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Phage mediated horizontal transfer of the *sopE1* gene increases enteropathogenicity of *Salmonella enterica* serotype Typhimurium for calves

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Abstract

Epidemiological evidence shows that the *sopE1* gene is associated with *Salmonella* Typhimurium phage types causing epidemics in cattle. In this study we demonstrate that horizontal transfer of the *sopE1* gene by lysogenic conversion with the SopEΦ increased enteropathogenicity of *S.* Typhimurium in the bovine ligated ileal loop model. These data support the hypothesis that phage mediated horizontal transfer of the *sopE1* gene contributes to the emergence of epidemic cattle-associated *S.* Typhimurium clones.

Keywords: Enteritis; Type III secretion; Bacteriophage; Horizontal gene transfer; *Salmonella*

1. Introduction

Horizontal gene transfer has been proposed to be a major driving force in the evolution of virulence in the genus *Salmonella*. The phylogenetic distribution of *Salmonella* pathogenicity island 1 (SPI1), which encodes the invasion-associated type III secretion system, suggests that this virulence factor was acquired by a common ancestor of the genus *Salmonella* after its divergence from the *Escherichia coli* lineage [1]. The type III secretion system encoded by SPI1 is the prime virulence factor of *Salmonella* Typhimurium for the pathogenesis of diarrheal disease in cattle [2]. It has therefore been proposed that acquisition of SPI1 was a key event during the evolution of enterocolitis, the disease syndrome caused the vast majority of *Salmonella* serotypes in man [1]. However, it is difficult to test this hypothesis experimentally since we cannot reconstruct events that led to acquisition of SPI1 by a common ancestor of the genus *Salmonella* some 50 to 100 million years ago.

The main function of the invasion-associated type III secretion system of *S.* Typhimurium is to translocate effector proteins into the cytosol of the host cell [3]. A number of these type III secreted effector proteins are encoded by genes that are located outside SPI1. These include *sopA*, *sopB* (*sigD*), *sopD*, *sopE1*, *sopE2*, *slrP* and *sspH1* [4–10]. The genes *sopB*, *sopD* and *sopE2* are present in *Salmonella bongori* and in all subspecies of *Salmonella enterica*, suggesting an acquisition early in the evolution of the genus *Salmonella* [11]. However, different combinations of *sopE1*, *slrP* and *sspH1* are present in the genomes of *Salmonella* serotypes, indicative of a more recent shuffling of these genes by lateral transfer [9,11]. Inactivation of *slrP* reduces the ability of *S.* Typhimurium to colonize Peyer's patches of the mouse while colonization of the calf intestinal mucosa is not altered [9]. This experimental evidence suggests that the presence of specific combinations of type III secreted effector proteins generated by horizontal transfer may be among the mechanisms involved in

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determining the host range of *Salmonella* serotypes. However, the *slrP* gene is present in a large number of *Salmonella* serotypes and is not associated with an intact mobile genetic element [9]. It is thus likely that the *slrP* pathogenicity islet was acquired by an ancestral *Salmonella* serotype in the distant past, thereby making it difficult to test the notion that acquisition of *slrP* altered the host range of this hypothetical organism.

In contrast, *sopE1* and *sspH1* are only present in a small number of *Salmonella* serotypes [9,12] and are carried by the SopEΦ and *Gifsy*-3 prophages of *S.* Typhimurium, respectively [6,13]. Upon induction, both phages can be released from *S.* Typhimurium and are capable of horizontally transferring the *sopE1* and *sspH1* genes, respectively, by lysogenic conversion of a susceptible recipient [12,13]. While the function of SspH1 is currently unknown, purified SopE1 protein induces membrane ruffling and nuclear responses in human cell lines presumably by acting as a nucleotide exchange factor in two Rho GTPases, Rac-1 and CDC42 [14]. Inactivation of *sopE1* does not alter the virulence of *S.* Typhimurium for mice and reduces its invasiveness for tissue culture cells only modestly [6,15]. The modest invasion defect of an *S.* Typhimurium *sopE1* mutant can be explained by the presence of a homologous gene, *sopE2* [7,8]. However, SopE2 and SopE1 do not activate identical sets of Rho GTPase signaling cascades, since SopE2 acts as a nucleotide exchange factor only for CDC42 but not for Rac-1 [16].

The presence of *sopE1* and *sspH1* on intact prophages suggests that the generation of new combinations of type III secreted effector proteins may be an ongoing process that may be involved in more recent evolutionary events related to host adaptation, such as the emergence of epidemic *S.* Typhimurium strains. For instance, surveillance of isolates from cattle in Britain and Germany reveals that the persistence of *S.* Typhimurium in this animal reservoir is characterized by a series of small epidemics, each caused by a distinct bacterial clone. After dominating for a period of time in the bovine animal reservoir, each epidemic *S.* Typhimurium strain is replaced by a new clone as indicated by the dominance of a new phage type [17]. Although the successive epidemics caused by *S.* Typhimurium phage types among cattle populations are well documented, the factors responsible for the temporal preponderance of individual clones are poorly understood.

