Salmonella Typhimurium utilizes a T6SS-mediated antibacterial weapon to establish in the host gut

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Title: ***Salmonella*** **Typhimurium utilizes a T6SS-mediated antibacterial weapon to establish in the host gut**

Short title: Pathogen gut invasion using antibacterial weapon

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Abstract

The mammalian gastrointestinal tract is colonized by a high-density polymicrobial community where bacteria compete for niches and resources. One key competition strategy includes cell-contact-dependent mechanisms of interbacterial antagonism, such as type VI secretion system (T6SS), a multiprotein needle-like apparatus that injects effector proteins into both prokaryotic and/or eukaryotic target cells. However, the contribution of T6SS antibacterial activity during pathogen invasion of the gut has not been demonstrated. We report that successful establishment in the gut by the enteropathogenic bacterium *Salmonella enterica* serovar Typhimurium, requires a T6SS encoded within *Salmonella* Pathogenicity Island-6 (SPI-6). In an *in vitro* setting, we demonstrate that bile salts increase SPI-6 antibacterial activity and that *S*. Typhimurium kills commensal bacteria in a T6SS-dependent manner. Furthermore, we provide evidence that one of the two T6SS nanotube subunits, Hcp1, is required for killing *Klebsiella oxytoca in vitro,* and that this activity is mediated by the specific interaction of Hcp1 with the antibacterial amidase Tae4. Finally, we show that *K. oxytoca* is killed in the host gut in an Hcp1-dependent manner and that the T6SS antibacterial activity is essential for *Salmonella* to establish infection within the host gut. Our findings provide the first example of pathogen T6SS-dependent killing of commensal bacteria as a mechanism to successfully colonize the host gut.

Significance statement

Gram-negative bacteria use the type VI secretion system (T6SS) to deliver effectors into adjacent cells. *Salmonella* Typhimurium is an enteric pathogen that causes disease in millions of individuals each year. Its ability to infect the mammalian gut is a key factor that contributes to its virulence and transmission to new hosts. However, many of the details on how *Salmonella* successfully colonizes the gut and persists amongst members of the gut microbiota remain to be deciphered. In this work, we provide evidence that *Salmonella* uses an antibacterial weapon, the Type VI secretion system, to establish infection in the gut. In addition, our results suggest that *S.* Typhimurium selectively targets specific members of the microbiota in order to invade the gastrointestinal tract.

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Introduction

Infections by enteric microbial pathogens begin upon invasion of the intestinal tract where survival and replication is necessary for transmission to occur. This environment, however, is already colonized by a high-density population of commensals and other microorganisms that directly interact with the pathogen and modulate its colonization. Previous work has shown that the microbiota provides colonization resistance against pathogens through a myriad of roles involving host tissue development, physiology, and mucosal immunology (1, 2). This phenomenon is mediated by secretion of antimicrobial peptides, competition for nutrients, and immune modulation by specific phylogenetic groups (3-6). In addition, bacteria often exhibit direct antagonistic behavior towards each other in microbial communities by delivering antibacterial toxins into competitors (7).

To survive in a multispecies environment such as the gastrointestinal tract, bacterial pathogens have developed various strategies to compete with other species and acquire access to nutritional and spatial niches. For example, some bacteria exert long-range inhibitory effects by secreting diffusible molecules such as antibiotics, bacteriocins and H_2O_2 (8). Interestingly, previous studies have shown that one molecular mechanism mediating such behavior is the widely conserved type VI secretion system (T6SS) (9). Many sequenced genomes of Gram-negative bacteria encode a T6SS, which could be present in more than one copy (10, 11). The T6SS is widespread in Gram-negative bacteria with an overrepresentation in γ-*Proteobacteria*, particularly in *Enterobacteriaceae* (11, 12). T6SS are versatile systems that deliver toxins into either eukaryotic or prokaryotic cells, or both (10). For example, the *V. cholerae* Vas and the *P. aeruginosa* H2-T6SS target and kill bacteria, but also inject toxins into host cells to prevent phagocytosis or to facilitate invasion, respectively (13-18). The T6SS is a multi-protein machine that uses a contractile mechanism for toxin secretion (19). In short, the T6SS comprises a trans-envelope complex that docks a contractile tail composed of an inner tube, made of stacked Hcp protein hexamers, tipped by the VgrG syringe, and surrounded by a sheath, comprised of polymerized TssB and TssC subunits (20-22) . Sheath contraction provides the energy necessary for the injection of the toxins that are confined into the Hcp tube or bound to VgrG (23). After injection, the ClpV ATPase recycles the contracted sheath to permit a new assembly/injection step to occur (24).

Salmonella enterica serovar Typhimurium is a leading cause of human gastroenteritis worldwide and causes a typhoid-like disease in mice. As this pathogen is transmitted by the fecal-oral route, it spends a significant part of its life cycle within intestinal microbial communities. While absence of the microbiota allows the pathogen to multiply to high densities (25), a high-complexity microbiota facilitates *S.* Typhimurium clearance (26). These studies not only illustrate the critical role of the intestinal microbiota in modulating *Salmonella* infection, but suggest that *Salmonella* must modulate its interactions with the microbiota (27). Several reports have shown that acute inflammation triggered by *S.* Typhimurium modifies the gut bacterial community to facilitate pathogen colonization in a mouse model (28-32). However, it is currently unknown whether *Salmonella* can directly target commensal bacteria with an antibacterial activity.

