, separated by 53–58 bp-long non-repeated DNA segments. Because they are usually located near a gene encoding for a phage integrase, it is thought that the DRSs work as an attachment site for phages. Similarly, prophage ϕSE12 is also bounded by the same DRS and probably used these sequences as an attachment site for integrating in the bacterial chromosome.

To evaluate whether prophage ϕSE12 could also be present in other strains of *S.* Enteritidis, we performed PCR amplification assays for *sopE*, *sodC* and the boundaries of this prophage in a collection of 30 clinical isolates. The expected PCR products were

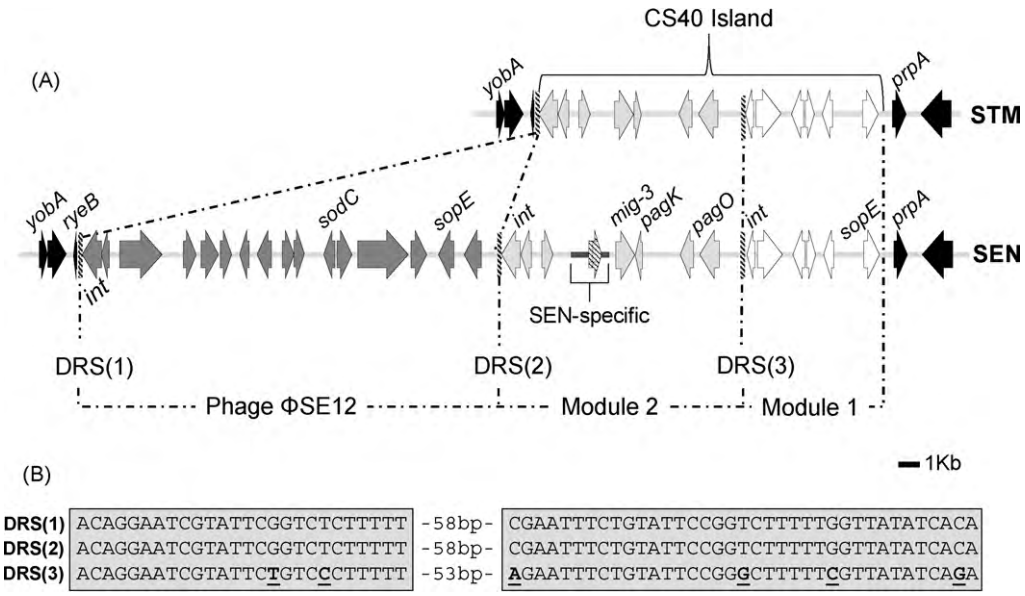

Fig. 1. Characterization of prophage ϕSE12 in the *S.* Enteritidis genome. (A) A comparative sequence analysis between *S.* Enteritidis (SEN) PT4 NCTC13349 and *S.* Typhimurium (STM) LT2 genomes was performed in order to identify the genetic location of ϕSE12. CS40 island, a degenerated prophage shared by *S.* Enteritidis and *S.* Typhimurium, is shown beside prophage ϕSE12 (dark arrows). According to the presence of direct repeated sequences (DRS), CS40 island was divided into two modules of DNA (modules 1 and 2, light gray and white arrows, respectively). Genes with assigned functions within modules 1 and 2, as well as in prophage ϕSEN12, are shown. Also a DNA segment found only in *S.* Enteritidis is observed. Note that beside each DRS, a phage integrase gene is found. (B) The sequences of the DRS found in CS40 island module 1, module 2, and in prophage ϕSE12 were aligned, to determine their percentage of identity. DRS have two conserved segments, separated by 53–58 bp of non-conserved DNA. Non-conserved nucleotides in these sequences are shown as bold and underlined letters. It seems that these DRS are attachment sites for lambdoid prophage insertion in the *Salmonella* genome.

Table 1
Detection of ϕSE12 in clinical strains of *S.* Enteritidis.

Coordinates[a]	Genes in the amplified DNA fragment	Number of positive strains
1246367–1246978	SEN1159	30/30
1243136–1243858	sopE (SEN1155)	30/30
1237763–1238296	sodC (SEN1149)	30/30
1225735–1226088	SEN1130	30/30

[a] Coordinates are those of the *S. enterica* serovar Enteritidis PT4 NCTC13349 sequence.