The *sopE1* gene was first described in the bovine adapted *S. enterica* S. Dublin [15] and later in SL1344, a bovine isolate of *S.* Typhimurium [6]. A screen performed with a representative set of *S.* Typhimurium phage types isolated in Germany for the presence of strains that are lysogenized by the SopEΦ established an association of the *sopE1* gene with epidemic bovine isolates. This study showed that the *sopE1* gene was only present in five phage types, including the epidemic cattle-associated DT204, DT204c, and DT49. However, the *sopE1* gene was not present in the remaining 31 *S.* Typhimurium phage types investigated in this study [12]. The finding that the *sopE1* gene is present in epidemic cattle-associated phage types is intriguing because it suggests that recent SopEΦ mediated lateral transfer of *sopE1* may have contributed to the emergence of these clones [12]. However, since the role of *sopE1* during *S.* Typhimurium infection in calves has not yet been examined there is currently no causal link between lysogenic conversion with the SopEΦ and the epidemiological success of phage types in this animal reservoir. To address this question, we performed experiments to determine whether lysogenic conversion with the SopEΦ has an effect on the ability of *S.* Typhimurium to cause enterocolitis in calves.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. Typhimurium strain 14028 is a bovine isolate from the American Type Culture Collection (ATCC) which carries the *sopE2* gene but does not harbor the SopEΦ. Strains used in this study are listed in Table 1. Plasmid pM149 carries the cloned *sopE2* gene (W.-D. Hardt, unpublished data). Plasmid pWK-sopE1 carries the *sopE1* gene cloned into the vector pWSK29 [18]. SopEΦ^{aphT} and SopEΦ$^{sopE1::aphT}$ are phage derivatives in which a kanamycin resistance cassette either is inserted directly downstream of the *sopE1* gene or replaces the *sopE1* open reading frame, respectively (W.-D. Hardt, unpublished data). Strain ZA25 was derived from M119 (*sopE2*::tet^{R}) by lysogenic conversion with SopEΦ$^{sopE1::aphT}$ using methods described previously [12]. To prepare bacterial inocula, strains were cultured aerobically in Luria–Bertani (LB) broth at 37°C. Overnight cultures were diluted 1:100 with sterile LB and incubated for 4 h at 37°C, harvested and resuspended to a concentration of approximately 0.75×10^{9} colony forming units (CFU) ml^{-1} in sterile LB broth prior to infection of loops.

2.2. Animal experiments

Male Holstein calves ($n=4$), 4–5 weeks of age, weighing 45–55 kg were used. They were fed milk replacer twice a day and water ad libitum. The calves were clinically healthy before the experiment and were culture negative for fecal excretion of *Salmonella* serotypes. Detection of *Salmonella* serotypes in fecal swabs was performed by enrichment in tetrathionate broth (Difco) and subsequent streaking on brilliant green agar (BBL).

The calves were fasted for 24 h prior to the surgery. Anesthesia was induced with Propofol (Propoflo, Abbot Laboratories, Chicago, IL, USA) followed by placement of an endotracheal tube and maintenance with Isofluorane (Isoflo, Abbot Laboratories, Chicago, IL, USA). A lapa-

Table 1
S. Typhimurium strains used in this study

Strain	Genotype	Source
14028	wild-type, bovine isolate	ATCC
M119	14028, *sopE2*::tet^R	[19]
M30	14028, SopEΦ^{aphT}	Hardt Laboratory
M31	14028, SopEΦ$^{sopE1::aphT}$	Hardt Laboratory
M32	14028, *sopE2*::tet^R SopEΦ^{aphT}	Hardt Laboratory
ZA25	14028, *sopE2*::tet^R SopEΦ$^{sopE1::aphT}$	this study

rotomy was performed, the ileum exposed and 21 loops with length ranging from 6 to 9 cm ligated leaving 1-cm loops between them. The loops were infected by intraluminal injection of 3 ml of sterile LB broth or a bacterial suspension. The loops were replaced into the abdominal cavity. Fluid accumulated in loops was collected and samples for bacteriologic culture and histopathology were collected at 8 h after inoculation. Tissue samples from Peyer's patches were weighed, homogenized in phosphate buffered saline (PBS), serially diluted and plated onto LB agar plates for counting CFU. Fragments from the Peyer's patches were fixed in formalin, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin and examined for histopathological changes.

