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Salmonella enterica serovar Typhi has a 4.1 kb genetic island inserted within the *sapABCDF* operon that causes loss of resistance to the antimicrobial peptide protamine

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Objectives: To investigate the association between the presence of a genetic island inserted within the *sapABCDF* operon of *Salmonella* Typhi and the susceptibility to antimicrobial peptides.

Methods: Genetics and bioinformatics approaches were used to study the genomic organization of the *sap* operon of *Salmonella* Typhi and several serovars of *Salmonella enterica*. PCR was used to confirm the information obtained from these analyses. Deletion of the entire genetic island of *Salmonella* Typhi was achieved by the red swap method. RT–PCR amplification and antimicrobial peptide susceptibility tests were used to evaluate expression of the *sap* genes and bacterial resistance to protamine.

Results: Inspection of the genomes of *Salmonella* Typhi and 10 serovars of *Salmonella enterica* showed an insertion of a genetic island located between the *sapB* and *sapC* genes of the *sap* operon. This genetic element was referred to as GICT18/1. Unlike *Salmonella* Typhimurium, the bacterial susceptibility to protamine is increased in *Salmonella* Typhi wild-type. Deletion of GICT18/1 resulted in protamine susceptibility levels similar to those of *Salmonella* Typhimurium, suggesting that restoration of the *sap* operon occurred in the *Salmonella* Typhi ΔGICT18-1 mutant strain. RT–PCR experiments supported this assumption because an amplicon containing a fragment of *sapD*–*sapF* was detected in *Salmonella* Typhi ΔGICT18/1, whereas it was not detected in *Salmonella* Typhi wild-type.

Conclusions: The presence of GICT18/1 seems to be a natural feature of *Salmonella* Typhi. This genetic island is found only in 10 out of 32 *Salmonella enterica* serovars included in this study. Removal of GICT18/1 has an impact in the susceptibility of *Salmonella* Typhi to the antimicrobial peptide protamine.

Keywords: *sap* operon, protamine susceptibility, enteric bacteria.

Introduction

Salmonella enterica serovars are a group of Gram-negative pathogens that infect animals and humans. Systemic infections, gastroenteritis and septicaemia can occur depending on the animal species and the bacterial serovar. *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) can infect a wide range of hosts causing different symptoms, including a systemic disease in mice. In contrast, *Salmonella enterica* serovar Typhi (*Salmonella* Typhi) infects only humans and is the aetiological agent of typhoid fever, a disease that causes significant morbidity and mortality worldwide.[1–4]

Antimicrobial resistance is a major public health problem in *Salmonella* Typhi.[5] Timely treatment with appropriate antimicrobials is important for reducing the mortality associated with enteric fever.[5] The rapid emergence of resistant bacterial strains requires the development of alternative treatments to control this disease. Antimicrobial peptides (APs) are essential elements of innate immunity that have been identified in organisms ranging from bacteria to humans.[6,7] The main features of APs include short length (<100 amino acids), positive charge and amphiphilicity. Despite these similarities APs show significant differences in both structure and function.[8] Initial contact of APs with the target cell is mediated by electrostatic interactions between the positively charged peptides and the negatively charged bacterial membrane. While several APs interact with the cytoplasmic membrane and then intracellularly, others exert their effect by acting on specific intracellular targets.[9]

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It has been suggested that APs may be useful for the treatment of bacterial infections as several of them exhibit high antibacterial efficacy *in vitro*. Nevertheless, some bacteria have developed systems to tolerate the antimicrobial effect of APs. One such element is the *sapABCDF* operon that was first described in *Salmonella* Typhimurium.[10,11] This operon is present in numerous Gram-negative organisms such as *Erwinia chrysanthemi*,[12,13] *Haemophilus influenzae*,[14,15] *Vibrio fischeri*[16,17] and *Proteus mirabilis*.[18] The *sap* operon of *Salmonella* Typhimurium contains five genes that are polycistronically transcribed and encodes a peptide transporter system of the 'ATP-binding cassette' family (ABC), also known as traffic ATPases.[11] ABC transporters are involved in a variety of physiological functions in both prokaryotes and eukaryotes, including detoxification of noxious compounds and ion transport.[19]

The *Salmonella* Typhimurium SapABCDF transporter system confers resistance to small cationic peptides such as protamine.[10] This molecule is a highly cationic peptide of 32 amino acids containing 21 arginine residues and is found in fish sperm nuclei.[20] Protamine exerts a broad-spectrum activity against Gram-positive and Gram-negative microorganisms,[10,21,22] representing an attractive model for studying the antimicrobial effects of APs.[12,13,21–24]

Unlike *Salmonella* Typhimurium, the *sapABCDF* operon of *Salmonella* Typhi has not yet been characterized. However, it has been reported that this human pathogen is more sensitive to protamine than *Salmonella* Typhimurium.[25] Moreover, preliminary studies in our laboratory suggested the presence of a genetic island inserted within the *sap* operon of this bacterium. The aims of this research were to determine the prevalence of the genetic island GICT18/1 among *Salmonella* isolates and to determine whether a relationship exists between the genetic island inserted within the *sapABCDF* operon of *Salmonella* Typhi and the AP susceptibility phenotype reported for this pathogen.