The genome of *S.* Typhimurium encodes a T6SS within the *Salmonella* Pathogenicity Island 6 (SPI-6) locus that is well conserved among *S. enterica* serovars (33). In addition to the structural components of the T6SS, the SPI-6 locus encodes two Hcp subunits, Hcp1 and Hcp2 (33), as well as Tae4, an

antibacterial amidase with homologs in other bacteria (34) (Fig. S1). Tae4 induces bacterial lysis by cleaving the γ-D-glutamyl-L-*meso*-diaminopimelic acid amide bond of peptidoglycan, and is toxic when expressed in a laboratory strain of *E. coli* (34, 35). Furthering this idea, it has recently been reported that the SPI-6 T6SS is required for *S.* Typhimurium to outcompete a laboratory strain of *Escherichia coli in vitro* (36). However, the contribution of the T6SS anti-prokaryotic activity of *S.* Typhimurium during infection of the gut remains unknown.

In this work, we show that a SPI-6 deficient *S.* Typhimurium mutant is impaired in intestinal colonization. We show that SPI-6 mediated killing is magnified by the presence of bile salts, and is required for killing members of the gut microbiota *in vitro,* such as *Klebsiella oxytoca.* We observe that the SPI-6-mediated antibacterial activity against *K. oxytoca* requires the Hcp1 protein but is independent of Hcp2. We report that only Hcp1 binds to the Tae4 antibacterial toxin, suggesting that Hcp proteins select the effectors to be secreted. Additionally, we demonstrate that the antibacterial activity of SPI-6 against *K. oxytoca* occurs *in vivo* in the mouse gut and we provide evidence that Hcp1-mediated antibacterial activity is necessary for *Salmonella* establishment within the gut.

Results

S. Typhimurium SPI-6 is required for colonization in the mouse gut

Although the type VI secretion system has been proposed to shape bacterial communities *in vitro,* little is known about its role *in vivo*. To test whether the *S.* Typhimurium SPI-6-encoded T6SS plays a role in the colonization of the gastrointestinal tract, we orally infected 129X1/SvJ mice with either wild-type *S.* Typhimurium or an isogenic mutant lacking the entire SPI-6 locus, represented in Fig. S1 (Δ*SPI-6*). We have previously shown that the level of *S.* Typhimurium excreted in the feces is identical to the levels in the cecum and colon during the first 35 days post-infection (37). Thus, we quantitated the levels of wild-type and mutant bacteria excreted in the feces of individual mice over time. The levels of wild-type *S.* Typhimurium were significantly higher than the Δ*SPI-6* mutant at 5 days post-infection (Fig. 1A). In addition, we noted a striking difference in the ability of these strains to expand in the gut. While the levels of wild-type bacteria increased 100-fold during the first 18 days of infection, the Δ*SPI-6* mutant did not significantly expand (Fig. 1A). We next tested the role of SPI-6 in *S.* Typhimurium colonization of systemic tissues after oral infection. The levels of wild-type and SPI-6-deficient bacteria were similar in Peyer's patches (PP), mesenteric lymph nodes (MLN), spleen and liver at 7 days post-infection (Fig. 1B). Collectively, our results demonstrate that SPI-6 plays an important role in the colonization of the gut but not in systemic tissues. This raises the question of whether the T6SS encoded on SPI-6 is able to target commensal bacteria within the gut in order for *S.* Typhimurium to successfully establish itself in this niche.

S. Typhimurium T6SS kills microbiota members and is enhanced by bile salts *in vitro*

Previous studies have highlighted that T6SS gene clusters are tightly regulated (38-40). The *S.* Typhimurium SPI-6 T6SS is repressed by the DNA-binding protein H-NS and thus limits its antibacterial activity (36). However, H-NS is a global regulator that controls the expression of multiple secretion systems and iron acquisition systems. In addition, *S.* Typhimurium H-NS-deficient bacteria grow very slowly compared to wild-type bacteria and accumulate compensatory mutations over time. Thus, the pleiotropic effects that are associated with H-NS-deficient strains prohibit the use of the *hns* mutant for *in*

vivo and *ex vivo* experiments. This prompted us to identify signal molecules that activate S. Typhimurium T6SS encoded on SPI-6. A previous study demonstrated that the T6SS from *Vibrio cholerae*, an intestinal pathogen, is activated by bile salts (41). Since we found that SPI6 is important for S. Typhimurium to colonize the guts of mice, we tested whether bile salts increase the activity of *S.* Typhimurium SPI-6 T6SS. To test this we mixed either wild-type or Δ*clpV* (ATPase essential for T6SS function) bacterial strains with *E. coli* K-12 on plates containing bile salts. The presence of bile salts increases the ability of *S.* Typhimurium to outcompete *E. coli* K-12 *in vitro* in a T6SS-dependent manner (Fig. 2A).

In the mammalian gut, *S.* Typhimurium shares this niche with commensal bacteria from diverse genera, including *Proteobacteria* and *Bacteroidetes*. We first tested the ability of wild-type or SPI-6-deficient strains to kill representative commensals of the gut microbiota *in vitro*. Using the conditions described above, we show that *S.* Typhimurium kills *Klebsiella oxytoca* and *Klebsiella variicola* in a SPI-6-dependent manner, but not *Enterobacter cloacae* or the *Escherichia coli* JB2 mouse commensal strain (28) (Fig. 2B). Finally, *S.* Typhimurium did not outcompete gut commensals such as *Bacteriodes fragilis*, *Bifidobacterium longum*, *Parabacteriodes distasonis* and *Prevotella copri* when grown anaerobically on blood plates, a condition in which the SPI-6 T6SS is active as shown by *S.* Typhimurium killing of *K. oxytoca* (Fig. S2).