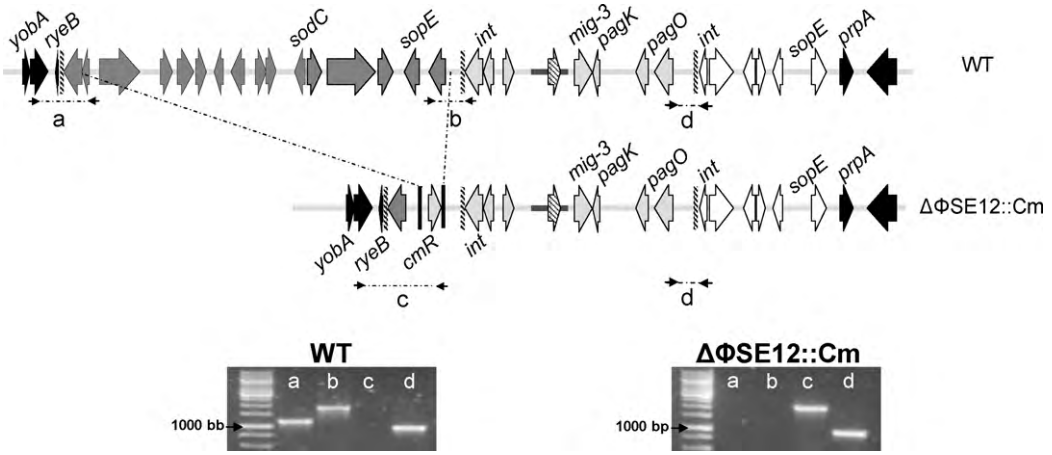

Fig. 2. Generation of a *S.* Enteritidis strain lacking genes contained in ϕSE12. The whole prophage ϕSE12 was replaced for a chloramphenicol-resistance gene, as described in materials and methods. Arrows show the location of PCR primers used to corroborate the replacement of ϕSE12. PCR detection of prophage ϕSE12 in the *S.* Enteritidis genome was performed using primer pairs that aligned in the left (PCR product "a") and right (PCR product "b") boundaries of ϕSE12. In addition, a primer that aligned within the chloramphenicol-resistance gene was used with another primer that aligned beside prophage ϕSE12 insertion, to detect by PCR the appropriate insertion of the chloramphenicol-resistance gene (PCR product "c"). A primer pair that aligned in the boundaries of modules 1 and 2 of CS40 island was used as a positive control for the PCR reaction (PCR product "d"). As shown in the lower panels, the PCR products "a" and "b" were only detected in the wild type strain, while the PCR product "c" was detected only in the mutant strain, which lacks the prophage ϕSE12 (ΔϕSE12::Cm).

specifically amplified for all these strains (Table 1). These data suggest that prophage ϕSE12 can be found in the chromosome of several *S.* Enteritidis strains and phagotypes.

3.2. Deletion of prophage ϕSE12-encoded genes reduces S. Enteritidis capacity to cause systemic disease in mice

Next, we evaluated the contribution of prophage ϕSE12 to the virulence of *S.* Enteritidis in mice. With this aim, we generated a strain lacking this prophage by deleting genes SEN1132 to SEN1175 by means of a chloramphenicol-resistance gene replacement, as described in materials and methods. As a result, the mutant strain *S.* Enteritidis ΔϕSE12::Cm was generated, in which deletion of prophage ϕSE12 was confirmed by PCR analyses (Fig. 2). The mutant *S.* Enteritidis strain showed an equivalent growth capacity than did the WT strain in culture broth (data not shown), suggesting that deletion of prophage ϕSE12 genes did not cause an unspecific metabolic deficiency.

Next, we tested whether deletion of prophage ϕSE12 could influence the capacity of *S.* Enteritidis to cause lethal disease in mice. C57BL/6 mice were orally infected with 1×10^6 CFUs of either wild type or ΔϕSE12::Cm *S.* Enteritidis strains and the survival rate was registered on a daily basis. As shown in Fig. 3A, 100% of mice infected with WT *S.* Enteritidis died after 9 days of infection. In contrast, over 75% of mice infected with mutant *S.* Enteritidis ΔϕSE12::Cm survived until the end of the experiment (day 28).

Consistently with these data, a significant reduction of the capacity of colonizing internal organs, such as liver and spleen, was shown by the *S.* Enteritidis ΔϕSE12::Cm mutant strain, as compared to WT *S.* Enteritidis (Fig. 3B). At various time points during infection, mice infected with WT *S.* Enteritidis showed significantly more bacteria colonizing liver and spleen, as compared to animals challenged with the *S.* Enteritidis ΔϕSE12::Cm mutant strain (Fig. 3B). In addition, we evaluated whether *S.* Enteritidis ΔϕSE12::Cm can be cleared after 28 days post-immunization. We observed that the bacteria could still be recovered from livers ($38{,}250 \pm 11{,}250$ in total liver) and spleens (2005 ± 845 in total spleen) of infected animals, suggesting that although this mutant strain did not cause lethal disease, it remained capable of persisting in tissues of immunized animals.

