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Membrane topology of *Salmonella* invasion protein SipB confers osmotolerance

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ABSTRACT

Salmonella enterica serovar Typhimurium is a major cause of human gastrointestinal illness worldwide. This pathogen can persist in a wide range of environments, making it of great concern to public health. Here, we report that the *salmonella* pathogenicity island (SPI)-1 effector protein SipB exhibits a membrane topology that confers bacterial osmotolerance. Disruption of the *sipB* gene or the *invG* gene (SPI-1 component) significantly reduced the osmotolerance of *S.* Typhimurium LT2. Biochemical assays showed that NaCl osmolarity increased the membrane topology of SipB, and a neutralising antibody against SipB reduced osmotolerance in the WT strain. The WT strain, but not the *sipB* mutant, exhibited elevated cyclopropane fatty acid C19:0 during conditions of osmotic stress, correlating with the observed levels of survival and membrane integrity. This result suggests a link between SipB and the altered fatty acid composition induced upon exposure to osmotic stress. Overall, our findings provide the first evidence that the *Salmonella* virulence translocon SipB affects membrane fluidity and alters bacterial osmotolerance.

1. Introduction

Salmonella enterica subsp. *enterica* serovar Typhimurium is one of the major causes of foodborne diseases worldwide. Central to *S.* Typhimurium pathogenesis are two Type 3 Secretion Systems (TTSSs) encoded within *salmonella* pathogenicity islands (SPI)-1 and -2. TTSSs are responsible for the secretion and translocation of a set of bacterial proteins known as effectors into host cells. This process facilitates host cell invasion and inflammation (by SPI-1) or survival and replication within phagocytes (for SPI-2) to establish a systemic infection [1]. The expression of SPI apparatus genes is known to be regulated by multiple environmental signals, including osmolarity, oxygen tension, pH, and the growth rate of bacteria [2–5].

This pathogen is known to be widespread in host and hostile environments and can survive for long periods of time in aquatic environments, making it a significant public health concern [6]. There is mounting evidence that SipB, a SPI-1-mediated effector protein that promotes host cell apoptosis and necrosis via caspase-1-mediated activation of IL-1β and IL-18 [7,8], exhibits bacterial outer membrane topology [9,10]. More recent studies have shown that this surface-association is mediated through SPI-1 assembly when bacteria are cultivated with NaCl osmolarity [11]. The osmo-responsible SipB translocation was shown to be regulated through the upregulation of *hilAD* systems [12] under the control of the ATP-dependent Lon protease [13].

Considering that bacterial membranes first sense environmental changes and then trigger a variety of response mechanisms to adapt [14], we hypothesised that the membrane localisation of SipB might affect bacterial osmotic stress tolerance. To examine this issue, we investigated the role of SipB membrane fusion in the osmotolerance of *S.* Typhimurium. Our studies revealed that the disruption of the *sipB* gene or the *invG* gene (encoding the SPI-1 multiring base required for SPI-1 mediated protein secretion) [15] clearly impaired the osmotolerance of this pathogen. Biochemical assays then provided evidence that the disruption of SipB weakened bacterial osmotolerance upon exposure to osmotic stimuli, coinciding with reduced membrane integrity and no clear upshift in the level of cyclopropane fatty acid C19:0.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *Salmonella enterica* serovar Typhimurium LT2 strain was used as the wild type (WT) strain and for constructing the knockout mutants. *Escherichia coli* DH5α was used for genetic cloning.

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Except where indicated otherwise, bacteria were routinely grown at 37 °C in Luria–Bertani (LB) broth or on LB agar (LA) plates with the appropriate antibiotics.

2.2. Disruption and complementation of the sipB/invG genes

The *S.* Typhimurium LT2-derived *invG* mutant and *sipB* mutant were constructed by the λRed recombinase method as described [16]. The primer sequences used for the replacement of the target genes are listed in Table S1. The antibiotic cassette was removed by FRT-mediated FLP recombination after transformation with pCP20 [16], and unmarked mutants were selected by sensitive antibiotics and verified by DNA sequencing. For complementation, the *invG* and *sipB* genes were PCR-amplified with primer sets (Table S1) and cloned into the *Bam*HI/*Sal*I site of the plasmid pACYC184 in *E. coli* DH5α. The plasmids were then used to transform the *invG* mutant or *sipB* mutants by electroporation. Successful transformants were screened on chloramphenicol (30 μg ml^{-1})-agar plates at 37 °C.