Hcp1, but not Hcp2, binds Tae4 and is required for inter-bacterial antagonism

T6SS effectors are delivered into target cells by a cargo mechanism using the Hcp hexamer or the VgrG/PAAR spike as a carrier (10, 23, 42-45). The SPI-6 T6SS encodes two distinct Hcp proteins, STM0276 and STM0279, hereafter called Hcp1 and Hcp2 respectively. To test the contribution of these two distinct Hcp proteins for the antibacterial activity, we engineered Δ*hcp1* and Δ*hcp2* mutant strains and performed *in vitro* competition assays against *K. oxytoca* (Fig. 3A). In addition, we constructed a strain deficient for the Tae4 muramidase, Δ*tae4*. The Δ*clpV*, Δ*hcp1* and Δ*tae4* mutant bacterial strains were attenuated in their ability to kill *K. oxytoca*. In contrast, the Δ*hcp2* mutant outcompeted *K. oxytoca* at levels comparable to the wild-type *S.* Typhimurium strain (Fig. 3A). The ability of the Δ*hcp1* and Δ*tae4* mutant strains to kill *K. oxytoca* was rescued by providing plasmid-borne wild-type copies of *hcp1* and *tae4* under an arabinose-inducible promoter, respectively (Fig. S3). Taken together, our data indicate that the Hcp1 and Tae4 proteins are specifically required for anti-bacterial antagonism against commensal *K. oxytoca*.

Silverman *et al*. previously reported that a subset of T6SS effector proteins including *Pseudomonas aeruginosa* Tse1, Tse2 and Tse3 bind the luminal side of the Hcp hexamer. This interaction between Hcp and the effector stabilizes the effector and allows for proper delivery upon sheath contraction (23). To test the interaction of Tae4 with the Hcp1 and Hcp2 proteins we performed a bacterial two-hybrid assay. Our data indicate that Tae4 binds to Hcp1, but does not interact with Hcp2 (Fig. 3B). In addition, the Tae4 and 6×His-tagged Hcp1 and Hcp2 proteins were purified to homogeneity and interactions were assessed by co-purification experiments. The biotinylated Tae4 protein co-purified with 6×His-tagged Hcp1 using immobilized metal affinity chromatography but did not precipitate with 6×His-Hcp2 (Fig. 3C). Collectively, these results show that the Tae4 effector binds specifically to Hcp1 and suggest that Hcp1 delivers this amidase effector into target cells, which is consistent with the role of Hcp1 in inter-bacterial antagonism.

The T6SS antibacterial activity of *S.* Typhimurium is required for early establishment within the host gut

Based on our *in vitro* findings, we hypothesized that *S.* Typhimurium outcompetes *K. oxytoca* within the host gut in a T6SS-dependent manner. To test this notion we utilized a mouse model to measure T6SS contact-dependent activity (9). However, *K. oxytoca* does not represent a large portion of the gut microbiota (Fig.S4A). Thus, we increased *K. oxytoca* levels in the guts of 129X1/SvJ mice by first treating the mice with a cocktail of antibiotics for two weeks to reduce the amount of indigenous commensal bacteria (Fig.S4A), followed by oral inoculation of mice with 10^8 *K. oxytoca*. After confirming *K. oxytoca* colonization by plating feces on MacConkey agar (Fig. S4A), we orally infected mice with 10^8 wild-type or Δ*hcp1 S.* Typhimurium. *K. oxytoca* and *S.* Typhimurium levels were enumerated in the cecum and colon of co-infected mice three days post infection. There was a dramatic decrease in the levels of *K. oxytoca* in the cecum (Fig. 4A) and the colon (Fig. S4B) of mice co-infected with wild-type *S.* Typhimurium. In contrast, the levels of *K. oxytoca* remained similar to input when the mice were co-infected with the Δ*hcp1* mutant bacteria. There were no significant differences in the levels of wild-type versus Δ*hcp1 S.* Typhimurium in the guts of the antibiotic-treated mice (Fig. 4A). We therefore conclude that the antibacterial activity of SPI-6 is active against *K. oxytoca* in the host gut.

To test whether Hcp1-mediated antibacterial activity mediates establishment of *Salmonella* within unperturbed guts, we orally infected 129X1/SvJ mice with WT, Δ*hcp1,* or Δ*hcp2* bacterial strains and measured CFU in feces over time. Although the levels of wild-type and T6SS mutant strains were similar for the first two days of infection, there were significantly lower levels of the Δ*hcp1* mutant bacterial strain compared to the wild-type strain 5 days post-infection (Fig. 4B). In contrast, the levels of the Δ*hcp2* mutant strain were not significantly different from wild-type levels (Fig. 4B).