As an additional control, we generated a *S.* Enteritidis ΔϕSE12 lacking the choramphenicol-resistance gene, to rule out the possibility that the expression of this antibiotic resistance gene could have been influencing the virulence of the mutant strain. Survival assays corroborated that both chloramphenicol-resistant and chloramphenicol-sensitive strains were equally attenuated in mice (data not shown). These results suggest that genes in prophage ϕSE12 are required by *S.* Enteritidis for colonizing internal organs and causing a systemic disease in mice.

3.3. sodC gene complementation restores the capacity of S. Enteritidis to cause systemic disease in mice

To determine whether the absence of *sopE* and *sodC* genes, which have been previously involved in the virulence of *S.* Typhimurium, were responsible for the attenuation observed in the *S.* Enteritidis ΔϕSE12 mutant strain, we cloned these genes in the expression vector pKK233.2 to complement the mutant strain. The chloramphenicol-sensitive *S.* Enteritidis ΔϕSE12 mutant strain was electrotransformed with plamids pKK233.2 harboring *sopE*, *sodC* or OVA (irrelevant antigen) genes, to generate strains ΔϕSE12/sopE, ΔϕSE12/sodC and ΔϕSE12/OVA. The resulting strains were used to orally infect mice, as described above. To avoid growth of bacteria that have lost the plasmid in the infective process, mice were treated daily with ampicillin. As observed in Fig. 3C, ΔϕSE12/sodC showed the most virulent phenotype and caused a lethal disease in mice after 10 days of infection. ΔϕSE12/sopE showed a partial recovery of the virulence, as compared to the non-complemented ΔϕSE12 strain, although this complemented bacterium was not as virulent as was the ΔϕSE12/sodC strain. These results suggest that the absence of both, *sopE* and *sodC* genes due to the deletion of ϕSE12, may lead to the attenuation shown by the *S.* Enteritidis ΔϕSE12.