Materials and methods

Bacterial strains, growth media and culture conditions

Clinical strains of *Salmonella* Typhi were obtained from the Hospital of Infectious Disease Lucio Córdova of Santiago, Chile, and have been described elsewhere.[26–28] Twenty-six strains were isolated from blood samples of patients with typhoid fever. *Salmonella* Typhi Ty2 was obtained from the Instituto de Salud Pública of Santiago, Chile. Serovars of *Salmonella enterica* subspecies I were from the *Salmonella* reference collection B (SARB)[29] and are listed in Table S1 [available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. Bacterial strains were grown routinely in Luria–Bertani (LB) broth (Bacto Tryptone, 10 g/L; Bacto Yeast Extract, 5 g/L; NaCl 5 g/L) at 37°C with shaking. Media were supplemented with kanamycin (50 mg/L), ampicillin (100 mg/L) and L-arabinose (2 mg/mL) as required. Solid media were prepared by addition of agar (15 g/L). PBS buffer contained 55 mg/mL $NaH_2PO_4 \cdot 7H_2O$, 15 mg/mL K_2HPO_4 and 4.25 mg/mL NaCl, and was adjusted to pH 7.4.

Genomic structure analyses of the sapABCDF operon

Salmonella Typhi CT18 (GenBank accession no. AL627270.1)[30] was used as the genome reference to compare a number of *Salmonella enterica* genome sequences. Genome sequences of *Salmonella* Typhi Ty2,[31] *Salmonella* Typhimurium LT2,[32] *Salmonella* Typhimurium 14028s,[33] *Salmonella* Gallinarum 287/91,[34] *Salmonella* Choleraesuis SC-B67,[35] *Salmonella* Enteritidis PT4,[34] *Salmonella* Paratyphi A ATCC 9150,[36] *Salmonella* Agona SL483, *Salmonella* Dublin CT-02021853, *Salmonella* Heidelberg SL476-SL486, *Salmonella* Javiana GA-MM04042433, *Salmonella* Kentucky CDC191-CVM29188, *Salmonella* Newport SL254-SL317, *Salmonella* Saintpaul SARA23-SARA29 and *Salmonella* Schwarzengrund CVM19633-SL480 were obtained from the National Center for Biotechnology Information (NBCI, http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Genomic sequences of *Salmonella* Typhimurium DT2, *Salmonella* Typhimurium DT104, *Salmonella* Typhimurium DT2358, *Salmonella* Typhimurium SL1344 and 17 *Salmonella* Typhi isolates[37] were obtained from the Sanger Institute (http://www.sanger.ac.uk/Projects/Salmonella/). *Salmonella* Dublin and *Salmonella* Pullorum were obtained from the University of Illinois (www.Salmonella.org/genomics/). Nucleotide sequence comparisons and alignments were made using bioinformatics tools provided by the web sites cited above.

PCR analyses and deletion of the GICT18/1 genetic island of Salmonella Typhi

PCR amplifications were performed in a standard volume of 25 μL, except for the amplification of GICT18/1 which was performed in 12.5 μL. Primers E12D (5′-AAA AGG AGA CTT CGC CAT AGC G-3′) and E12R (5′-CAG CAC TAT TGA GGT CTA CG-3′) were used to amplify GICT18/1 of *Salmonella* Typhi. Pairs of primers SB-1 (5′-AGC GAC CCA ATG ACC ATC ACG C-3′), SB-2 (5′-ACG CTT ACA GGC TTA CCG CTA CGA T-3′) and SC-1 (5′-TCC ACC CCC GCA ATA ATC GC-3′), SC-2 (5′-TCG GCA AGC AAT GTG TAC ACT CCA-3′) were used to amplify PCR products corresponding to the *sapB* and *sapC* genes of the *Salmonella* Typhi STH2370 clinical strain, respectively. The reaction mixture contained 1× buffer, 3 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 1 μM of each primer, 100 ng of template DNA and 2 U of DNA polymerase (Invitrogen). Amplification conditions included 30 cycles (94°C for 30 s, 58°C for 45 s and 72°C for 3 min) and a final extension step at 72°C for 10 min. *Salmonella enterica* chromosomal DNA was prepared as described previously[26–28] and used as template in the PCR experiments.

Deletions of *Salmonella* Typhi GICT18/1 and GICT18/1+*sapCDF* chromosomal elements were achieved by the method described by Datsenko and Wanner.[38] These deletions were constructed in the *Salmonella* Typhi STH2370 clinical strain, yielding the *Salmonella* Typhi ΔGICT18/1 and *Salmonella* Typhi ΔGICT18/1-*sapCDF* mutant strains, respectively (Table S1). PCR primers were designed with 40 nucleotide sequences homologous to the respective flanking regions of the targets (GICT18/1 genetic island or GICT18/1-*sapCDF*) followed by 20 nucleotides complementary to the template plasmid pKD4.

The direct and reverse primers used for deleting GICT18/1 were H1P1R12 (5′-TAC TCG TTT TAT TTT TGT TTA CCA TGC TTT ATG TCT TTT GTG TAG GCT GGA GCT GCT TCG-3′) and H2P2R12 (5′-AAT AAA AAT ATC ATT AAA TCA AAA AGT TAT GCT TTT ATT TCA TAT GAA TAT CCT CCT TAG-3′), respectively. The primers used for deletion of ΔGICT18/1-*sapCDF* were H1WR12+sap (5′-GGG TAT TTG TGT CGC TTA GCG ATC CTT TCG CCA TGC GCT ATG TAG GCT GGA GCT GCT TCG-3′) and H2P2-sapCDF (5′-CAA AAG ACA TAA AGC ATG GTA AAC AAA AAT AAA ACG AGT ACA TAT GAA TAT CCT CCT TAG-3′). PCR products were purified using Qiagen mini columns and used to transform *Salmonella* Typhi/pKD46 grown at 30°C in LB supplemented with 10 mM L-arabinose and 100 mg/L ampicillin. Bacteria were spread on LB agar plates supplemented with kanamycin and incubated at 37°C to select for the allelic exchange and cure plasmid pKD46. Subsequent deletion of the antibiotic-resistant cassette was confirmed by PCR.