Since the intestinal microbiota composition in 129X1/SvJ and C57BL/6 mice are different (46), we wondered whether SPI-6 T6SS is important for *S.* Typhimurium colonization in the gut of C57BL/6 mice. Since *S.* Typhimurium infection of C57BL/6 mice induces high levels of inflammation in the gut at the early time points that we were interested in, we performed a competition assay in which mice were infected with an equal mixture of *S.* Typhimurium wild-type strain and either an *hcp1* or *hcp2* mutant strain. Feces were collected one and two days post-infection, and cecum and colons were harvested on day three. Wild-type and mutant bacteria were enumerated in each sample by plating on regular and kanamycin-containing plates (to select for the mutant strain). One day post-infection, levels of both wild-type and mutant bacterial strains were similar in the feces (Fig. 4C). Two days post-infection, 10-fold lower levels of the Δ*hcp1* mutant bacteria were recovered compared to wild-type bacteria (Fig. 4C). Similarly, at 3 days post-infection, Δ*hcp1* mutant bacteria were outcompeted in the cecum and colon (Fig. 4D, E). In contrast, Δ*hcp2* mutant bacteria were present at comparable levels to the wild-type strain in the feces, cecum and colon (Fig. 4C). Collectively, these results suggest that Hcp1-dependent antibacterial activity is essential for successful colonization of the gut.

From these results, we conclude that the Hcp1-mediated antibacterial activity of the SPI-6 T6SS confers a competitive advantage to *S.* Typhimurium against the gut microbiota and facilitates *S.* Typhimurium establishment in multi-species communities. Since these results could be due to different bacterial burdens between our mutants, we decided to treat mice orally with a cocktail of antibiotics for two weeks, which has been previously shown to reduce the inherent colonization resistance against *S.* Typhimurium

mediated by the indigenous microbiota (47). After antibiotic treatment, we orally gavaged equivalent amounts of wild-type, Δ*hcp1*, Δ*hcp2* or Δ*tae4* mutant bacteria. Wild-type and mutant bacteria colonized the guts of the antibiotic-treated mice at high, comparable levels (Fig. S5). These results indicate that in the absence of an intact commensal microbial community, the antibacterial weapon of *S.* Typhimurium is not required to establish in the gut.

Discussion

In nature or within hosts, microbes often exist in complex communities, and hence they must compete with other species for limited resources. Many Gram-negative bacteria encode a molecular machine called the T6SS that is dedicated to target and kill other bacteria and sometimes to inject effector proteins into eukaryotic cells (10). For example, the human pathogen *Vibrio cholerae* employs the T6SS to kill *E. coli* or to disable the amoebae *Dictyostelium discoideum* and macrophage cell lines (14, 48, 49). Recent studies demonstrated that T6SS organelles in *V. cholerae*, *P. aeruginosa* and entero-aggregative *E. coli* cells are very dynamic and likely expel their T6SS spike/tube VgrG/Hcp complex towards prokaryotic cells and cause target cell lysis by delivering anti-bacterial toxins (50, 51). A broad diversity of anti-bacterial toxins has been described so far (10, 42). In this work, we show that the SPI-6 T6SS is required for efficient *Salmonella* establishment in the gut and that it is the antibacterial activity of the T6SS that contributes to this phenotype, suggesting a direct interaction between the pathogen and the indigenous microbiota. *In vitro* competition assays demonstrated a SPI-6 T6SS-dependent killing of members of the gut microbiota *in vitro*, which was confirmed by *in vivo* survival assays. Finally, we provide details on the molecular mechanism underlying SPI-6-mediated competition by showing that the Tae4 amidase interacts with Hcp1, facilitating its delivery and activity within prey bacterial cells. This is the first report demonstrating that the antibacterial activity of a T6SS is important for a pathogen to establish within the host gut, and opens new exciting perspectives.

In addition to the core set of 13 genes required for T6SS assembly and function, the *S.* Typhimurium SPI-6 locus contains two copies of the *hcp* gene, *hcp1* and *hcp2* (33). Hcp proteins assemble hexamers that stack on each other to form the T6SS tail tube (52, 53). In addition to be a structural component of the apparatus, Hcp hexamers are delivered into target cells and serve as chaperone and cargo for toxin effectors (23, 53). The *S.* Typhimurium Hcp1 and Hcp2 proteins share 94% identity and only differ by 10 residues (Fig. S6A). However, *S.* Typhimurium has evolved to specifically utilize the Hcp1 protein to target bacterial cells, as *hcp1* - but not *hcp2* - is required for bacterial killing. This specificity is conferred by the ability of Hcp1 to specifically bind the anti-bacterial Tae4 toxin (Fig. 3B, C). Based on electron micrographs, Silverman *et al*. recently proposed that effectors bind inside the lumen of Hcp tube (23). Intriguingly, molecular modeling of the Hcp1 and Hcp2 hexamers demonstrate that 4 out of the variable 10 residues, including residues 124 and 125, are predicted to face the lumen (Fig. S6B). These differences might explain the specificity of interaction with different effectors. Interestingly, Zhou and colleagues previously reported that Hcp1 and Hcp2 of *E. coli* K1 have differential roles in binding and invading human endothelial cells and actin cytoskeleton rearrangement, respectively, suggesting a similar mechanism of binding to different effectors (54). Therefore, this mechanism of specificity could be common among T6SS. It is also important to note that two variable residues, at position 115 and 116, located at the hexamer-hexamer interface. These differences might mediate specificity between Hcp interactions and prevent assembly of Hcp1/Hcp2 hetero-tubes. Future investigations will focus on the role of Hcp2 and on the identification of the putative effectors that it may bind.

