REPORT

INFECTION

Inflammation boosts bacteriophage transfer between *Salmonella* spp.

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Bacteriophage transfer (lysogenic conversion) promotes bacterial virulence evolution. There is limited understanding of the factors that determine lysogenic conversion dynamics within infected hosts. A murine *Salmonella* Typhimurium (S.Tm) diarrhea model was used to study the transfer of SopEΦ, a prophage from *S.*Tm SL1344, to *S.*Tm ATCC14028S. Gut inflammation and enteric disease triggered >55% lysogenic conversion of ATCC14028S within 3 days. Without inflammation, SopEΦ transfer was reduced by up to 10^5-fold. This was because inflammation (e.g., reactive oxygen species, reactive nitrogen species, hypochlorite) triggers the bacterial SOS response, boosts expression of the phage antirepressor Tum, and thereby promotes free phage production and subsequent transfer. Mucosal vaccination prevented a dense intestinal *S.*Tm population from inducing inflammation and consequently abolished SopEΦ transfer. Vaccination may be a general strategy for blocking pathogen evolution that requires disease-driven transfer of temperate bacteriophages.

Bacteriophages (phages) often encode virulence factors and are important drivers of bacterial pathogen evolution (*1*, *2*). This also holds true for pathogenic enterobacteriaceae, such as *Salmonella enterica* Typhimurium (*S.*Tm). *S.*Tm genomes typically harbor several prophages (*1*). Phage transfer (lysogenic conversion) is a key driver of genomic diversification between closely related enterobacteriaceae and is thought to allow rapid adaptation to new host species, notably by reassorting the virulence factor repertoire (*1*). Phage transfer has been studied extensively in vitro. This has established its molecular basis and delivered numerous tools for molecular biology. In hosts infected by *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, or *Streptococcus pyogenes*, the reactivation or transfer of phages has also been observed (*3*–*8*). However, we have limited information about the factors controlling phage-transfer dynamics in vivo.

To address this knowledge gap, we characterized SopEΦ transfer between two well-established *S.*Tm strains (SL1344 and ATCC14028S) commonly used in virulence studies in mice (fig. S1, A and B). SopEΦ belongs to the P2 family of temperate phages (*9*, *10*). Its tail-fiber region carries a gene encoding SopE, a virulence factor enhancing *S.* Tm's host cell invasion and exacerbating enteropathy (*11*, *12*). Lysogenic conversion by *sopE*-encoding phages has been observed during epidemic strain evolution (*13*), and a SopEΦ lysogen of ATCC14028S elicits stronger enteropathy in the bovine gut than does the isogenic parental strain (*14*). On the basis of these observations, it has been speculated that phage-mediated *sopE* transfer may promote expansion of epidemic *Salmonella* strains (*1*, *13*). However, so far, nothing is known about the pace of SopEΦ transfer in vivo and the factors that could influence its dynamics.

To study SopEΦ-transfer dynamics in vivo, we used SL1344 as the phage donor. In SL1344, the SopEΦ prophage is inserted at the 3′ end of the *ssrA* gene (*10*). Specifically, we used a variant of this strain, in which SopEΦ carried a kanamycin resistance cassette [*aphT*; SL1344 (SopEΦ^{aphT}); Fig. 1A, fig. S1A, and table S1]. This allows quantification of lysogenic conversion by selective plating. *S.*Tm ATCC14028S (14028S) was chosen as recipient because it does not carry any bacteriophages at the integration site (*attB*) targeted by the SopEΦ integrase (Fig. 1A and fig. S1A). Moreover, 14028S colonizes the gut lumen of mice as efficiently as does SL1344.