DNA sequencing of sapB and sapC genes

PCR products obtained for the *sapB* and *sapC* genes of *Salmonella* Typhi STH2370 were used for DNA sequencing at Macrogen Corp. (Rockville,

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MD, USA). DNA sequences were compared with the genome sequences of *Salmonella* Typhi CT18,[30] *Salmonella* Typhi Ty2,[31] *Salmonella* Typhimurium LT2[32] and *Salmonella* Typhimurium 14028s[33] using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Cloning of the GICT18/1 genetic island of Salmonella Typhi

The PCR product of the *Salmonella* Typhi GICT18/1 genetic island was directly cloned into the pCC1 vector according to the manufacturer's instructions (CopyControl™ PCR Cloning Kit, Epicentre) to yield the plasmid pRP012 (Table S1). The presence of GICT18/1 was confirmed by PCR and restriction endonuclease assays.

Detection of the sapDF transcript

RNA samples were obtained as described previously.[39] Briefly, cells were grown overnight in LB broth at 37°C with shaking. Total RNA was extracted and purified by TRIzol (Invitrogen) and treated with RNase-free DNAse (Promega). RT–PCR amplification was performed with 3 μg of DNase I-treated RNA using Superscript II RT (Invitrogen) following the instructions provided by the vendor. Amplification included 35 cycles (94°C for 30 s, 58°C for 45 s and 72°C for 90 s) followed by a 5 min extension at 72°C to ensure full extension of the amplified fragments. Primers SF-3d (5′-GAA GTC AAG AAT CTG TGA AA-3′) and SF-2 (5′-GGC GCG AAA AAT CAT CTC TAT GCC-3′), designed on the basis of the *Salmonella* Typhi strain CT18,[30] were used to amplify the hybrid *sapD* and *sapF* fragment. Reverse transcription of 16S rRNA was used as a positive control.[40] A negative control consisted of the same amplification mixture except that the reverse transcriptase enzyme was omitted. Amplified products were fractionated on 1.5% agarose gels, stained with ethidium bromide and visualized under a UV source.

Antimicrobial susceptibility

To test the bacterial susceptibility to APs, protamine sulphate, mellitin and human defensin-1 (HNP-1) were used as described previously.[10,25,41] Stock solutions of protamine sulphate and mellitin (Calbiochem) were made by dissolving appropriate amounts of solid peptide in PBS (pH 7.4). Solid peptide HNP-1 (Sigma) was dissolved in 0.01% acetic acid. Cells were grown at 37°C for 16 h in LB broth, diluted in LB (1:20) and grown until mid-exponential phase. Cells were centrifuged, washed twice with PBS (pH 7.4) and diluted (1:20 000) in the same buffer. Aliquots of 50 μL were added into a 96-well microtitre tray and mixed with 50 μL of peptide solution. Controls included bacterial cells (50 μL) diluted in PBS (50 μL), and peptide solution (50 μL) diluted in PBS (50 μL). Trays were incubated at 37°C with shaking for 1 h (protamine and mellitin) or 2 h (HNP-1) and placed on ice. Incubation mixtures were diluted 10-fold in PBS and aliquots of 100 μL spread on LB plates and incubated at 37°C overnight. Results were expressed as the percentage of bacteria surviving compared with those in the control with no peptide added. Statistical analyses were performed using one-way analysis of variance (ANOVA) with P values of <0.05 considered significant.

Results

Insertion of GICT18/1 within the sapABCDF operon of Salmonella Typhi

Previous studies in our laboratory indicated that *Salmonella* Typhi has a genetic insertion interrupting the *sapABCDF* operon. To further investigate this finding we looked at the genomic structure of the *sapABCDF* operon reported for the CT18 strain[30] that is currently available in GenBank. Results showed that the *Salmonella* Typhi *sapABCDF* operon contains a 4.1 kb region that is absent in *Salmonella* Typhimurium, which has an intact operon (Figure 1). Nine open reading frames (ORFs) of low G+C content (42.5%) and encoding peptides from 45 to 166 residues [STY1358 through STY1367; Table S2, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)] are found in the 4.1 kb DNA insertion. This insertion is located between the *sapB* and *sapC* genes of the *sap* operon and does not affect the coding sequences of these two genes as determined by PCR and DNA sequencing (data not shown). This 4.1 kb insertion is a genetic island that we named GICT18/1.