Previous studies of the mechanisms of *S.* Typhimurium colonization of the mouse gut have shown that *Salmonella* exploits intestinal inflammation to compete with the resident microbiota and to thrive in the inflamed gut (28-32). All of these previous studies have been performed in mice that have been pre-treated with an antibiotic (e.g., streptomycin) that perturbs the indigenous microbiota. We have shown that in the absence of antibiotic pretreatment, *S.* Typhimurium utilizes its T6SS to colonize the gut within the first 2 to 5 days of infection. Furthermore, we observed the contribution of the antibacterial-specific factors at times in which we do not detect signs of inflammation based on histopathology, fecal cytokine levels and flow cytometry (55). Our results strongly suggest that an active Hcp1-dependent delivery of Tae4 directly targets the resident microbiota leading to an efficient establishment in the host gut. Surprisingly, this phenotype is seen in both 129X1/SvJ and C57BL/6 mice, although their respective commensal microbiota is profoundly different (46), suggesting a mechanism that has evolved to face a wide variety of bacteria in order to establish within the gut. Finally, the anti-bacterial action of the *S.* Typhimurium SPI-6 T6SS within the gut is supported by the observation that killing of bait bacteria is enhanced in the presence of bile salts (Fig. 2A), a result similar to that observed for mucin- and bile salts-activation of the *V. cholerae* T6SS within the mouse gut (41).

One can ask whether SPI-6 specifically delivers effectors to selected commensal bacteria. Amongst the strains tested, we identified two commensals that are killed in a SPI-6 dependent manner, *K. oxytoca* and *K. variicola*. In a recent report, it appears that *K. oxytoca* and *S.* Typhimurium are potentially metabolizing the same oxidized sugars within the gut post-antibiotic treatment (56). In this scenario, it would be advantageous for the pathogen to kill competitors of the same food. This would only work for some species that are sensitive to *Salmonella's* T6SS, however, as we found that *E. cloacae* was not killed by the SPI-6 attack. This result is in agreement with the fact that the genome of *E. cloacae* encodes a Tai4 immunity protein which has been shown to bind and inhibit *S.* Typhimurium Tae4 activity (35, 57). In our study we found that the ability of S. Typhimurium to kill *E. coli* was strain-dependent. We were surprised that, in contrast to the *E. coli* K-12 DH5α or W3110 laboratory strains (this study and (36)), the commensal *E. coli* JB2 strain was resistant to SPI-6 attack. This suggests that a defense mechanism may be present in the commensal strain, such as the presence of a T6SS gene cluster or cross-immunity via a Tai4 homologue. Alternatively, it was shown that resident commensal *E. coli* and *S.* Typhimurium may transiently cooperate in the gut during inflammation (31) suggesting that perhaps a secondary mechanism is at play. Although we have shown that *K. oxytoca* is one target of the *S.* Typhimurium T6SS *in vivo*, future studies are necessary to determine whether other members of the gut microbiota are also targeted via this secretion machinery *in vivo*.

Intriguingly, other gut pathogens also have a T6SS and it would be interesting to determine whether gut colonization is mediated through a potential anti-bacterial activity. For example, *Campylobacter jejuni*, *Helicobacter hepaticus*, and *V. cholerae* have a T6SS that plays a role in colonization of the host (58-60). Whether these T6SS encode an antibacterial activity has not been determined yet. Altogether, this new colonization strategy could be a common theme among Gram-negative gut pathogens and T6SS-mediated antibacterial interactions should be further studied in order to have a more comprehensive understanding of the interaction between the pathogen and the host in its entirety. The role of T6SS in host colonization is a new and exciting area of exploration. Recently, it has been shown that some members of the microbiota have a T6SS that is active against other bacteria (61). Moreover, it was reported that about half of the *Bacteroidales* genomes, the most prevalent Gram-negative bacterial order of the human gut, encode at least one T6SS (62). Finally, a recent report shows that the T6SS is important for *Bacteroides fragilis* to

outcompete other commensal bacteria *in vitro* and that this T6SS is active *in vivo*, suggesting a role in the gut colonization for this commensal (63).

In our work, we provide the first evidence of a pathogen using a T6SS against the microbiota, leading to establishment in the host gut. Therefore, this work provides strong evidence for considering a new important player for the battle in the gut that is directed by the T6SS, which facilitates a fine tuned killing mechanism that selectively kills competing members of the microbiota (e.g., *K. oxytoca*), but leaves cross-feeding bacterial members alive. In addition, members of the microbiota that possess a functional T6SS may implement a counter attack in this war against the pathogen. We could then imagine a T6SS-directed war in the gut between commensal microbiota and pathogenic bacteria. It is interesting to note that the *hcp1* mutant is not rescued by wild-type *S.* Typhimurium in the coinfection model (Fig. 4C). Even if the wild-type and mutant bacteria were occupying the same spatial niche, the *hcp1* mutant would still be at a competitive disadvantage if it is targeted by other commensal bacterial species that have a T6SS apparatus. In this scenario the *hcp1* mutant would not be able to counter-attack $T6SS^+$ commensals. Our results highlight the importance of T6SS-mediated antibacterial activity in host gut colonization. A deeper understanding of pathogen strategies in the battle to colonize the gut could lead to new therapeutic strategies.