In vivo phage-transfer experiments were initiated by orogastric infection of mice first with 200 colony-forming units (CFU) of the donor [SL1344 (SopEΦ^{aphT}); kan^R; amp^R; table S1] and immediately thereafter with 200 CFU of the recipient (14028S; *marT*::*cat*; cm^R, amp^R; table S1) (*15*). Washing, dilution, and sequential inoculation ensured that phage transfer could only occur in vivo. Donors and recipients colonized the gut lumen with comparable efficiencies [mean competitive index $CI_{donor\ vs\ recipient}$ day 1 postinfection (p.i.) = −0.10 ± 0.13; not significantly different from 0 (*t* test, $P = 0.475$)] (Fig. 1B), and all mice developed pronounced intestinal inflammation (Fig. 1C). Phage transfer [i.e., recipient lysogens; denoted as 14028S (SopEΦ^{aphT}); Fig. 1A] was detected as early as 24 hours p.i. and verified by multiplex polymerase chain reaction (PCR) (Fig. 1B, fig. S1B, and table S2). Recipient lysogen frequencies continued to rise, and 14028S (SopEΦ^{aphT}) outnumbered the parental recipient in half of the animals by day 3 p.i. (Fig. 1B). Equivalent results were obtained in a second mouse strain (129 SvEv; *Nramp1*$^+$), which could be infected for longer periods (fig. S1, C and D). Here, 14028S (SopEΦ^{aphT}) could completely replace the recipient population by day 15 p.i. (fig. S1C). Thus, phage transfer proceeds independently of the mouse line used.

Competitive infection experiments verified that recipient lysogens arose from frequent lysogenic conversion events and not from increased fitness of the lysogens. To address if 14028S (SopEΦ^{aphT}) might outcompete the ancestral recipient strain 14028S, we "locked" the prophage in the recipient lysogen. Deleting *attR* [14028S (SopEΦ$^{aphT\Delta attR}$); fig. S1A] prohibited prophage excision and de novo phage transfer. Both strains remained equally represented through days 1 to 3 p.i. (Fig. 1D). Thus, the presence of SopEΦ did not provide a detectable benefit to lysogens.

To address whether there is any potential cost associated with SopEΦ excision, we performed competitions between 14028S SopEΦ^{aphT} (excisable prophage) and 14028S (SopEΦ$^{aphT\Delta attR}$) (locked prophage; resistant to superinfection by SopEΦ^{aphT}). The final CI at day 3 p.i. did not significantly deviate from 0, excluding significant effects of excision on the overall fitness of the lysogen population (Fig. 1E). Finally, bar-coded recipient strain mixtures confirmed high numbers of independent phage-transfer events (fig. S2, A and B). These observations highlight the efficiency of phage-mediated horizontal gene transfer in the intestinal microenvironment and permitted us to further identify factors affecting phage-transfer dynamics in vivo.

On the basis of established in vitro data, we reasoned that prophage activation (lytic induction) might be of key importance. In vitro, most prophages are activated by the stress-induced SOS response of the host bacterium (*16*), and this can increase phage release and transfer to new recipient bacteria. Much less is known about prophage activation in vivo. Previous work had shown that *S.*Tm induces antimicrobial peptide secretion and recruits granulocytes into the gut lumen, which kills a substantial fraction of the *S.*Tm cells (*17*, *18*). Thus, we hypothesized that damage inflicted by the mucosal innate immune defenses may stimulate *S.*Tm's SOS response and thereby trigger lytic induction and subsequent transfer of SopEΦ. This led to three testable predictions: (i) Disrupting the signaling from SOS response to SopEΦ's lytic control should diminish phage transfer; (ii) *S.*Tm mutants failing to elicit gut inflammation should show reduced rates of phage transfer; and (iii) interventions preventing enteropathy should prevent phage transfer.

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To test our first hypothesis, we used a transcriptional reporter and a SopEΦ derivative that is "blind" for the bacterial SOS response. The SopEΦ-encoded *tum* gene was chosen to design the transcriptional reporter. This gene encodes the Tum antirepressor that links the bacterial SOS response to the lytic induction of the prophage (fig. S3A) (*19*–*21*). Indeed, mitomycin C, a well-known inducer of DNA damage, the SOS response, and prophage lytic cycles, enhanced not only phage transfer (fig. S3, B and C) but also *gfp* (green fluorescent protein) expression by the *tum*-reporter plasmid in vitro (pP*tum*GFP; fig. S3, D to F). Moreover, in vitro phage transfer was increased in the presence of sublethal concentrations of SOS-inducing stressors elicited by inflammatory immune defenses (i.e., reactive nitrogen species, reactive oxygen species, or hypochlorite; fig. S3, B and C).

To assess the relevance of *tum* induction for phage transfer in vivo, we constructed a SopEΦ variant that carries a *tum* deletion [SL1344 (SopEΦ$^{aphT\Delta tum}$); fig. S1A and table S1]. In in vivo phage-transfer experiments using SL1344 (SopEΦ$^{aphT\Delta tum}$) as the donor and 14028S as recipient, both strains colonized the gut; the infection triggered pronounced gut inflammation, but no phage transfer was detectable in any of the mice tested (Fig. 2, A and B; for *tum* complementation, see fig. S4). Thus, Tum controls SopEΦ transfer in vivo.