To determine whether GICT18/1 is a common feature of *Salmonella* Typhi, PCR experiments were conducted using

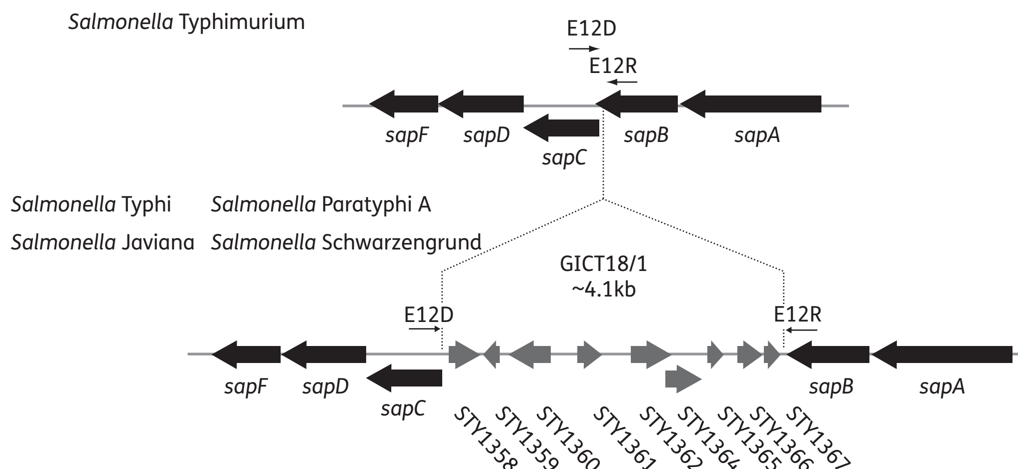

Figure 1. Genomic organization of the *sapABCDF* operon of *Salmonella* Typhi. Schematic representation of the genetic alignment between the *sap* operon of *Salmonella* Typhi and the genomes of *Salmonella* Typhimurium and *Salmonella enterica* serovars described in the Materials and methods section. The GICT18/1 genetic island of *Salmonella* Typhi is indicated. Thin arrows represent forward and reverse primers used to amplify the chromosomal insert located between *sapB* and *sapC* (∼4.5 kb). The predicted size of amplified fragments lacking GICT18/1 is ∼0.5 kb.

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DNA isolated from the reference strain Ty2 and 26 Chilean clinical strains. Forward and reverse primers flanking the GICT18/1 insertion site (Figure 1) were designed to assess the presence of this genetic island. Results showed an amplicon of ~4.5 kb in all PCR amplifications, suggesting that GICT18/1 is found in all *Salmonella* Typhi strains analysed (data not shown). The nucleotide sequence of the amplicon obtained from *Salmonella* Typhi STH2370 was determined and compared with the *Salmonella* Typhi CT18 sequence, showing an identical genetic island composed of 4057 nucleotides (data not shown).

Detection of the GICT18/1 genetic island in other Salmonella enterica serovars

The presence of GICT18/1 in the genomes of 15 *Salmonella enterica* serovars was determined using nucleotide sequences obtained from GenBank. Results showed that GICT18/1 was only found in *Salmonella* Paratyphi A (98% identity), *Salmonella* Schwarzengrund (96% identity) and *Salmonella* Javiana (96% identity) (Figure 1). These results suggest that GICT18/1 is infrequent in *Salmonella enterica* serovars. PCR amplifications using DNA from *Salmonella* Typhimurium, *Salmonella* Pullorum, *Salmonella* Gallinarum, *Salmonella* Montevideo and 32 other serovars of the *Salmonella enterica* reference collection B (SARB)[29] showed amplicons of ~0.5 kb in *Salmonella* Typhimurium and 23 other serovars. Amplicons of ~4.5 kb were detected in *Salmonella* Montevideo, *Salmonella* Brandenburg, *Salmonella* Enteritidis (SARB30), *Salmonella* Panama, *Salmonella* Paratyphi A, *Salmonella* Reading, *Salmonella* Rubislaw, *Salmonella* Schwarzengrund, *Salmonella* Typhi (SARB63), *Salmonella* Typhisuis and *Salmonella* Wien (Table 1), suggesting that the GICT18/1 island is present in these strains interrupting the *sapABCDF* operon.

Salmonella Typhi susceptibility to APs associated with the sapABCDF operon

Because *Salmonella* Typhi has GICT18/1 inserted within the *sap* operon, the function of this operon in regard to bacterial tolerance to the APs protamine, mellitin and HNP-1 was investigated. Bacterial susceptibility to these peptides has been associated with the *sapABCDF* operon in *Salmonella* Typhimurium.[10] A *Salmonella* Typhi mutant was constructed in which GICT18/1 was fully removed by the method of Datsenko and Wanner.[38] This mutant, referred to as *Salmonella* Typhi ΔGICT18/1, was exposed to protamine and mellitin for 1 h[10,25] and to HNP-1 for 2 h.[41] *Salmonella* Typhi ΔGICT18/1 showed a susceptibility pattern similar to that of *Salmonella* Typhimurium and a much lower susceptibility to protamine than that of *Salmonella* Typhi STH2370 (Figure 2). This suggests that removal of the GICT18/1 genetic island reinstates the *sap* operon AP resistance function. We supported this affirmation by testing protamine susceptibility in the *Salmonella* Typhi ΔGICT18/1-*sapCDF* mutant strain, which showed a similar pattern to that of *Salmonella* Typhi STH2370 wild-type. Moreover, complementation of *Salmonella* Typhi ΔGICT18/1 with the recombinant plasmid pRP012 showed the same phenotype as that shown by the mutant (Figure 2), suggesting that the susceptibility pattern observed initially in *Salmonella* Typhi STH2370 is not a contribution of the nine ORFs encoded in this genetic island but is a consequence of the insertion itself. No significant differences in the AP pattern were detected when melittin and HNP-1 were used (data not shown).