Material and methods

Ethics statement. Experiments involving animals were performed in accordance with NIH guidelines, the Animal Welfare Act, and US federal law. All animal experiments were approved by the Stanford University Administrative Panel on Laboratory Animal Care (APLAC) and overseen by the Institutional Animal Care and Use Committee (IACUC) under Protocol ID 12826. Animals were housed in a centralized research animal facility certified by the Association of Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Mouse strains and husbandry. 129X1/SvJ and C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Male and female mice (5–8 weeks old) were housed under specific pathogen-free conditions in filter-top cages that were changed weekly by veterinary personnel. Sterile water and food were provided ad libitum. Mice were given 1 week to acclimate to the Stanford Research Animal Facility prior to experimentation. When specified, an antibiotic cocktail (ampicillin 1g/L, vancomycin 0.5 g/L, neomycin 1 g/L, and metronidazole 1g/L (AVNM)) was added in drinking water for two weeks to reduce microbiota concentration in the host gut, and was then removed 16 hours before mice infection.

Mouse infections. Food and antibiotics in drinking water were removed 16 hours prior to all mouse infections. Mice were infected via oral gavage with 10^8 CFU and 10^9 CFU in 100 µl PBS for 129X1/SvJ and C57BL/6 mice, respectively. In the coinfection model, mice were inoculated via oral gavage with an equal mixture of strains in 100 µl PB*S.* For *S.* Typhimurium and *K. oxytoca* co-infection experiments, mice were first treated with an antibiotic cocktail (AVNM) for 2 weeks. After two weeks, mice were switched to regular drinking water and fasted for 16 hours. After fasting, mice were inoculated with 10^8 *K. oxytoca* orally. After 8 hours of recovery, mice were fasted once again for 16 hours prior to oral inoculation with 10^8 *S.* Typhimurium. *K. oxytoca* was plated on MacConkey plates immediately prior to inoculation with *S.* Typhimurium to check colonization levels.

Bacterial strains and growth conditions. The *S.* Typhimurium strains used in this study were derived from the streptomycin-resistant parental strain SL1344. The different mutant strains were engineered by replacing the target gene with that of a kanamycin (or chloramphenicol)-resistance cassette using the one-step inactivation method (64). Genetic manipulations were originally made in the *S.* Typhimurium LT2 background before being transferred to SL1344 by P22 transduction. All deletions were constructed as described by Maier *et al*., with P22 phage transduction to insert the deleted genomic region into the wild-type strain (65) (Table S1). All constructs were verified by PCR. All *S.* Typhimurium strains were grown at 37°C with aeration in Luria-Bertani (LB) medium containing the appropriate antibiotics: streptomycin (200 μg/mL), kanamycin (40 μg/mL), and chloramphenicol (8 μg/mL). For mouse inoculation, an overnight culture of bacteria was spun down and washed with phosphate-buffered saline (PBS) before resuspension to obtain the desired concentration.
Escherichia coli JB2 commensal strain was kindly provided by M. Raffatellu (28). Commensal bacterial strains used in this study were isolated from wild-type C57Bl/6 and 129X1/SvJ mice housed at Stanford (see Table S1). Fecal pellets were streaked on BHI agar (BD Difco) plates supplemented with 5% defibrinated sheep's blood (Hemostat Laboratories), followed by aerobic incubation at 37°C for 24h. Individual colonies were picked, grown up in Luria broth and frozen at -80C in 10% glycerol. Individual isolates were characterized by 16S rDNA sequencing. Briefly, colonies were resuspended in 100ul PBS and boiled for 10min at 95°C. The 16S gene was amplified by PCR by using Phusion High-Fidelity DNA polymerase (Thermo/Fisher) and primers 63F (CAG GCC TAA CAC ATG CAA GTC) and 1387R (GCC CGG GAA CGT ATT CAC CG). PCR products were sequence using the 63F and 1387R primers and classified using the Michigan State University Ribosomal Database Project classifier function and NCBI BLAST program.

***S.* Typhimurium burden in tissues**. Following collection of fresh fecal pellets, animals were sacrificed at the specified time points. Animals were euthanized by cervical dislocation. Sterile dissection tools were used to isolate individual organs, which were weighed prior to homogenization. Visible PPs (3–6/mouse) were isolated from the small intestine using sterile fine-tip straight tweezers and scalpels. PP, mLN, spleens, and livers were collected in 1 ml PBS. The small intestine, cecum, and colon were collected in 3 ml PBS. Homogenates were then serially diluted and plated onto LB agar containing the appropriate antibiotics to enumerate colony-forming units (CFU) per g of tissue. For co-infections, several dilutions were plated to ensure adequate colonies (>100 CFU per sample) for subsequent patch plating to determine strain abundance.

Bacterial two-hybrid assay. The adenylate cyclase-based bacterial two-hybrid technique (66) was used as previously published (67). Briefly, pairs of proteins to be tested were fused to the isolated T18 and T25 catalytic domains of the *Bordetella* adenylate cyclase. Plasmids encoding protein fusion between the Hcp/Tae4 proteins and the T18 or T25 domain were obtained by restriction-free cloning as previously published (68) using pairs ECO2255/ECO2257 (T18-Hcp1), ECO2256/ECO2257 (T18-Hcp2) and ECO2253/ECO2254 (T25-Tae4). After transformation of the two plasmids producing the fusion proteins into the reporter BTH101 strain, plates were incubated at 30°C for 24 hours. Three independent colonies for each transformation were inoculated into 600 μL of LB medium supplemented with ampicillin, kanamycin and IPTG (0.5 mM). After overnight growth at 30°C, 10 μL of each culture were dropped onto LB plates supplemented with ampicillin, kanamycin, IPTG and Bromo-Chloro-Indolyl-

Galactopyrannoside (40 µg/ml) and incubated for 16 hours at 30 °C. The experiments were done at least in triplicate and a representative result is shown.