To test the impact of inflammation on phage-transfer dynamics, we constructed isogenic, avirulent variants of the donor and the recipient strains by disrupting the type III secretion systems 1 and 2 (T1 and T2) [Δ*invG* Δ*sseD*; SL1344^{avir} (SopEΦ^{aphT}); 14028S^{avir}; table S1]. Such T1/T2 double mutants cannot trigger inflammation but normally colonize the mouse gut lumen for at least 3 days (*22*). In sequential infections as described above, both strains efficiently colonized the intestinal lumen (Fig. 3A; open blue and open green symbols). However, in contrast to virulent strains, the mice did not develop gut inflammation (median pathological score of 1 versus 9, Fig. 3B; compare open and closed symbols) and yielded significantly fewer recipient lysogens [14028S^{avir} (SopEΦ^{aphT}); ≈10^3 per gram of feces versus up to 10^9 per gram of feces; Fig. 3A, compare open and closed red symbols].

Further experiments established that *tum* is also expressed in the inflamed gut (Fig. 3, C and D). GFP-positive bacteria were only detected if mice were infected with *S*.Tm strains eliciting gut inflammation [i.e., SL1344 (SopEΦ^{aphT}) and 14028S carrying pP*tum*GFP; Fig. 3, C and D]. No GFP was detected in mice infected with avirulent *S*.Tm mutants that fail to elicit disease [i.e., Δ*invG* Δ*sseD* derivatives of SL1344 (SopEΦ^{aphT}) and 14028S carrying pP*tum*GFP; >10^3 bacteria examined]. These observations provide evidence that SopEΦ's lytic cycle is induced in response to intestinal inflammation.

To substantiate this finding, we tested if reestablishing gut inflammation would rescue phage transfer by replacing either the donor or the recipient with the original virulent strain. Indeed, the sequential infection with either the avirulent