3. Results and discussion

To assess the contribution of phage mediated horizontal gene transfer to calf virulence we characterized *S.* Typhimurium strain 14028 and derivatives of 14028 that were lysogenized with the SopEΦ, a bacteriophage which carries the *sopE1* gene and can be transferred horizontally among *S.* Typhimurium isolates. Two male Holstein calves were used to assess the contribution of *sopE1* and its homolog, *sopE2*, to enteropathogenicity in a bovine ligated ileal loop model (Fig. 1). No overt differences in the severity of lesions or CFU recovered from tissue were observed between different *S.* Typhimurium strains used in this study (data not shown). Lysogenic conversion of 14028 (wild-type) with the SopEΦ (M30, SopEΦ^{aphT}) resulted in a small but significant increase in fluid accumulation ($P < 0.05$) induced by *S.* Typhimurium in bovine ligated ileal loops (Fig. 1). In contrast, lysogenic conversion of *S.* Typhimurium strain 14028 with a SopEΦ phage derivative which carried a deletion of the *sopE1* gene (M31, SopEΦ$^{sopE1::aphT}$) did not significantly alter fluid accumulation ($P > 0.1$). Inactivation of the *sopE2* gene (M119, *sopE2*::tet^R) present in *S.* Typhimurium strain 14028 results in a small but significant reduction in fluid accumulation ($P = 0.024$) in bovine ligated ileal loops which has been reported previously [19]. The defect in fluid accumulation could be complemented to wild-type levels either by introducing the cloned *sopE2* gene on a plasmid (M119(pM149)) or by introduction of *sopE1* by lysogenic conversion with the SopEΦ (M32, SopEΦ^{aphT}) (Fig. 1). These data suggested that the translocation of nucleotide exchange factors for Rho GTPases into host cells is a virulence mechanism of *S.* Typhimurium involved in the pathogenesis of enterocolitis and provided evidence for a role of the SopEΦ phage in calf virulence. However, results from this experiment raised two questions. Firstly, the degree of fluid accumulation caused by strain M30 (SopEΦ^{aphT}) did not differ significantly from that caused by strain M31 (SopEΦ$^{sopE1::aphT}$), thereby raising the question as to whether *sopE1* alone is sufficient for causing this effect. Secondly, it was not clear from these data whether or not SopE1 and SopE2 have redundant functions during the pathogenesis of enterocolitis. A second set of experiments was performed to address these points.

To determine whether the cloned *sopE1* gene is sufficient to increase fluid accumulation in bovine ligated ileal loops,

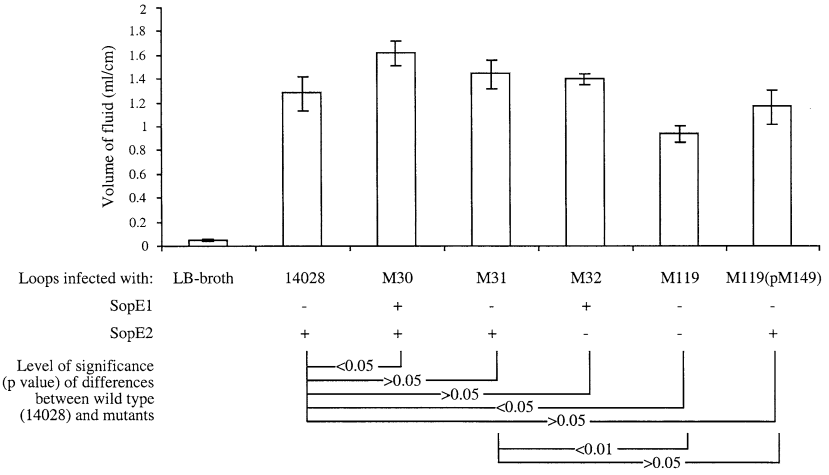

Fig. 1. Fluid accumulation 8 h post infection of bovine ligated ileal loops with *S.* Typhimurium strains. Data are presented as averages from six loops (three loops/animal) ± standard error. A Student's *t*-test was performed to assess whether the difference in fluid accumulation between strains was statistically significant and the level of significance (*P*-value) is given below the graph. The presence or absence of SopE1 and SopE2 in strains used in this study is indicated by + or −, respectively.