2.3. NaCl sensitivity test

Approximately 1.0×10^9 cells grown in TY medium (1% (w/v) tryptone and 0.5% (w/v) yeast extract) for 18 h at 37 °C were washed with phosphate-buffered saline (PBS) (pH 7.2) twice, followed by incubation in 50 ml of 1.36 M (equivalent to 8% (w/v)) NaCl in PBS (pH 7.2) at 37 °C for up to 9 days. At 24 h intervals, 100 μl of the suspension and serial dilutions was spread onto Tryptic soy agar (TSA) plates to count the number of viable cells. Simultaneously, the suspensions were fluorescently stained with Bac Light Bacterial viability kit (Invitrogen-Molecular Probes, Eugene, OR, USA) to measure the membrane integrity as described [17].

2.4. Real-time PCR

Total RNA was prepared from the bacterial cells with a RiboPure bacterial RNA isolation kit (Life Science Technologies, Carlsbad, CA) and subjected to cDNA synthesis using a first strand cDNA synthesis kit (Roche Diagnostics, Burgdorf, Switzerland). Real-time PCR assays were performed with oligonucleotide primers for *hilA*, *hilD*, *sipB*, and 16S rRNA (Table S1) using a Light Cycler 480 DNA analyser (Roche Diagnostics, Burgdorf, Switzerland). The transcript levels for each gene were determined according to the manufacturer's instructions by calculating the relative quantification and using 16s rRNA as a standard.

2.5. Expression and purification of SipB recombinant protein

The *sipB* coding sequence derived from *S.* Typhimurium LT2 was amplified by PCR with primers (Table S1), cloned into pBAD202-D-Topo (Life Science Technologies, Carlsbad, CA), and transformed into *E. coli* KRX competent cells (Promega, Fitchburg, WI, USA). Successful transformants were grown in 500 ml of LB broth at 37 °C to an A_{600} of 1.0 and induced with 0.1% L-arabinose (Sigma Aldrich, St. Louis, MO, USA) for 6 h. The recombinant SipB was purified with Ni–NTA agarose (QIAGEN, Hilden, Germany). The resultant rSipB protein was then used to produce rabbit anti-SipB antisera by in-house immunisation of white rabbits.

2.6. Subcellular fractionation, SDS–PAGE, and Western blot

Subfractions of *Salmonella* cells were obtained essentially as described [11]. Approximately 30 μg of the protein samples was loaded onto 10% acrylamide gels. The proteins on the gels were visualised with CBB (Coomassie Brilliant Blue) stain. For the western blot protocol, the proteins were simultaneously transferred onto PVDF membrane (Millipore). Rabbit anti-SipB or anti-OmpW antisera [17] were used as the primary antibodies. HRP-conjugated anti-rabbit IgG antibody (GE HealthCare, Little Chalfont, UK) was used as the secondary antibody. The protein signals were detected with the ECL detection system (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions.

2.7. Antibody neutralisation assay

Approximately 10^9 CFU of WT strain grown in TY medium for 18 h at 37 °C were washed twice with PBS (pH 7.2), followed by incubation at 37 °C in 50 ml of 1.36 M NaCl/PBS (pH 7.2) supplemented with 0, 1, 5, 10, and 20 μl of anti-SipB antiserum. At days 3, 5, and 7 post incubation, 100 μl of the suspension and serial dilutions was spread onto TSA plates to count the number of viable cells.

2.8. Fatty acid methyl ester (FAME) assay

Bacterial cells were harvested, washed three times with sterile PBS, and freeze-dried. The pellets were then used to extract and measure fatty acid methyl ester (FAME) using the Sherlock Microbial ID system (MIDI Inc., Newark, DE, USA) according to the manufacturer's instructions. Each sample was tested twice, and the mean values were used for a comparative analysis.

2.9. Statistics

Data for plate counts, membrane integrity, and real-time PCR represented means ± standard deviations (SD) from three independent experimental sets. Fatty acid profiling data represent a means from two testing.