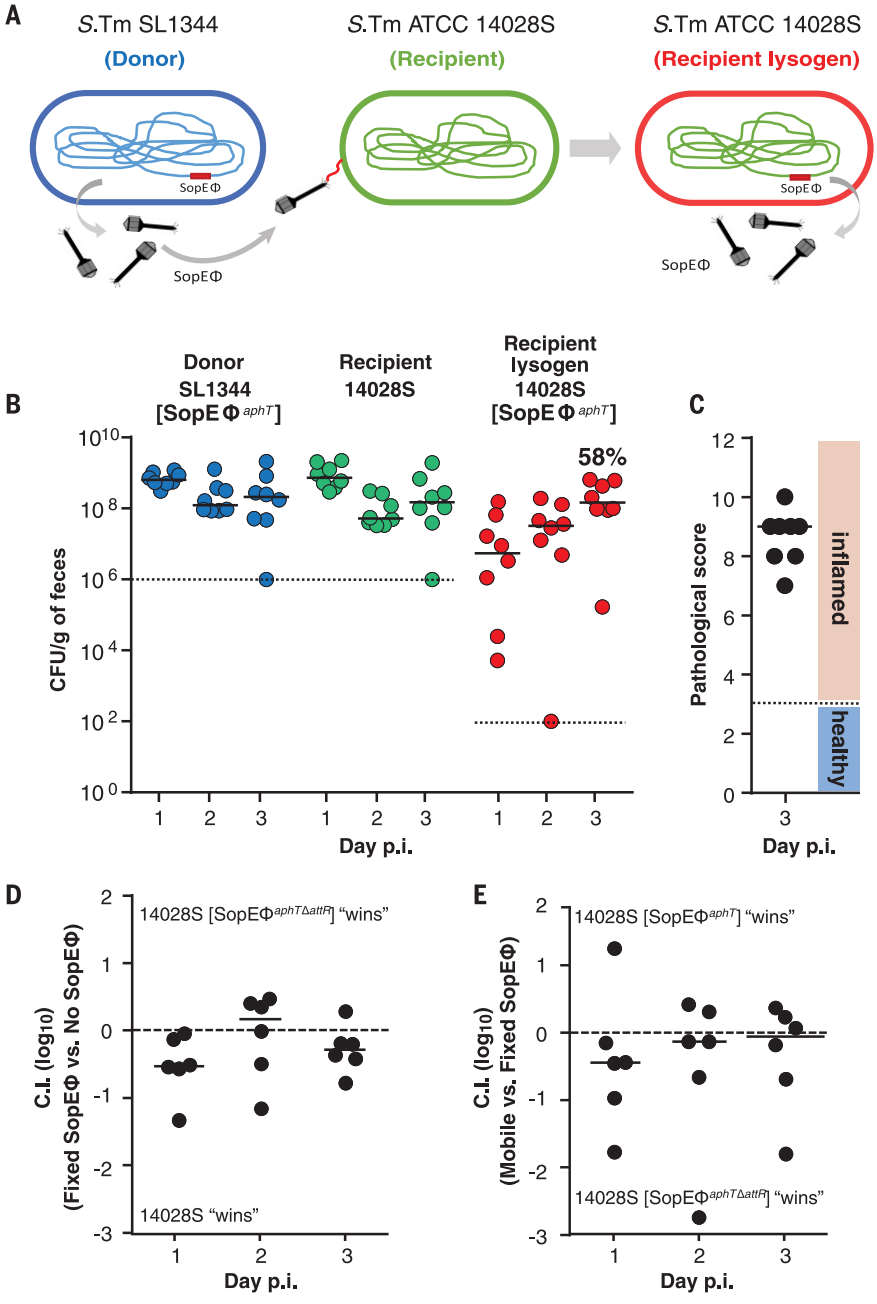

Fig. 1. In vivo, the bacteriophage SopEΦ is efficiently transferred between two *S*.Tm strains. (**A**) Phage-transfer experiment. SopEΦ^{aphT} is released from the donor strain SL1344 and infects the recipient 14028S to yield the recipient lysogen 14028S (SopEΦ^{aphT}) ("lysogenic conversion"). (**B**) *S*.Tm populations, as determined by plating of feces from mice that were sequentially infected with the donor [SL1344 (SopEΦ^{aphT}); kan^R; amp^R; table S1] and the recipient (14028S; *marT*::*cat*; cm^R, amp^R; table S1). Dotted lines: detection limits of the selective plating procedure. Fifty-eight percent of 14028S bacteria were lysogens by day 3 p.i. (median value) (n = 8; three independent experiments). (**C**) Gut inflammation at day 3 p.i., determined by scoring tissue sections as described in (*15*). (**D**) Control experiment 1: Competitive infection with a 1:1 mixture of 14028S (SopEΦ$^{aphT\Delta attR}$) (kan^R; amp^R) and 14028S (*marT*::*cat*; cm^R, amp^R; table S1) indicated that the phage did not confer a growth advantage (n = 6; two independent experiments). (**E**) Control experiment 2: Competitive infection with a 1:1 mixture of 14028S (SopEΦ$^{aphT\Delta attR}$) and 14028S (SopEΦ^{aphT}) (*marT*::*cat*; cm^R, kan^R, amp^R) indicated that prophage excision conferred no detectable fitness cost to the recipient lysogen (n = 6; two independent experiments). (D and E) dashed lines: CI = 0, both strains are equally represented (*15*). No significant deviation of the mean from 0 at day 3 p.i. (t test, P > 0.05). In all experiments, strains were inoculated by sequential gavage with 200 CFU each.

donor [SL1344^{avir} (SopEΦ^{aphT})] plus the virulent recipient (14028S) or vice versa not only elicited pronounced gut inflammation as previously shown in (*23*) but also promoted phage transfer {up to 10^9 per gram of recipient lysogens [14028S (SopEΦ^{aphT}) or 14028S^{avir} (SopEΦ^{aphT})]; fig. S5, A and B}. These data verified that intestinal inflammation was required for efficient phage transfer.

We also asked if a particular arm of the host's inflammatory response would be the main stimulus of phage transfer. However, mice with a NO synthase– and NADPH (reduced nicotinamide adenine dinucleotide phosphate) oxidase–deficient immune system (reduced reactive oxygen species, nitrogen species, and hypochlorite) and myeloperoxidase-deficient mice (no hypochlorite production) displayed intense intestinal inflammation and the same high levels of phage transfer as wild-type control mice (fig. S6). In combination with the in vitro data demonstrating that these are sufficient (fig. S3, B and C), this suggests that phage transfer is boosted in vivo by SOS-inducing compounds, including, but not restricted to, reactive oxygen species, nitrogen species, and hypochlorite.