Hcp1, Hcp2 and Tae4 protein purification. Plasmids encoding the Hcp proteins fused to an N-terminal 6×His tag or the Tae4 protein fused to a 6×His-TRX-TEV tag were obtained by restriction-free cloning as previously published (68) using pairs ECO2217/ECO2219 (Hcp1), ECO2218/ECO2219 (Hcp2) and ECO2220/ECO2221 (Tae4). 6×His-tagged Hcp proteins were purified by ion metal affinity chromatography (IMAC) from 0.5 liter of culture of *E. coli* BL21(DE3) cells bearing the pRSF-1 plasmid derivatives grown at 37°C to an OD_{600}=0.6 and gene induction with IPTG (500 mM) for 18 hours at 16°C. Bacteria were harvested, resuspended to an OD_{600}=80 in buffer A (50 mM Tris-HCl pH 8.0, 150 mM NaCl) supplemented with EDTA (1 mM), lysozyme (100 µg/ml), DNase (100 µg/ml), $MgCl_2$ (10 mM) and protease inhibitors (Complete, Roche). Bacteria were broken using a Emulsiflex apparatus and the insoluble material was discarded by centrifugation for 30 min at 55,000 × *g*. All the subsequent purification steps were performed using an AKTA FPLC system. First, the soluble fraction was loaded into a 5-mL HisTrap column (GE Health Sciences). After extensive washing with a 0-20 mM-gradient of imidazole, the Hcp proteins were eluted using 500 mM imidazole in Buffer A. The pooled fractions were dialyzed overnight at 4°C on 3,500-Da pore membrane tubing (Spectra/Por, Spectrumlabs) in buffer A supplemented with imidazole 10 mM. The 6×His-TRX-TEV-tagged Tae4 protein was purified using an identical protocol except that purified 6×His-tagged Tobacco Etch Virus (TEV) protease and dithiotreitol (1.2 mM) were added in the membrane tubing during dialysis. The cleaved, untagged Tae4 protein was obtained in the flow-through of a second IMAC.

Complementation of *tae4* and *hcp1* To complement *tae4* and *hcp1*, both genes were individually amplified by PCR from *S*. Typhimurium (SL1344) genomic DNA using Phusion DNA polymerase (NEB). After digestion with the appropriate enzymes, the products were ligated to the plasmid pDiGc using T4 ligase (NEB). Following heat-shock transformation into competent cells, clones were screened by PCR using primers TSD107 along with either KL1 or KL3, for *hcp1* and *tae4* respectively. Positive clones inserts were sequenced using TSD107 and then electroporated in *S*. Typhimurium mutant strains. Competition was performed as described except 0.05% arabinose or 0.1% glucose added to respectively induce or repress the corresponding cloned gene. Empty pDiGc was used as a negative control.

Hcp/Tae4 co-purification assay. 200 µg of the purified Tae4 protein were diluted 10 times in PBS buffer and biotinylated using EZ-Link™ NHS-PEG4-Biotin (Thermo-Fischer Scientific), mixed with 100 µg of purified 6×His-tagged Hcp1 or Hcp2 protein and 60 µL of Pureproteome™ Nickel magnetic beads (Millipore). After incubation for one hour at 4°C on a wheel, the flow through was collected, the beads were washed three times with 8 volumes of buffer A, and eluted in buffer A supplemented with imidazole 0.5 M. The total, flow-through, 1st wash and elution samples were then resuspended in Laemmli buffer, boiled for 10 minutes and the proteins were resolved by SDS-PAGE. After transfer onto nitrocellulose, the Hcp and Tae4 proteins were detected using monoclonal anti-5×His antibody (QIAGEN) and streptavidin coupled to alkaline phosphatase (Molecular Probes, Life technologies) respectively.

Hcp1 and Hcp2 structure modeling. The homology models of Hcp1 and Hcp2 were built using C*oot* (69) according to a Multalin alignment with the closest homologue, the EAEC Hcp protein (PDB: 4HKH; (70)).

***In vitro* competition**. Overnight cultures of bacteria were spun down and washed with phosphate-buffered saline (PBS) before resuspension at 10^{10} bacteria/ml. 10µl of each resuspension were then spotted on a LB plate supplemented with 0.05% of porcine bile salts (Sigma-Aldrich) and grow at 37°C for 48 hours. Spots were serially diluted and plated on LB supplemented with the appropriate antibiotic, or MacConkey Agar (Fisher Scientific). Bait recovered is calculated as the ratio of total bait CFU divided by total *Salmonella* CFU recovered after scraping and counting on selective plates. For *in vitro* competition in anaerobic conditions, a similar protocol was followed with the exception that bacteria were grown at 37°C in an anaerobic chamber for 96 hours on blood plates (horse blood from Sigma-Aldrich at 10% in BHI agar medium).

Monitoring fecal shedding of *S.* Typhimurium. Individual mice were identified by distinct tail markings and tracked throughout the duration of infection. Between 2 to 3 fresh fecal pellets were collected directly into eppendorf tubes and weighed at the indicated time points. Pellets were resuspended in 500 ml PBS and CFU/g of feces were determined by plating serial dilutions on LB agar plates with the appropriate antibiotics.