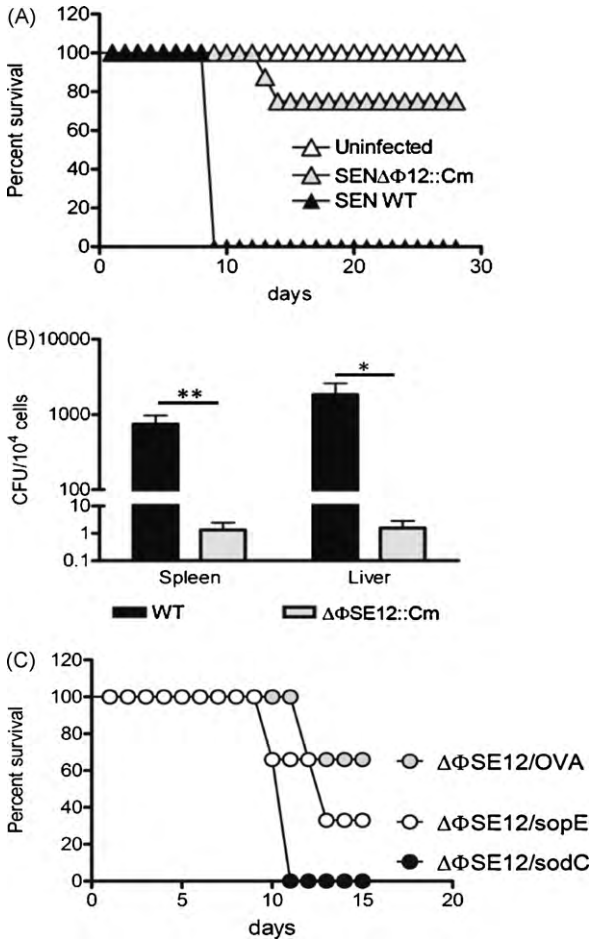

Fig. 3. Deletion of prophage ϕSE12 reduces the capacity of *S.* Enteritidis to cause disease in mice. (A) C57BL/6 mice were orally infected with 1×10^6 colony forming units (CFUs) of either WT *S.* Enteritidis or *S.* Enteritidis ΔϕSE12::Cm. The survival rate was recorded daily. Uninfected mice were used as a control. The graph shows the average of three experiments that included four mice per group. (B) After 9 days of oral infection with either WT *S.* Enteritidis or *S.* Enteritidis ΔϕSE12::Cm, mice were euthanized, and liver and spleen were recovered to detect the bacterial load per 10,000 cells. Bars show the average result of three independent experiments ± SE. $^{**}p$ value <0.01; $^{*}p$ value <0.05, Student's *t* test. (C) *S.* Enteritidis ΔϕSE12 was electrotransformed with pKK233.2 plasmid containing either the *sodC*, *sopE* or OVA (irrelevant) genes to generate strains *S.* Enteritidis ΔϕSE12/pKK233.2-sopE (ΔϕSE12/sopE), *S.* Enteritidis ΔϕSE12/pKK233.2-sodC (ΔϕSE12/sodC) and *S.* Enteritidis ΔϕSE12/pKK233.2-OVA (ΔϕSE12/OVA). Group of 3 C57BL/6 mice were orally infected with 1×10^6 CFU of these transformed bacteria and survival rate was recorded on a daily basis.

3.4. Infection with the S. Enteritidis strain lacking prophage ϕSE12 protects mice against virulent S. Enteritidis

Next, we evaluated whether infection with *S.* Enteritidis ΔϕSE12::Cm could confer immune protection against WT *S.* Enteritidis in mice. After 28 days of infection with *S.* Enteritidis ΔϕSE12::Cm, mice were re-infected with 1×10^6 CFUs of WT *S.* Enteritidis. As a control, uninfected (naïve) mice were also challenged with an equivalent dose of the virulent WT *S.* Enteritidis strain. In these mice, the survival rate was monitored on a daily

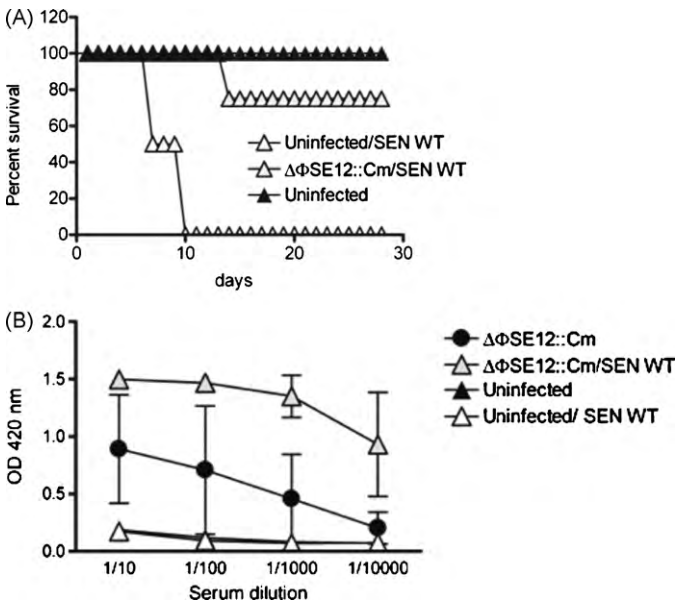

Fig. 4. Immunization with *S.* Enteritidis ΔϕSE12::Cm prevents a further challenge with WT *S.* Enteritidis. (A) Groups of four C57BL/6 mice received either 1×10^6 CFUs of *S.* Enteritidis ΔϕSE12::Cm (ΔϕSE12::Cm) or 20 μl 1X PBS (uninfected). Twenty-eight days after infection, a challenge with 1×10^6 CFUs of WT *S.* Enteritidis was performed. Survival rate was recorded daily. Uninfected mice were included as negative controls. The graph shows the average of three independent experiments, including four mice per group. (B) Anti-*Salmonella* IgG titers were detected in mice sera 28 days after infection with *S.* Enteritidis ΔϕSE12::Cm (ΔϕSE12::Cm, dark circles) and 28 days after a challenge with WT *S.* Enteritidis (ΔϕSE12::Cm/SEN WT, grey triangles). As a control, anti-*S.* Enteritidis IgG was detected in the sera of uninfected control mice, both before (uninfected, dark triangles) and 9 days after WT *S.* Enteritidis infection (uninfected/SEN WT, white triangles). The graph shows the average absorbance at 450 nm obtained in two independent experiments.