Finally, we tested whether interventions preventing inflammatory disease would reduce SopEΦ transfer in vivo. For this, we used an inactivated oral vaccination protocol that induces high titers of *S*.Tm O-antigen–specific immunoglobulin A (IgA) (*24*). This vaccine protects efficiently from tissue invasion and intestinal inflammation, without affecting the total luminal loads of *S*.Tm in the antibiotic pretreated intestine (*24*). This protocol should therefore provide an ideal assay system to examine whether vaccination can limit inflammation-dependent phage transfer.

Vaccinated mice or mock-vaccinated "naïve" controls were sequentially infected with the donor [SL1344 (SopEΦ^{aphT}); table S1] and the recipient (14028S *marT*::*cat*; table S1). Both strains efficiently colonized the intestinal lumen of naïve and of vaccinated mice (Fig. 4A; closed and open, blue and green symbols). In sharp contrast to the naïve controls, the vaccinated mice were not only protected from disease for up to 3 days (Fig. 4B) but also yielded much lower numbers of recipient lysogens [14028S (SopEΦ^{aphT}); Fig. 4A, compare closed and open red symbols]. In half of the mice, recipient lysogens remained below the detection limit throughout the entire experiment. There was a correlation between vaccinated mice developing some degree of mucosal inflammation by day 3 p.i. and the presence of recipient lysogens (Fig. 4, A and B; marked in gray). These data verified that gut inflammation controls phage-transfer

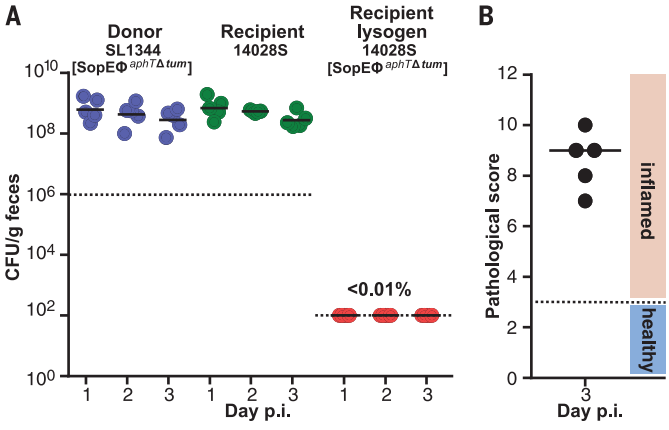

Fig. 2. Tum controls phage transfer in vivo. (**A**) *S*.Tm populations, as determined by plating of feces from mice that were sequentially infected with the donor [SL1344 (SopEΦ^{aphTΔtum}); kan^R; amp^R; table S1] and the recipient (14028S; *marT*::*cat*; cm^R, amp^R; table S1; 200 CFU each, by gavage). Less than 0.01% of 14028S bacteria were lysogens by day 3 p.i. (median value) (n = 5 mice from two independent experiments). Dotted lines: detection limits of the selective plating procedure. (**B**) Gut inflammation at day 3 p.i., as determined by scoring tissue sections (*15*).