3. Results

3.1. Disruption of the sipB and invG genes impaired osmotolerance of S. Typhimurium

To investigate the potential role of SipB in the osmotolerance of *S.* Typhimurium, we constructed a *sipB* mutant and an *invG* mutant (lacking functional SPI-1) with the backbones of the *S.* Typhimurium LT2 strain. Each strain's survival in 8% NaCl solution was examined by conducting a plate count each day for 9 days. As shown in Fig. 1A, the two mutants showed impaired survival under the high osmotic condition when compared with the wild-type (WT) strain (i.e., after 5 days of incubation, the WT strain exhibited a 1.3 log-reduction, and the *sipB* mutant exhibited a 3.1 log-reduction). Complementation of each gene restored the osmotolerance, while the introduction of the *sipB* gene into the *invG* mutant did not alter its phenotype (Fig. 1). A parallel measurement for membrane integrity using BacLight staining showed similar trends; 14.1% of the WT strain, 1.7% of the *sipB* mutant strain, and 1.5% of the *invG* mutant strain retained integrity after 5 days of incubation in the 8% NaCl solution (Fig. 1B). Thus, we demonstrated that both the *sipB* gene and the SPI-1 system are required for the osmotolerance of *S.* Typhimurium.

3.2. NaCl osmolarity enhances the translocation of SipB to the membrane

Given that the addition of NaCl to a broth culture enhances the secretion of SipB *in vitro* [12], we examined whether SipB translocates to the bacterial surface in response to stringent NaCl osmolarity (8% NaCl). After a 20 h incubation of the WT strain in 8% NaCl solution, protein subfractionation and western blot assays

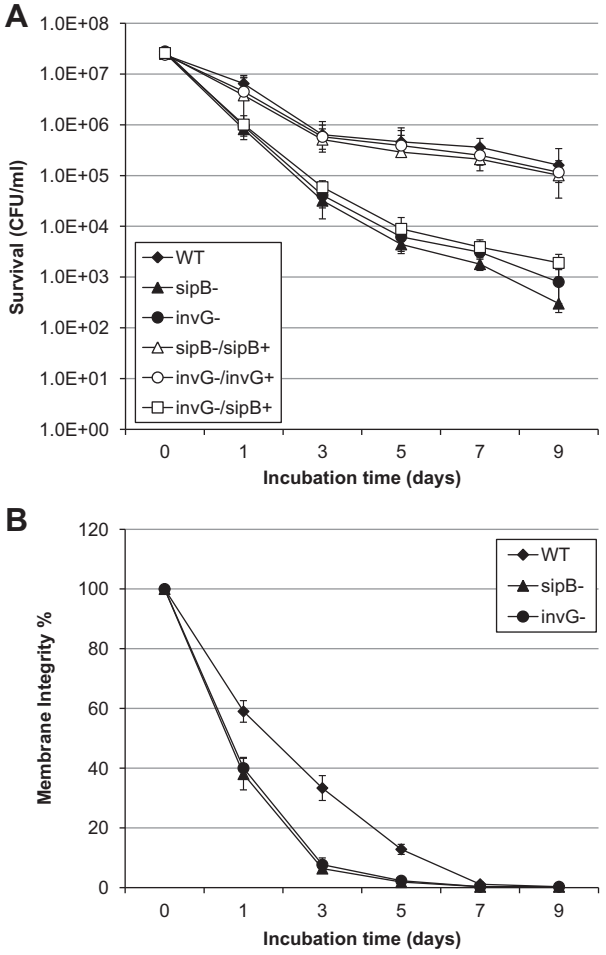

Fig. 1. The effect of *sipB* and *invG* gene disruption on the viability of *S.* Typhimurium under osmotic stress is shown. Bacteria were grown in TY medium for 20 h and were then incubated in 8% NaCl solution at 37 °C. At the indicated time point after incubation, 100 µl aliquots of the suspensions were serially diluted and plated on TSA agar plates to measure viable cell counts (section A, left *y*-axis). In parallel, the suspensions of the WT, sipB−, and invG− strains were subjected to LIVE/DEAD Bac Light viability stain to determine the percentage of cells with intact membranes (section B, right *y*-axis). The data in sections A and B represent the standard mean ± standard deviation from three independent tests. WT, wild-type LT2 strain; sipB−, *sipB* mutant; invG−, *invG* mutant; sipB−/sipB+, *sipB* mutant complemented with *sipB* gene; invG−/invG+, *invG* mutant complemented with *invG* gene; invG−/sipB+, *invG* mutant complemented with *sipB* gene.