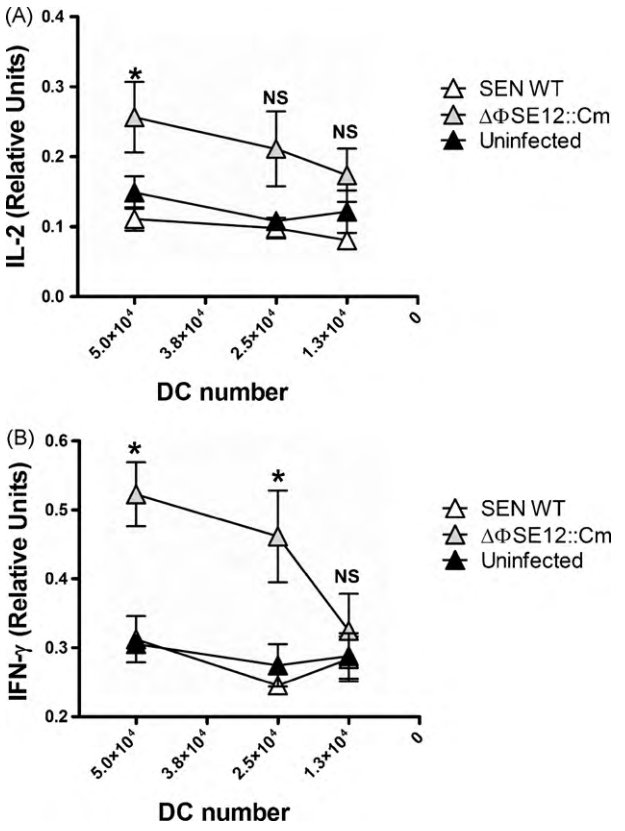

Fig. 5. Immunization with *S.* Enteritidis ΔϕSE12::Cm promotes the expansion of IL-2 and IFN-γ secreting T cells in the spleen. Groups of four C57BL/6 mice received either 1×10^6 CFUs of *S.* Enteritidis ΔϕSE12::Cm (ΔϕSE12::Cm) or 20 μl 1X PBS (uninfected). Twenty-eight days after infection, a challenge with 1×10^6 CFUs of WT *S.* Enteritidis was performed. After 30 days of infection, spleens were removed and single cell suspension were obtained and co-cultured with decreasing amounts of HK *S.* Enteritidis-pulsed DCs (MOI 50). After 24 h, cell supernatants were recovered and the amount of IL-2 (A) and IFN-γ (B) was measured by sandwich ELISA. As a control, the results obtained for spleen cells derived from unimmunized mice challenged with WT *S.* Enteritidis and uninfected mice were included. **p* value <0.05, Student's *t* test.

basis. As shown in Fig. 4A, 100% of naïve (control) mice that were challenged with WT *S.* Enteritidis died by day 9 of infection. In contrast, most of the mice that had been previously infected with the mutant *S.* Enteritidis ΔϕSE12::Cm strain survived the challenge with the WT *S.* Enteritidis strain.

Consistently with the notion that challenge with the mutant *S.* Enteritidis ΔϕSE12::Cm strain was conferring immune protection to mice, significantly increased titers of anti *S.* Enteritidis IgG antibodies were detected in the serum samples derived from surviving mice, both before and after the infection with virulent WT *S.* Enteritidis (Fig. 4B). Moreover, cells derived from the spleen of mice immunize with *S.* Enteritidis ΔϕSE12::Cm and infected with the WT strain secreted significant amounts of IL-2 and IFN-γ after co-culture with DCs pulsed with HK *S.* Enteritidis (Fig. 5).

To evaluate whether the protective capacity shown by *S.* Enteritidis ΔϕSE12::Cm strain was equivalent to a standard auxotrophic attenuated strain, we generated a *S.* Enteritidis strain lacking the *aroA* gene. It has been previously suggested that strains of *S.* Enteritidis lacking genes involved in the biosynthesis of aromatic aminoacids can be considered safe and effective vaccine candidates [17]. Thus, mice were orally infected with 10^6 CFU of either *S.* Enteritidis ΔϕSE12::Cm or *S.* Enteritidis ΔaroA::Kn, and the survival rate was registered on a daily basis. We observed that 100% of mice survived after immunization with *S.* Enteritidis ΔaroA::Kn (data not shown). This result suggests that *S.* Enteritidis ΔaroA::Kn is less virulent that *S.* Enteritidis ΔϕSE12::Cm. We also compared the humoral and cellular immune response promoted by the immunization with both strains 15 days after challenging with a virulent *S.* Enteritidis strain. We observed that mice immunized with the *S.* Enteritidis ΔϕSE12::Cm strain showed higher anti-*Salmonella* IgG antibodies titers than did mice immunized the *S.* Enteritidis ΔaroA::Kn strain, at equal times of infection (Fig. 6A). In addition, we observed higher IL-2 secretion by spleen cells from mice immunized with the *S.* Enteritidis ΔϕSE12::Cm strain than *S.* Enteritidis ΔaroA::Kn strain when stimulated with DCs pulsed with HK *S.* Enteritidis (Fig. 6B). These data suggest that an oral challenge with the mutant *S.* Enteritidis ΔϕSE12::Cm strain can confer protective immunity that prevents susceptibility to systemic disease caused by the virulent *S.* Enteritidis strain. Such an immune response was stronger for the ΔϕSE12::Cm strain than it was for the *S.* Enteritidis ΔaroA::Kn strain. However, *S.* Enteritidis ΔaroA::Kn appeared to be more attenuated and thus safer than was the *S.* Enteritidis ΔϕSE12::Cm strain.