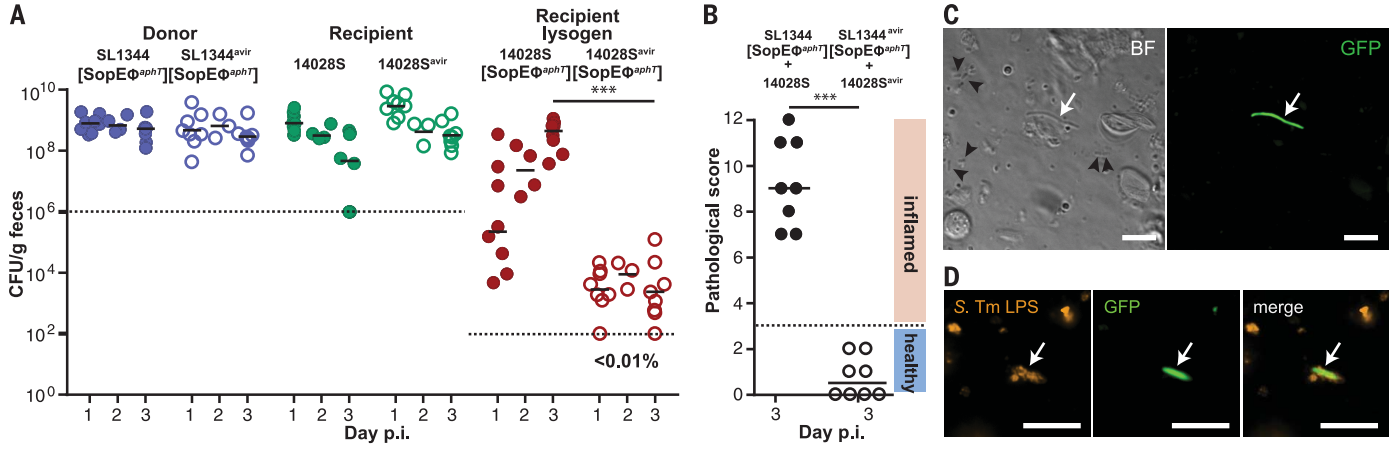

Fig. 3. Reduced phage transfer in the absence of intestinal inflammation. (**A**) *S*.Tm populations, as determined by plating of feces from mice that were sequentially infected with the avirulent donor [open blue symbols; SL1344^{avir} (SopEΦ^{aphT}); kan^R; amp^R; table S1] and the avirulent recipient (open green symbols; 14028S^{avir}; *marT*::*cat*; cm^R, amp^R) (n = 8 mice; three independent experiments) or (as control) with the virulent donor [closed blue symbols; SL1344 (SopEΦ^{aphT}); kan^R; amp^R; table S1] and the virulent recipient (closed green symbols; 14028S; *marT*::*cat*; cm^R, amp^R; table S1; 200 CFU each, by gavage) (n = 8 mice; three independent experiments). Red symbols: fecal loads of recipient lysogens [open symbols: 14028S^{avir} (SopEΦ^{aphT}); closed symbols: 14028S (SopEΦ^{aphT})]. Less than 0.01% of 14028S^{avir} bacteria were lysogens by day 3 p.i. (median value). Dotted lines: detection limits of the selective plating procedure. (**B**) Gut inflammation at day 3 p.i. (*15*). (A and B) ***Mann Whitney U-test, $P < 0.001$. (**C** and **D**) The P*tum* controlled *gfp* expression in the gut contents from mice infected for 24 hours with SL1344 (SopEΦ^{aphT}) pP*tum*::*gfp* (200 CFU by gavage) was analyzed by light microscopy. (C) Left panel: bright field (BF); right panel: GFP; white arrow indicates a filamenting GFP-positive bacterium, black arrowheads indicate *Salmonella*-shaped bacteria negative for GFP. (D) Left panel: anti–*S*.Tm LPS antibody staining; middle panel: GFP; right panel: overlay. Arrow indicates the GFP-positive bacterium counterstained with antibodies against lipopolysaccharide (LPS). Scale bars, 10 μm.