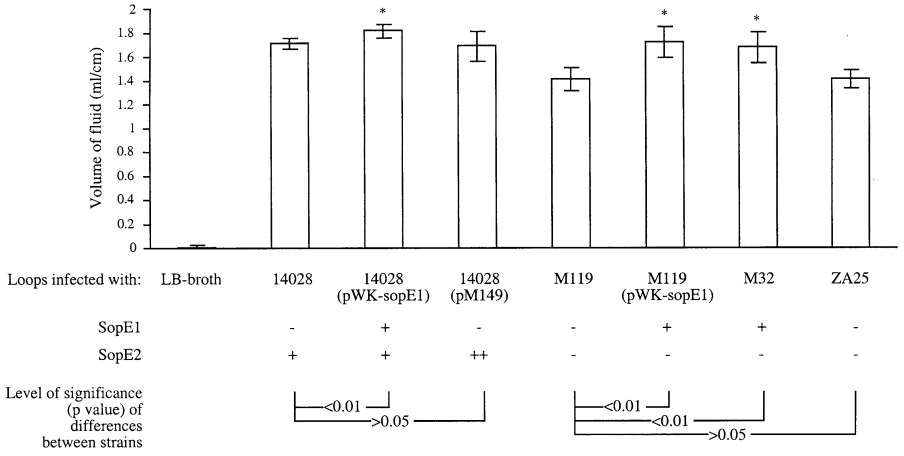

Fig. 2. Fluid accumulation at 8 h post infection of bovine ligated ileal loops with *S.* Typhimurium strains. Data are presented as averages from six loops (three loops/animal) ± standard error. A Student's *t*-test was performed to assess whether the difference in fluid accumulation between strains ATCC14028 (wild-type) and its derivatives or between strain M119 and its derivatives was statistically significant (*) and the level of significance (*P*-value) is given below the graph. The presence or absence of SopE1 and SopE2 in strains used in this study is indicated by + or −, respectively.

plasmid pWK-sopE1 (*sopE1*) was transformed into strain M119 (*sopE2*::tet^{R}) and virulence of the resulting strain compared with that of strains M32 (*sopE2*::tet^{R} SopEΦ^{aphT}), ZA25 (*sopE2*::tet^{R} SopEΦ$^{sopE::aphT}$) and its isogenic parent (M119) in the ligated ileal loop assay (Fig. 2). Introduction of the cloned *sopE1* gene (pWK-sopE1) or lysogenic conversion of strain M119 (*sopE2*::tet^{R}) with the SopEΦ phage (M32) resulted in a significant increase in fluid accumulation ($P < 0.01$) (Fig. 2). In contrast, lysogenic conversion of strain M119 (*sopE2*::tet^{R}) with a SopEΦ phage derivative which carried a deletion of the *sopE1* gene (SopEΦ$^{sopE1::aphT}$) did not significantly alter fluid accumulation ($P > 0.1$). Furthermore, strain M32 (*sopE2*::tet^{R} SopEΦ^{aphT}) caused significantly more fluid accumulation than strain ZA25 (*sopE2*::tet^{R} SopEΦ$^{sopE::aphT}$) ($P < 0.01$) (Fig. 2). These data supported the idea that the increase in fluid accumulation observed for strains M32 (*sopE2*::tet^{R} SopEΦ^{aphT}) and M119(pSW-sopE1) was due to introduction of the *sopE1* gene.

To further assess the roles of *sopE1* and *sopE2* during enteropathogenesis, we constructed ATCC14028 derivatives which either carried the cloned *sopE1* gene (pWK-sopE1) or the cloned *sopE2* gene (pM149) and compared the virulence of these strains with that of their parent using the ligated ileal loop assay as described above. Introduction of the cloned *sopE1* gene (pWK-sopE1) into strain 14028 resulted in a small but significant increase in secretory response ($P < 0.01$) (Fig. 2), thereby further supporting its role in enteropathogenesis. Interestingly, introduction of a plasmid encoded copy of *sopE2* (PM149) into a strain which carries an intact chromosomal copy of this gene (14028) did not lead to an increased secretory response ($P > 0.1$) (Fig. 2). Thus, the increase in enteropathogenicity observed in a strain carrying both *sopE1* and *sopE2* (M30) (Fig. 1) is not likely to be the result of an increased copy number resulting from the introduction of a functionally redundant gene.

In summary, these data provided direct evidence that lateral transfer of the *sopE1* gene by lysogenic conversion of a wild-type *S.* Typhimurium strain with the SopEΦ increases enteropathogenicity in the calf. The role of SopE1 in enteropathogenicity may be the stimulation of pro-inflammatory cytokine production by activating Rho GTPase signaling cascades, thereby contributing to fluid accumulation by an inflammatory mechanism [19]. The presence of the SopEΦ in epidemic cattle-associated phage types, including DT204, DT204c and DT49 [12], and its effect on enteropathogenicity in the calf (Figs. 1 and 2) suggest that phage mediated transfer of type III effector genes may be among the mechanisms involved in the epidemiological success of *S.* Typhimurium clones. Although lysogenic conversion with the SopEΦ mediates only a small increase in enteropathogenicity, it is likely that even a small increase in transmissibility of an *S.* Typhimurium clone may translate into an increased prevalence among cattle populations over time. Lysogenic conversion with the SopEΦ may, however, may not be the only factor involved in the emergence of epidemic phage types since the *S.* Typhimurium clone currently dominating among cattle, DT104, does not harbor this phage [12]. The epidemiological success of DT104 may instead be related to its chromosomal encoded multiple antibiotic resistance [17].

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