3.5. Genes in prophage ϕSE12 are not required for S. Enteritidis to survive inside phagocytic cells

It has been previously shown that SodC is required for *S.* Typhimurium survival inside the *Salmonella* containing vacuole (SCV) in macrophages [9]. Since the prophage ϕSE12 contains a gene encoding for a SodC precursor protein that restores the virulence of the *S.* Enteritidis ΔϕSE12::Cm strain, it is likely that the impaired capacity shown by the mutant *S.* Enteritidis ΔϕSE12::Cm strain to cause a lethal disease in mice could be due to a reduced survival inside phagocytic cells. To evaluate this hypothesis, intracellular survival in J774.3 macrophages was measured for both WT

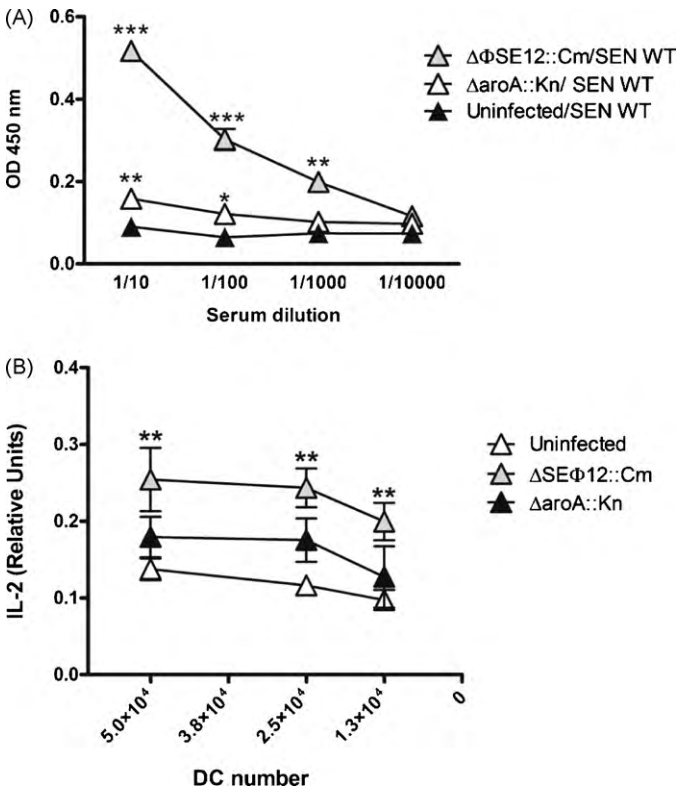

Fig. 6. *S.* Enteritidis ΔϕSE12::Cm promotes a stronger immune response than *S.* Enteritidis ΔaroA::Kn. Groups of three C57BL/6 mice received either 1×10^6 CFUs of *S.* Enteritidis ΔϕSE12::Cm (ΔϕSE12::Cm) or 1×10^6 CFUs of *S.* Enteritidis ΔaroA::Kn (ΔaroA::Kn). Twenty-eight days after infection, a challenge with 1×10^6 CFUs of WT *S.* Enteritidis was performed and, after 15 days of infection, mice were sacrificed. (A) Serum was obtained after challenge with WT *S.* Enteritidis to measure titers of anti-*Salmonella* IgG antibodies. As a control, serum from uninfected mice was included. (B) Spleens of immunized mice were removed after 15 days of challenge with WT *S.* Enteritidis and single cell suspension were obtained and co-cultured with decreasing amounts of HK *S.* Enteritidis-pulsed DCs (MOI 50). After 24 h, cell supernatants were recovered and the amount of IL-2 was measured by sandwich ELISA. As a control, the results obtained for spleen cells derived from uninfected mice were included. ***p value <0.001, **p value <0.01, *p value <0.05, Student's t test.