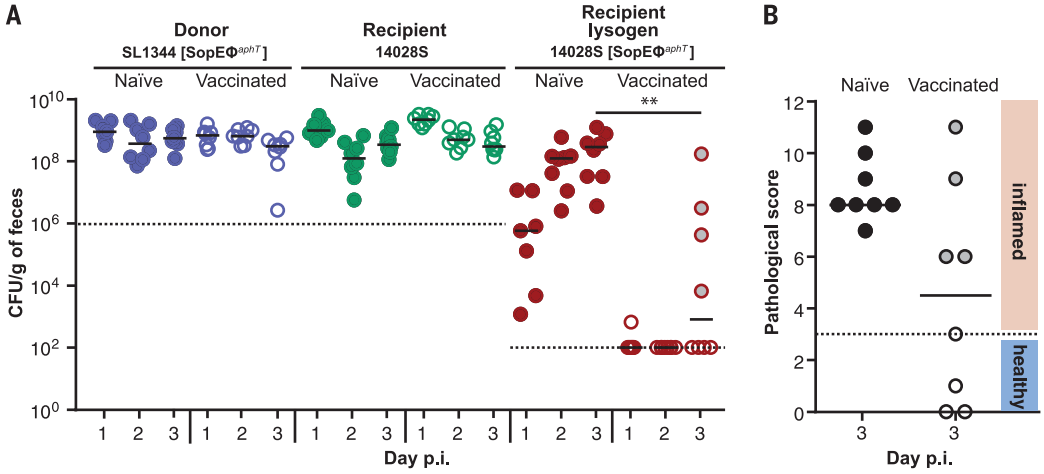

Fig. 4. Vaccination prevents gut inflammation and phage transfer. For vaccination, mice were treated 4 weeks before infection with gavages of peracetic acid–killed *S*.Tm, whereas naïve mice received phosphate-buffered saline (material and methods). (**A**) *S*.Tm populations, as determined by plating of feces from naïve (closed symbols; $n = 8$, three independent experiments) and vaccinated (open symbols; $n = 8$, three independent experiments) mice that were sequentially infected with the donor [SL1344 (SopEΦ^{aphT}); kan^R; amp^R; table S1] and the recipient (14028S; *marT*::*cat*; cm^R, amp^R, table S1, 200 CFU each, by gavage). Note that some mice did not defecate at each sampling point. Dotted lines: detection limits of the selective plating procedure. (**B**) Gut inflammation at day 3 p.i. Open symbols highlighted with light gray: four vaccinated mice that featured detectable densities of recipient lysogens and mucosal inflammation (detected on tissue sections as described (*15*). **Mann Whitney test, $P < 0.01$.

kinetics and showed that vaccination not only prevented inflammatory disease but also reduced phage-mediated horizontal gene transfer. This was further supported by the increased densities of free phage particles in the lumen of the inflamed (but not the uninflamed) mouse gut (fig. S7).

Our results show that an important mechanism of microbial evolution—horizontal gene transfer by temperate phages—is promoted by gut inflammation (i.e., disease symptoms elicited by enteropathogenic bacteria). This is attributable to phage regulators, which link the bacterial SOS response that is elicited by gut inflammation to lytic induction and phage transfer. The process operates in addition to inflammation-associated bacterial blooms known to drive contact-dependent plasmid transfer (fig. S8). The direct requirement for inflammation implies that vaccination could reduce phage transfer and thereby can be used to slow down pathogen evolution.

From the pathogen's perspective, the elicitation of gut inflammation has two different effects. *S*.Tm growth is favored by the environmental conditions provided by the inflamed gut (*22*, *25*). However, as inflammation produces many stressors (*17*, *26*), it also stimulates the microbial SOS response, induces the lytic cycle, and boosts horizontal gene transfer and reassortment of virulence factors, but also raises the risk of death by lysis. From the perspective of the phage, the link between lytic induction and the environmental cues of the inflamed gut may represent an adaptation by amplifying phage copy numbers and increasing chances to reach novel bacterial hosts. It is also tempting to speculate that the disease-accelerated phage transfer may explain why enteropathogenic bacteria such as *Salmonella* spp., *Vibrio cholerae* (*27*), or Shiga-toxin–producing *E. coli* strains (*28*) harbor so many prophages (*1*). It would be interesting to determine if such disease-driven spread of temperate phages might also affect the microbiota, e.g., by accelerating the emergence of strains with increased virulence in patients suffering from inflammatory bowel diseases or AIDS (*29*, *30*).

In conclusion, phage transfer is a dynamic process occurring in vivo with variable frequencies. The host's immune response was identified as a key factor that can drastically affect its pace. Notably, the innate (proinflammatory) and the adaptive IgA response of the infected host have opposing effects. Whereas gut inflammation (elicited by the innate response of the naïve host) boosts phage transfer, IgA-mediated mucosal adaptive immunity slows it down. This highlights an unexpected advantage of vaccination over antibiotic therapy, which is known to stimulate phage transfer and generalized transduction (*31*). This important beneficial aspect should receive particular attention in future vaccination trials and might open the door to managing pathogen evolution, e.g., in farm animal reservoirs of zoonotic enteropathogens.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/355/6330/1211/suppl/DC1
Materials and Methods
Figs. S1 to S8
Tables S1 and S2
References (*32*–*45*)

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Editor's Summary

The parasite of my parasite is my friend?

Virulence factors of pathogenic bacteria can be swapped by means of bacterial viruses called phages. In turn, the pathogenic bacteria are under attack by the hosts' immune responses. Diard *et al.* discovered that SopE φ, a phage parasite of pathogenic *Salmonella* species, is encouraged to spread between bacteria by the mouse host's inflammatory responses. Conversely, mucosal vaccination against *Salmonella* reduced inflammatory responses and curbed the transfer of SopEφ to naïve bacteria.

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