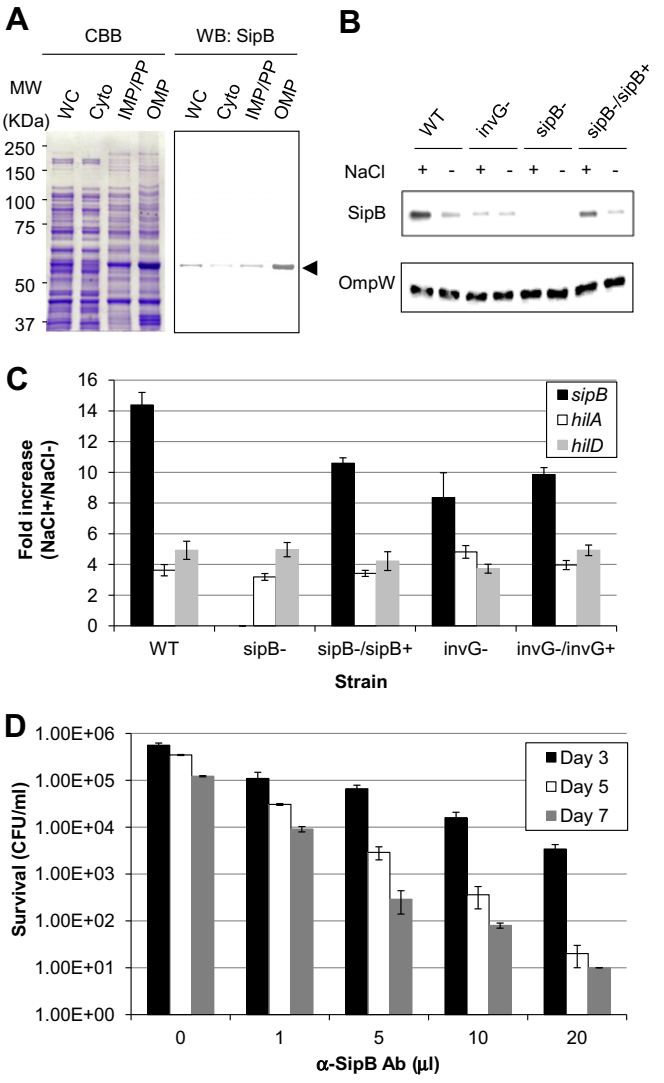

Fig. 2. NaCl osmolarity facilitates the membrane topology of SipB, affecting osmotolerance. (A) The SDS–PAGE and Western blot results of the *S.* Typhimurium LT2 strain incubated in 8% NaCl for 20 h are shown. Bacterial lysates (WC) were subfractionated into cytoplasmic proteins (Cyto), inner membrane/periplasmic proteins (IMP/PP), and outer membrane proteins (OMP), followed by separation on 10% acrylamide gels. Total proteins were visualised by CBB stain (left panel). The Western blot for the detection of SipB is shown in the right panel. (B) The membrane topology of SipB in *S.* Typhimurium in response to osmotic stimuli is shown. WT, *invG* mutant (invG−), *sipB* mutant (sipB−), and its complement (sipB−/sipB+) strains were incubated in 8% NaCl solution for 1 day. OMP fractions were separated before (NaCl−) and after (NaCl+) the incubation, and 30 µg proteins were subjected to Western blot analysis to detect SipB and OmpW. (C) Altered transcripts of *sipB*, *hilA*, and *hilD* genes in WT, invG−, sipB−, and sipB−/sipB+ strains after incubation in 8% NaCl solution for 2 h are shown. The data represent the fold increase compared with the transcript levels at the 0 h incubation. (D) The SipB-neutralisation antibody decreased survival of the WT strain under osmotic stimuli in a dose-dependent manner. The data represent plate counts (CFU/ml) on days 3, 5, and 7 post-incubation in 8% NaCl solution. The data in sections C and D represent the mean ± standard deviations (SD) from three independent tests.