Table 1. PCR amplification of *Salmonella* Typhi GICT18/1 in *Salmonella enterica* subspecies I

Strain/isolate	PCR product
Salmonella Typhimurium 14028s and LT2	−
Salmonella Pullorum SL2922	−
Salmonella Gallinarum SL2923	−
Salmonella Montevideo 3799-98	+
Salmonella Agona SARB1	−
Salmonella Anatum SARB2	−
Salmonella Brandenburg SARB3	+
Salmonella Choleraesuis SARB4	−
Salmonella Decatur SARB8	−
Salmonella Derby SARB9	−
Salmonella Dublin SARB12 and SARB14	−
Salmonella Duisberg SARB15	−
Salmonella Enteritidis SARB16	−
Salmonella Emek SARB20	−
Salmonella Gallinarum SARB21	−
Salmonella Haifa SARB22	−
Salmonella Heidelburg SARB23	−
Salmonella Infantis SARB26	−
Salmonella Miami SARB28	−
Salmonella Enteritidis SARB30	+
Salmonella Muenchen SARB32	−
Salmonella Newport SARB36	−
Salmonella Panama SARB39	+
Salmonella Paratyphi A SARB42	+
Salmonella Paratyphi B SARB43	−
Salmonella Paratyphi C SARB48 and SARB49	−
Salmonella Reading SARB53	+
Salmonella Rubislaw SARB54	+
Salmonella Schwarzengrund SARB57	+
Salmonella Sendai SARB58	−
Salmonella Senftenberg SARB59	−
Salmonella Stanley SARB60	−
Salmonella Thompson SARB62	−
Salmonella Typhi SARB63	+
Salmonella Typhisuis SARB70	+
Salmonella Wien SARB71	+

Detection of the Salmonella Typhi sapDF transcribed fragment

The products of *Salmonella* Typhimurium *sapD* and *sapF* exhibit similarity to ATP-binding proteins such as OppD and OppF that are involved in the uptake of oligopeptides.[11] In fact, it was reported that mutations in *sapD* and *sapF* result in hypersusceptibility to protamine in *Salmonella* Typhimurium.[11] The high susceptibility of *Salmonella* Typhi STH2370 to protamine suggests that this behaviour might be related to *sapD* and *sapF*. Amplified fragments suggestive of *sapD* and *sapF* transcription were not

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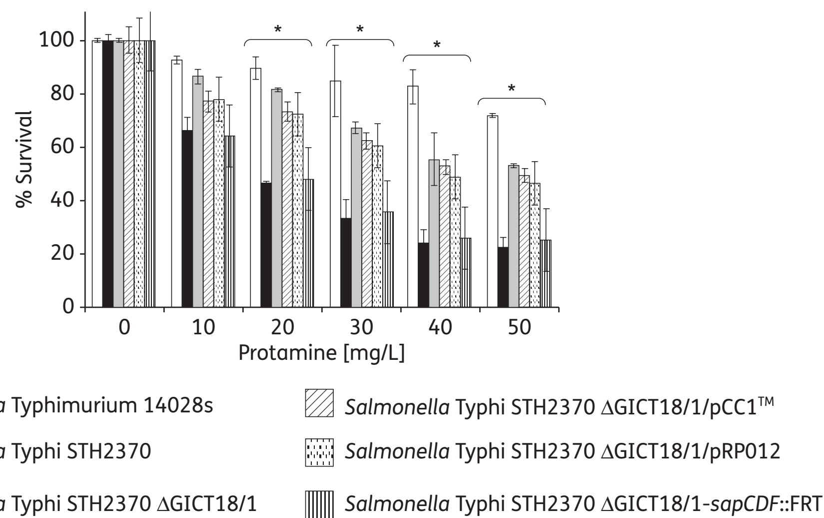

Figure 2. Resistance of *Salmonella* Typhi and *Salmonella* Typhimurium to the antimicrobial peptide protamine. Survival was determined as the ratio between the number of bacteria recovered after 1 h of exposure to different protamine concentrations and the initial bacterial number for *Salmonella* Typhimurium 14028s (white bars), *Salmonella* Typhi STH2370 (black bars), *Salmonella* Typhi STH2370 ΔGICT18/1 (grey bars), *Salmonella* Typhi STH2370 ΔGICT18/1/pCC1™ (diagonal line bars), *Salmonella* Typhi STH2370 ΔGICT18/1/pRP012 (segmented line bars) and *Salmonella* Typhi STH2370 ΔGICT18/1-*sapCDF*::FRT (vertical line bars) as described in the Materials and methods section. Bars indicate standard deviations from three independent experiments. Asterisks indicate significant values (*$P<0.05$).