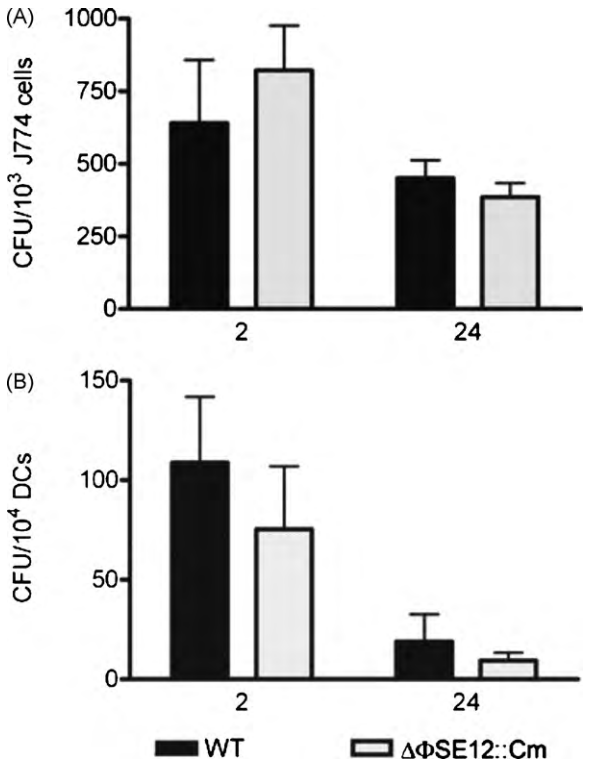

Fig. 7. Deletion of prophage ϕSE12 does not affect the capacity of *S.* Enteritidis to invade and survive inside phagocytic cells. Macrophages J774.3 cells (A) or bone marrow-derived dendritic cells (B) were infected with either WT *S.* Enteritidis or *S.* Enteritidis ΔϕSE12::Cm (MOI 25) for 1 h, and then were treated with gentamicin (50 μg/ml). 2 and 24 h after infection, cells were lysed with 0.25% Triton X-100 in PBS, and the volume containing 1×10^3 (A) or 1×10^4 (B) cells was seeded in LB agar plates, to quantify the amount of intracellular bacteria. Bars are the average result of 4 independent experiments ± SE.

S. Enteritidis and ΔϕSE12::Cm strains at 2 and 24 h post-infection. We observed that *S.* Enteritidis ΔϕSE12::Cm invaded these cells as efficiently as did the WT strain (Fig. 7A). Furthermore, equivalent amounts of both strains of bacteria were recovered after 24 h of infection. These data suggest that deletion of prophage ϕSE12genes did not impair the capacity of *S.* Enteritidis to survive inside macrophages.

In addition, we also tested whether the mutant *S.* Enteritidis ΔϕSE12::Cm strain was defective for invasion and survival inside dendritic cells (DCs). DCs are professional antigen presenting cells required to initiate an adaptive immune response against avirulent *Salmonella* [18]. Virulent strains of *Salmonella* are able to proliferate inside these cells and avoid bacterial degradation [13,19,20]. As shown in Fig. 7B, we recovered similar amounts of intracellular WT *S.* Enteritidis and *S.* Enteritidis ΔϕSE12::Cm after 2 and 24 h of infection.

Taken together, these data indicate that genes in prophage ϕSE12 might not be required for the capacity of *S.* Enteritidis to survive inside macrophages and DCs.