showed that SipB was largely present in the sarcosyl-insoluble fraction (mainly consisting of outer membranes) (Fig. 2A). Similarly, greater amounts of SipB were detected in the OMP fraction of the WT strain and the *sipB* mutant/*sipB*-complemented strain (sipB−/sipB+) after 20 h incubation in 8% NaCl solution. In contrast, the *invG* mutant did not exhibit an NaCl-induced increase in the level of membrane-associated SipB (Fig. 2B), indicating that SPI-1 mediated the osmo-inducible translocation of SipB onto the bacterial surface. The qRT-PCR assays showed that the inactivation of the *sipB* or *invG* genes did not alter the transcription of the *hilAD* genes (Fig. 2C), which are central regulators of the *sipB* gene transcript [12]. This indicated that SPI-1-mediated SipB translocation is essential to conferring the osmotolerance. This result was supported by the observation that blocking the surface-localised SipB with neutralising antisera significantly reduced bacterial osmotolerance in a dose-dependent manner (Fig. 2D). Together, our data indicated that the SPI-1-mediated translocation of SipB to the bacterial surface plays a prominent role in the osmotolerance of this pathogen.

3.3. SipB induces bacterial fatty acid compositional shift in response to osmotic stress

Bacterial membranes initially sense harsh environmental stresses and then trigger a variety of response mechanisms to adapt to the changing conditions [14]. Because fatty acids constitute the main body of bacterial membranes, and the disruption of the *sipB* gene accelerated the reduction of membrane integrity upon exposure to osmotic stimuli (Fig. 1B), we hypothesised that

Table 1
The osmotic stimuli-induced modulation in the fatty acid compositions in *S.* Typhimurium LT2 WT and *sipB* mutant strains.

Fatty acid	Percentage					
	WT			*sipB* mutant		
	0 h	6 h	20 h	0 h	6 h	20 h
10:0	0.12	0.04	–	0.09	0.03	–
12:0 Alde	1.08	0.74	0.56	0.93	0.77	–
12:0	3.86	2.74	1.88	3.94	2.78	1.68
13:0	0.15	0.12	–	0.17	0.12	–
12:0 2OH	0.15	0.08	–	0.15	0.08	–
14:0	5.04	4.09	3.22	5.05	4.16	3.19
15:0	0.87	0.77	0.53	0.82	0.73	0.52
14:0 2OH	1.95	1.34	0.84	1.95	1.29	0.26
Sum in Feature 2 (C14:0 3OH/C16:1)	9.12	5.34	3.91	9.36	5.09	1.77
Sum in Feature 3 (C16:1 ω7c/15 iso)	7.66	6.59	5.39	7.89	7.87	4.12
16:1 ω5c	0.19	0.18	0.19	0.20	0.17	0.19
16:0	35.60	37.36	37.01	35.40	36.71	28.47
17:0 CYCLO	10.74	14.66	17.25	10.48	13.63	10.90
17:0	0.94	1.05	0.32	0.91	0.95	0.39
18:1 ω7c	14.96	15.75	17.18	15.20	17.54	17.07
18:0	0.87	1.05	2.11	0.95	1.02	0.70
11 methyl 18:1 ω7c	0.29	–	–	0.32	–	0.19
19:0 CYCLO ω8c	3.86	6.27	8.44	3.63	4.10	2.39
20:2 ω6,9c	0.28	0.08	–	0.28	–	–
CFA ratio (%)	14.60	20.93	25.69	14.11	17.73	13.29

Fatty acid methyl esters were detected in *S.* Typhimurium WT and the *sipB* mutant strains incubated in 8% NaCl solutions for 0, 6, and 20 h.

membrane topology of SipB may alter the fatty acid composition of *S.* Typhimurium under conditions of osmotic stress. The fatty acid analyses were able to detect a total of 19 fatty acids, including C10:0, C12:0 Alde, C12:0, C12:0 2OH, C13:0, C14:0, C14:0 2OH, C15:0, Sum in Feature 2 (C14:0 3OH/C16:1 ISO I), Sum in Feature 3 (C16:1 ω7c/15 iso 2OH_C15:0 ISO 2OH/C16:1 ω7c), C16:1ω5c, C16:0, C17:0 cyclo, C17:0, C18:1ω7c, C18:0, 11-methyl C18:1 ω7c, C19:0 cyclo ω8c, and C20:2 ω6, 9c (Table 1). No apparent differences in fatty acid composition were found between the WT and *sipB* mutant strains before the introduction of osmotic stimuli; however, this profile was significantly altered upon incubation in 8% NaCl solution. The most notable change we observed was an elevation in the percentage of cyclopropane fatty acid (CFA) C19:0 in the WT strain. This is in contrast to the *sipB* mutant, which showed no alteration in this CFA (Table 1). Together, these data showed that stringent osmotic stress increases CFA in *S.* Typhimurium, which correlates with the presence of the *sipB* gene.