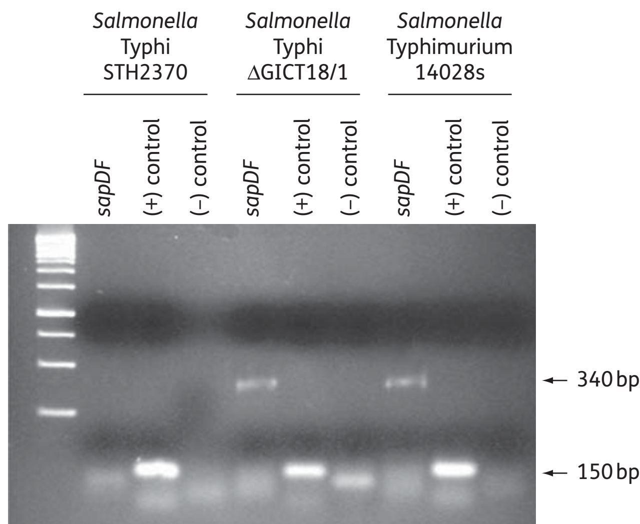

Figure 3. RT–PCR amplification of *sapDF* mRNA from *Salmonella* Typhi. Total RNA prepared from bacterial cultures grown in LB broth was used to amplify, by RT–PCR, a *sapDF* fragment of 340 bp. Amplification of a 16S rRNA nucleotide fragment of 150 bp was used as a positive control. Total RNA treated with DNase I and incubated without reverse transcriptase was used as a negative control. See the Materials and methods section for details.

detected in the *Salmonella* Typhi STH2370 clinical strain. A DNA fragment of ∼340 bp was observed in the mutant *Salmonella* Typhi ΔGICT18/1 (Figure 3), suggesting that incorporation of GICT18/1 within the *sap* operon of the *Salmonella* Typhi STH2370 genome affects expression of genes located downstream of the point of insertion.

Discussion

APs are a unique and diverse group of molecules that are divided into subgroups on the basis of their amino acid composition and structure.[7,42] These peptides are produced by many tissues and cell types in a variety of plant and animal species. Since APs have been demonstrated to kill both Gram-negative and Gram-positive bacteria, this property makes it difficult to come up with a universal model to study their mechanisms of action. Broad-spectrum peptides such as protamine have been used as a model to study the biological properties of APs.[10,21,22] *Salmonella* Typhimurium is the first microorganism in which a set of genes has been reported to be associated with antibacterial response to protamine.[10] One such element is the operon *sapABCDF* that has been found in several Gram-negative pathogens.[12–18] This study reports that a 4.1 kb genetic island (GICT18/1) interrupts the *sapABCDF* operon in the *Salmonella* Typhi genome as determined by bioinformatic analyses and PCR experiments. We also demonstrate that a relationship exists between the insertion of GICT18/1 within the *sapABCDF* operon and the enhanced susceptibility to the AP protamine exhibited by this pathogen.

The *sapABCDF* operon consists of genes organized in a single transcriptional unit.[11] These genes show similarity to the ABC transporter system, highlighting the evolutionary relevance of the *sap* operon.[13–18,43] The stable integration of the 4.1 kb genetic island in the *sapABCDF* operon of *Salmonella* Typhi raises the question of whether such a chromosomal insertion conferred any evolutionary advantage on this pathogen. This genetic island is rarely found in *Salmonella enterica* serovars included in this study. Interestingly, those *Salmonella enterica* serovars that harbour GICT18/1 also carry SPI-18, a pathogenicity island that encodes a gene for a haemolysin in *Salmonella* Typhi. All these serovars have been reported to cause a systemic

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infection in humans.[27,44–46] The insertion of GICT18/1 might be advantageous to *Salmonella* Typhi by providing novel functions to this bacterium. For instance, the product of STY1364 (one of the ORFs in the GICT18/1 island) has been found associated with the *Salmonella* Typhi proteome when the cells are grown under a low Mg^{2+} concentration, a condition related to the intracellular environment in the *Salmonella*-containing vacuole (SCV).[47] In addition, STY1362 and STY1364 exhibit a high degree of similarity to *artA* and *artB*, two genes displaying ADP-ribosyltransferase activity in *Salmonella* Typhimurium DT104.[48–50]

Although the nine ORFs encoded by GICT18/1 have not yet been well characterized, our results suggest that they have no role in protamine susceptibility because when *Salmonella* Typhi ΔGICT18/1 was complemented with pRP012 carrying GICT18/1 no modification of this susceptibility was observed. Therefore, the insertion of GICT18/1 within the *Salmonella* Typhi *sap* operon is responsible for the phenotype. In addition, the absence of an RT–PCR product from *sapDF* genes located downstream of GICT18/1 indicate that the polycistronic transcript is not produced.

The *Salmonella* Typhi wild-type strain used in this study is only susceptible to protamine and not to mellitin and HNP-1, suggesting that additional genes are involved in AP resistance. This is supported by observations that *Salmonella* Typhi expresses a number of genes, including *phoP*, *pqaB*, *uglT* and *pagP*, as part of the AP resistance response inside human macrophages.[51] In addition, Eswarappa *et al*.[52] have reported that another operon known as *yejABEF* is responsible for protamine resistance in *Salmonella* Typhimurium. The genes of the *Salmonella* Typhi *yejABEF* operon have not been characterized. However, our *in silico* nucleotide sequence analyses of the *Salmonella* Typhi genome indicate that a group of ORFs, STY2452, STY2453, STY2454 and STY2455, have a high level of identity with *Salmonella* Typhimurium *yejABEF*.

In summary, we report that insertion of the genetic island GICT18/1 in the *sap* operon of *Salmonella* Typhi is responsible for the high susceptibility to protamine in this pathogen. This genetic insertion is rarely found in other closely related *Salmonella enterica* serovars. To the best of our knowledge, this is the first report of a genetic island inserted within an operon.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Bäumler AJ, Tsolis RM, Ficht TA *et al*. Evolution of host adaptation in *Salmonella enterica*. *Infect Immun* 1998; **66**: 4579–87.

