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

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Abstract

Epidemiologic 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.

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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 celf 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
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 the gene encoding a nucleotide exchange factor only for CDC42 but not for Rac-1 modestly [6,15]. The modest invasion defect of an S. Typhimurium slrP mutant was let was acquired by an ancestral genetic element [9]. It is thus likely that the slrP gene is present in epidemic bovine-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]. SopEΦaphT and SopEΦsopE2::tetR 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::tetR) by lysogenic conversion with SopEΦsopE1::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 × 10⁸ 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 (Propofoil, Abbot Laboratories, Chicago, IL, USA) followed by placement of an endotracheal tube and maintenance with Isoflurane (Isofox, Abbot Laboratories, Chicago, IL, USA). A laparotomy was performed through a midline incision. 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.
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 loopswere infected by intraluminal injection of 3 ml of sterile LB broth or a bacterial suspension. The loopswere 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. Tissuesamples 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 para/fin, 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, SopEφaphT) 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, SopEφsopE1::aphT) did not significantly alter fluid accumulation (P > 0.1). Inactivation of the sopE2 gene (M119, sopE2::tetR) 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, SopEφaphT) (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 (SopEφaphT) did not differ significantly from that caused by strain M31 (SopEφsopE1::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,

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**Table 1**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>14028</td>
<td>wild-type, bovine isolate</td>
<td>ATCC</td>
</tr>
<tr>
<td>M119</td>
<td>14028, sopE2::tetR</td>
<td>[19] Hardt Laboratory</td>
</tr>
<tr>
<td>M30</td>
<td>14028, SopEφaphT</td>
<td>Hardt Laboratory</td>
</tr>
<tr>
<td>M31</td>
<td>14028, SopEφsopE1::aphT</td>
<td>Hardt Laboratory</td>
</tr>
<tr>
<td>M32</td>
<td>14028, sopE2::tetR SopEφ</td>
<td>Hardt Laboratory</td>
</tr>
<tr>
<td>M32</td>
<td>14028, sopE2::tetR SopEφ</td>
<td>this study</td>
</tr>
</tbody>
</table>

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**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.
plasmid pWK-sopE1 (sopE1) was transformed into strain M119 (sopE2::tetR) and virulence of the resulting strain compared with that of strains M32 (sopE2::tetR SopEαphT), ZA25 (sopE2::tetR SopEαophE::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::tetR) with the SopEα phage (M32) resulted in a significant increase in fluid accumulation (P < 0.01) (Fig. 2). In contrast, lysogenic conversion of strain M119 (sopE2::tetR) with a SopEα phage derivative which carried a deletion of the sopE1 gene (SopEαophE::aphT) did not significantly alter fluid accumulation (P > 0.1). Furthermore, strain M32 (sopE2::tetR SopEαphT) caused significantly more fluid accumulation than strain ZA25 (sopE2::tetR SopEαophE::aphT) (P < 0.01) (Fig. 2). These data supported the idea that the increase in fluid accumulation observed for strains M32 (sopE2::tetR SopEαphT) 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 SopEα 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 SopEα 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 SopEα 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 SopEα may, however, 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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