4. Discussion

Here we showed that the disruption of the *sipB* gene or the *invG* gene reduced osmotolerance in *S.* Typhimurium. The osmotic stress increased the levels of membrane-associated SipB protein in the WT strain, and antibody neutralisation assays provided further evidence of the important function of surface-associated SipB in conferring bacterial osmotolerance. This osmotic stress modulated the composition of fatty acids constituting the main body of the membrane, and SipB was shown to associate with the elevated levels of CFA C19:0. Our data thus indicated that surface-localised SipB plays a role in this pathogen's stabilised membrane integrity and osmotolerance.

To date, genetic and transcriptomic studies have implicated a substantial number of genes and environmental conditions in the regulation of SPI-1 genes [18]. Upon exposure to osmotic stimuli, Lon protease is involved in the upregulation of the *hilA*/*hilD* genes, which directly regulate transcription of the SPI-1 apparatus genes in *S.* Typhimurium [12]. We observed no altered expression of *hilAD* genes in the WT, *sipB* mutant, and *invG* mutant strains during incubation in stringent osmolarity, suggesting that the surface-localised SipB was sufficient for retaining osmotolerance.

Next, we examined whether the surface-localised SipB might affect fatty acid composition. In *E. coli*, the cyclisation of fatty acid acyl chains is generally regarded as a means of controlling the penetration of undesirable molecules to adapt the cells to adverse conditions [19,20]. A previous study additionally reported that fatty acid membrane composition was drastically modulated in response to osmotic stimuli in *Lactococcus lactis* [21]. The authors demonstrated that NaCl osmolarity increased cyclopropane fatty acid (CFA) C19:0. CFAs are known to be present in many bacteria, but the physiological consequences of their contribution to membrane properties are not yet understood, especially concerning the modifications of membrane fluidity in response to environmental stress. In *S.* Typhimurium, CFA formation is known to affect acid tolerance [22] and heat resistance [23]; in both conditions, *Salmonella* cells showed increased C19:0. Our data demonstrated that while the SipB-positive WT strain had an increased level of CFA C19:0 during incubation in the 8% NaCl solution, the *sipB* mutant's fatty acid composition remained unchanged. This novel finding indicated that *S.* Typhimurium increased CFA levels in response to osmotic stress, which correlated with SipB-related osmotolerance. The implications for the potential interaction between the altered fatty acid composition and SipB's polarity remain unclear; nevertheless, our data suggest that the increased CFA levels might be involved in the global stress response mechanism(s) of this pathogen.

The SPI-1 cluster is widely distributed among different *S. enterica* strains [24]. Nevertheless, the recent availability of genome sequences has revealed variation in SPI-1 homology among the different serovars of *S. enterica* [25]. The virulence factor database VFDB [26] showed high conservation of SipB, whereas conservation of one SPI-1 component, InvF protein, seemed to vary among different *S. enterica* serovars (data not shown). This suggested that the SPI-1-mediated membrane topology of SipB might be strain-dependent, and if so, it would determine the strain-to-strain variation in osmotolerance phenotypes. Considering that a variety of *Salmonella* serovars have been found in aquatic environments [27], our data provide new insight into the ecological role of the virulence determinant, potentially linking it to the public health threat.

Overall, our data presented herein demonstrate that *Salmonella* transports SipB to the bacterial surface, affecting bacterial osmotolerance. The altered CFA levels observed upon exposure to osmotic stimuli correlated with the reduced membrane integrity observed under stress conditions. Because we found that anti-SipB antisera significantly reduced bacterial survival, this protein may also become a potential target for the control of this pathogen in the food supply and the environment, providing an attractive strategy for preventing human infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.012.

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