2 Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. *Bull World Health Organ* 2004; **82**: 346–53.

3 Santos RL, Tsolis R, Bäumler AJ *et al*. Pathogenesis of *Salmonella*-induced enteritis. *Braz J Med Biol Res* 2003; **36**: 3–12.

4 Groisman EA, Ochman H. How *Salmonella* became a pathogen. *Trends Microbiol* 1997; **5**: 343–9.

5 Crump JA, Mintz ED. Global trends in typhoid and paratyphoid fever. *Clin Infect Dis* 2010; **50**: 241–6.

6 Cotter PD, Hill C, Ross RP. Bacteriocins: developing innate immunity for food. *Nat Rev Microbiol* 2005; **3**: 777–88.

7 Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002; **415**: 389–95.

8 Powers JP, Hancock RE. The relationship between peptide structure and antibacterial activity. *Peptides* 2003; **24**: 1681–91.

9 Hancock RE, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol* 2006; **24**: 1551–7.

10 Groisman EA, Parra-Lopez C, Salcedo M *et al*. Resistance to host antimicrobial peptides is necessary for *Salmonella* virulence. *Proc Natl Acad Sci USA* 1992; **89**: 11939–43.

11 Parra-Lopez C, Baer MT, Groisman EA. Molecular genetic analysis of a locus required for resistance to antimicrobial peptides in *Salmonella typhimurium*. *EMBO J* 1993; **12**: 4053–62.

12 Lopez-Solanilla E, Llama-Palacios A, Collmer A *et al*. Relative effects on virulence of mutations in the *sap*, *pel* and *hlp* loci in *Erwinia chrysanthemi*. *Mol Plant Microbe Interact* 2001; **14**: 386–93.

13 Lopez-Solanilla E, Garcia-Olmedo F, Rodriguez-Palenzuela P. Inactivation of the *sapA* to *sapF* locus of *Erwinia chrysanthemi* reveals common features in plant and animal bacterial pathogenesis. *Plant Cell* 1998; **10**: 917–24.

14 Mason KM, Munson RS Jr, Bakaletz LO. A mutation in the *sap* operon attenuates survival of nontypeable *Haemophilus influenzae* in a chinchilla model of otitis media. *Infect Immun* 2005; **73**: 599–608.

15 Mason KM, Bruggeman ME, Munson RS *et al*. The non-typeable *Haemophilus influenzae* Sap transporter provides a mechanism of antimicrobial peptide resistance and SapD-dependent potassium acquisition. *Mol Microbiol* 2006; **62**: 1357–72.

16 Chen HY, Wen SH, Lin JW. Identification and analysis of the *sap* genes from *Vibrio fischeri* belonging to the ATP-binding cassette gene family required for peptide transport and resistance to antimicrobial peptides. *Biochem Biophys Res Commun* 2000; **269**: 743–8.

17 Lupp C, Hancock RE, Ruby EG. The *Vibrio fischeri sapABCDF* locus is required for normal growth, both in culture and in symbiosis. *Arch Microbiol* 2002; **179**: 57–65.

18 McCoy AJ, Liu H, Falla TJ *et al*. Identification of *Proteus mirabilis* mutants with increased sensitivity to antimicrobial peptides. *Antimicrob Agents Chemother* 2001; **45**: 2030–7.

19 Davidson AL, Dassa E, Orelle C *et al*. Structure, function and evolution of bacterial ATP-binding cassette systems. *Microbiol Mol Biol Rev* 2008; **72**: 317–64.

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20 Miller BF, Abrams R, Dorfman A *et al*. Antimicrobial properties of protamines and histones. *Science* 1942; **96**: 428–9.

21 Johansen C, Verheul A, Gram L *et al*. Protamine-induced permeabilization of cell envelopes of Gram-positive and Gram-negative bacteria. *Appl Environ Microbiol* 1997; **63**: 1155–9.

22 Koo SP, Bayer AS, Yeaman MR. Diversity in antistaphylococcal mechanisms among membrane-targeting antimicrobial peptides. *Infect Immun* 2001; **69**: 4916–22.

23 Parra-Lopez C, Lin R, Aspedon A *et al*. A *Salmonella* protein that is required for resistance to antimicrobial peptides and transport of potassium. *EMBO J* 1994; **13**: 3964–72.

24 Aspedon A, Groisman EA. The antibacterial action of protamine: evidence for disruption of cytoplasmatic membrane energization in *Salmonella typhimurium*. *Microbiology* 1996; **142**: 3389–97.

25 Baker SJ, Daniels C, Morona R. PhoP/Q regulated genes in *Salmonella typhi*: identification of mellitin sensitive mutants. *Microb Pathog* 1997; **22**: 165–79.

26 Bueno SM, Santiviago CA, Murillo AA *et al*. Precise excision of the large pathogenicity island, SPI7, in *Salmonella enterica* serovar Typhi. *J Bacteriol* 2004; **186**: 3202–13.

27 Fuentes JA, Villagra NA, Castillo-Ruiz M *et al*. The *Salmonella* Typhi *hlyE* gene plays a role in invasion of cultured epithelial cells and its functional transfers to *S*. Typhimurium promotes deep organ infection in mice. *Res Microbiol* 2008; **159**: 279–87.