4. Discussion

Genes acquired by lateral transfer of pathogenicity islands are the main source of variation and evolution for pathogenic bacteria, such as *Salmonella* [1]. Importantly, these genetic regions might contain genes required for *Salmonella* to cause disease in a host-specific manner [1,13,21]. The data presented in this study are consistent with the notion that genes contained in the ϕSE12, a prophage-like element, are absolutely required for *S.* Enteritidis to cause systemic disease in mice. It is likely that the removed genes could work as virulence factors during the process of an infection by *S.* Enteritidis. Due to the recent characterization of prophage ϕSE12, only two genes have been annotated in this genetic region, which are similar to other virulence genes of *Salmonella*. One of these genes is 100% identical to the *sodC* gene found in the prophage Gifsy-2 that encodes for a Fe–Cu superoxide dismutase in *S.* Typhimurium [8,22]. Here we have provide data showing that complementation of the strain lacking prophage ϕSE12 with this gene restores the capacity of this mutant strain to cause a lethal disease in the mouse. A recent study has shown that this protein may protect an extracytoplasmatic *Salmonella* target from the action of phagocyte-produced ROS [9]. However, our results showed that *S.* Enteritidis strain lacking this gene failed to show significant differences in the capacity to invade and survive inside macrophages and DCs. This apparent discrepancy could be explained by a reduced dependency of *S.* Enteritidis on SodC activity to resist ROS inside phagocytes. However, this protein might be required during other stages of the infective cycle of *S.* Enteritidis. In agreement with this notion, a recent study described that a *S.* Enteritidis strain lacking *sodC* showed an important level of attenuation in BALB/c mice [23].

SopE is another prophage ϕSE12 gene that has been involved in virulence [10,11,24–26] However, a recent study showed that *S.* Enteritidis strains lacking *sopE* are rendered only mildly attenuated for virulence attenuation in mice [27]. Further, complementation

of *S.* Enteritidis ΔϕSE12 strain partially restores bacteria capacity to cause a lethal disease in mice. It is possible that the absence of both *sodC* and *sopE* results in the severe attenuation observed in our *S.* Enteritidis ΔϕSE12 strain. The additional 24 genes found in prophage ϕSE12 encode for either putative phage proteins or hypothetical proteins. Further studies will be required to evaluate the relative contribution of each of the other 24 genes contained in the prophage ϕSE12 in the capacity of *S.* Enteritidis to cause a lethal disease in mice and the potential polar effect that the deletion of the complete prophage might cause on adjacent genes.

In addition, we have shown that immunization with strains lacking the complete prophage ϕSE12 promote a protective humoral and cellular immune response in the mouse. In fact, we have shown that the immune response promoted by our attenuated strain was stronger than the immune response promoted by the metabolically impaired *S.* Enteritidis strain ΔaroA::Kn [17,28,29]. However, the ΔaroA::Kn strain resulted more attenuated than our ΔϕSE12 strain given that a percentage of mice infected with *S.* Enteritidis lacking the prophage ϕSE12 died after 14 days of infection and that bacteria were still recovered after 28 days after infection from the livers and spleens of infected mice. Therefore, additional mutations in other virulence genes in this strain would be required to generate a safer properly attenuated vaccine.

Prophage ϕSE12 was found in all the clinical strains of *S.* Enteritidis analyzed in this study. In agreement with our data, microarray analyses have shown that this prophage is also detected in another collection of 24 strains of *S.* Enteritidis [30]. In addition, a recent study has also described that *sodC* and *sopE* genes are found in 65 different isolates of *S.* Enteritidis [23]. These results suggest that prophage ϕSE12 is a conserved portion of DNA in *S.* Enteritidis. Therefore, genes in this location might be considered as molecular markers to identify or detect the presence of *S.* Enteritidis in different sources, such as food.

Prophage ϕSE12 is located beside the CS40 island, which seems to work as a hot-spot site for prophage acquisition in *Salmonella*. It seems that the DRS located in the left boundary of module 2 works as a preferred insertion site for lambdoid phages in several *Salmonella* serovars. For instance, strain DT104 of *S.* Typhimurium has acquired a lambdoid prophage, called ϕW104, which is found besides to the CS40 island and is bounded by the same DRS found in prophage ϕSE12 [16]. Furthermore, the strain CT18 of *S.* Typhi also contains a lambdoid prophage, known as ST18, integrated next to the CS40 island. Although this prophage is also bounded by similar DRSs, it is unrelated to prophage ϕSE12 [31]. Therefore, these data support the notion that CS40 can work as a source of variability for *S. enterica,* because recent prophage insertions at this genetic location are observed for several *Salmonella* serovars.

In summary, our data suggest that a strain of *S.* Enteritidis lacking the genes contained inside ϕSE12, a specific genetic region of this *Salmonella* serovar, showed a reduced capacity to spread from the intestine to deeper organs and failed to cause lethal systemic disease in mice. Furthermore, an infection with this mutant strain of *S.* Enteritidis promoted an anti-*Salmonella* immune response in mice, which protected animals against a lethal challenge with the WT virulent *S.* Enteritidis strain. These findings support the notion that strains lacking prophage ϕSE12 could be considered as potential attenuated vaccines candidates against virulent *Salmonella*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2010.05.073.

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