28 Santiviago CA, Toro CS, Bucarey SA *et al*. A chromosomal region surrounding the *ompD* porin gene marks a genetic difference between *Salmonella typhi* and the majority of *Salmonella* serovars. *Microbiology* 2001; **147**: 1897–907.

29 Boyd EF, Wang F, Beltran P *et al*. *Salmonella* Reference Collection B (SARB): strains of 37 serovars of subspecies I. *J Gen Microbiol* 1993; **139**: 1125–32.

30 Parkhill J, Dougan G, James KD *et al*. Complete genome of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature* 2001; **413**: 848–52.

31 Deng W, Liou S, Plunkett G III *et al*. Comparative genomics of *Salmonella enterica* serovar Typhi strains Ty2 and CT18. *J Bacteriol* 2003; **185**: 2330–7.

32 McClelland M, Sanderson KE, Spieth J *et al*. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* 2001; **413**: 852–6.

33 Jarvik T, Smillie C, Groisman EA *et al*. Short-term signatures of evolutionary change in the *Salmonella enterica* serovar Typhimurium 14028 genome. *J Bacteriol* 2010; **192**: 560–7.

34 Thompson NR, Clayton DJ, Windhorst D *et al*. Comparative genome analysis of *Salmonella* Enteritidis PT4 and *Salmonella* Gallinarum 287/91 provides insights into evolutionary and host adaptation pathways. *Genome Res* 2008; **18**: 1624–37.

35 Chiu CH, Tang P, Chu C *et al*. The genome sequence of *Salmonella enterica* serovar Choleraesuis, a highly invasive and resistant zoonotic pathogen. *Nucleic Acids Res* 2005; **33**: 1690–8.

36 McClelland M, Sanderson KE, Clifton SW *et al*. Comparison of genome degradation in Paratyphi A and Typhi, human-restricted serovars of *Salmonella enterica* that cause typhoid. *Nat Genet* 2004; **36**: 1268–74.

37 Holt KE, Parkhill J, Mazzoni CJ *et al*. High-throughput sequencing provides insights into genome variation and evolution in *Salmonella* Typhi. *Nat Genet* 2008; **40**: 987–93.

38 Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 2000; **97**: 6640–5.

39 Bucarey SA, Villagra NA, Fuentes JA *et al*. The cotranscribed *Salmonella enterica* sv. Typhi *tsx* and *impX* genes encode opposing nucleoside-specific import and export proteins. *Genetics* 2006; **173**: 25–34.

40 Bucarey SA, Villagra NA, Martinic M *et al*. The *Salmonella enterica* serovar Typhi *tsx* gene, encoding a nucleoside-specific porin, is essential for prototrophic growth in the absence of nucleosides. *Infect Immun* 2005; **73**: 6210–9.

41 Varkey J, Nagaraj R. Antibacterial activity of human neutrophil defensin HNP-1 analogs without cysteines. *Antimicrob Agents Chemother* 2005; **49**: 4561–6.

42 Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol* 2005; **3**: 238–50.

43 Harms C, Domoto Y, Celik C *et al*. Identification of the ABC protein SapD as the subunit that confers ATP dependence to the K^+-uptake systems Trk(H) and Trk(G) from *Escherichia coli* K-12. *Microbiology* 2001; **147**: 2991–3003.

44 Björkman P, Nilsson A, Riesbeck K. A pilot with pain in his leg: thigh abcess caused by *Salmonella enterica* serotype Brandenburg. *J Clin Microbiol* 2002; **40**: 3530–1.

45 Kim JY, Park YJ, Lee SO *et al*. Case report: bacteremia due to *Salmonella enterica* serotype Montevideo producing plasmid-mediated AmpC β-lactamase (DHA-1). *Ann Clin Lab Sci* 2004; **34**: 214–7.

46 Chiu CH, Lin TY, Ou JT. Predictors for extraintestinal infection of non-typhoidal *Salmonella* in patiens with AIDS. *Int J Clin Pract* 1999; **53**: 161–4.

47 Ansong C, Yoon H, Norbeck AD *et al*. Proteomic analysis of the causative agent of typhoid fever. *J Proteome Res* 2008; **7**: 546–57.

48 Satoi M, Tanaka K, Nishimori K *et al*. The *artAB* genes encode a putative ADP-ribosyltransferase toxin homologue associated with *Salmonella enterica* serovar Typhimurium DT104. *Microbiology* 2005; **151**: 3089–96.

49 Hermans AP, Abee T, Zwietering MH *et al*. Identification of novel *Salmonella enterica* serovar Typhimurium DT104-specific prophage and nonprophage chromosomal sequences among serovar Typhimurium isolates by genomic subtractive hybridization. *Appl Environ Microbiol* 2005; **71**: 4979–85.

50 Uchida I, Ishihara R, Tanaka K *et al*. *Salmonella enterica* serotype DT104 ArtA-dependent modification of pertussis toxin-sensitive G proteins in the presence of [32P]NAD. *Microbiology* 2009; **155**: 3710–8.

51 Faucher SP, Porwollik S, Dozois CM *et al*. Transcriptome of *Salmonella enterica* serovar Typhi within macrophages revealed through the selective capture of transcribed sequences. *Proc Natl Acad Sci USA* 2006; **103**: 1906–11.

52 Eswarappa SM, Panguluri KK, Hensel M *et al*. The *yejABEF* operon of *Salmonella* confers resistance to antimicrobial peptides and contributes to its virulence. *Microbiology* 2008; **154**: 666–78.

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