Statistical analyses. Prism (GraphPad) was used to perform all statistical analyses. Differences in CFUs and strain composition between groups were examined by unpaired nonparametric Mann-Whitney tests. Differences for vitro competition were examined by Student t test. Differences in Competitive Index were examined by Dunnett tests. Significance was defined by $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) and $P<0.0001$ (****).

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Figure Legends

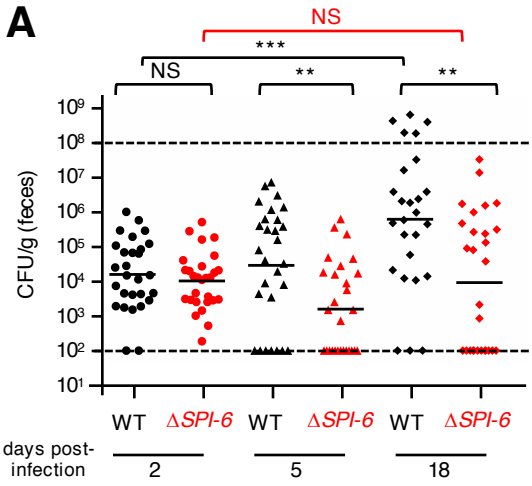
Figure 1. SPI-6 is important for colonization of the gut. (A) 129X1/SvJ Mice were infected orally with 10^8 wild-type (WT) or *ΔSPI-6* mutant *S.* Typhimurium bacterial strains. Feces were collected and plated over time on media that is selective for *Salmonella*. Every data point represents one mouse, and the bar represents the geometric mean. (B) 129X1/SvJ mice were orally infected with 10^8 wild-type or Δ*SPI-6 Salmonella* Typhimurium bacterial strains and tissues were harvested at day 7 post infection and plated on selective media. Data for mesenteric lymph nodes (MLN), Peyer's patches (PP), liver (L), and spleen (S) are shown. Statistical significance is shown based on Mann Whitney U test: NS, not significant; **, $P < 0.01$, ***, $P < 0.001$.

Figure 2. SPI-6 is activity is enhanced by bile salts and provides competitive advantage against members of the microbiota in vitro (A) wild-type and mutant *S.* Typhimurium strains were incubated with *E. coli* K-12 DH5α to assess T6SS-dependent killing. The indicated *S.* Typhimurium attacker strain was mixed with *E. coli* prey at a 1:1 ratio and incubated for 48 h on LB Agar plate supplemented or not with 0.05% porcine bile salts. Recovered mixtures were plated onto selective media. (B) wild-type and mutant *S.* Typhimurium strains were incubated with the indicated commensal strains (*Klebsiella oxytoca*, *Enterobacter cloacae*, *Klebsiella variicola*, and *Escherichia coli* JB2) for 48 hours on LB Agar plate supplemented with 0.05% porcine bile salts. Recovered mixtures were plated onto selective media. Statistical significance is shown based a Student's *t* test corresponding to the values of the WT strain: NS, not significant; *, $P < 0.05$; **, $P < 0.01$.

Figure 3. Hcp1 binds the Tae4 amidase effector and mediates *K. oxytoca* killing. (A) Wild-type and mutant *S.* Typhimurium strains were incubated with *K. oxytoca* for 48 hours on LB Agar plate supplemented with 0.05% porcine bile salts. Recovered mixtures were plated onto selective media. Statistical significance is shown based a Student's *t* test corresponding to the values of the wild-type strain: NS, not significant; *, $P < 0.05$; **, $P < 0.01$, ****, $P < 0.0001$. (B) Bacterial two-hybrid analyses. BTH101 reporter cells producing the Hcp1 or Hcp2 proteins fused to the T18 domain of the *Bordetella* adenylate cyclase and the Tae4 protein fused to the T25 domain were spotted on plates supplemented with IPTG and the chromogenic substrate X-Gal. Interaction between the two fusion proteins is attested by the blue color of the colony. The TolB-Pal interaction serves as a positive control. (C) Co-precipitation assay. Purified and biotinylated Tae4 was mixed with 6×His-tagged Hcp1 (left panels) or Hcp2 (right panels), and subjected to ion metal affinity chromatography on Ni^{2+} resin. The total (T), unbound (U), wash (W) and eluted (E) fractions were collected and subjected to SDS-PAGE and Western-blot analyses using anti-His antibody (upper panels) and streptavidin-conjugated alkaline phosphatase (lower panels). The position of the proteins and the molecular weight markers (in kDa) are indicated.

Fig. 4. *S.* Typhimurium SPI-6 antibacterial activity is active in vivo and mediates pathogen establishment in the host gut. (A) 129X1/SvJ mice were treated with antibiotics for two weeks prior to colonization with *K. oxytoca*. The day after, mice were infected orally with either 10^8 wild-type or Δ*hcp1* mutant *S.* Typhimurium bacterial strain. The cecum was harvested from each mouse, homogenized and plated at day three post-infection. The levels of *S.* Typhimurium and *K. oxytoca* were determined and

represented as paired bars for each mouse (B) 129X1/SvJ Mice were infected orally with either 10^8 wild-type or *Δhcp1* mutant *S*. Typhimurium bacterial strain. Feces are collected and plated over time on *Salmonella* selective medium. Every data point represents one mouse, and the bar represents the geometric mean. Statistical significance is shown based on Mann Whitney U test: NS, not significant; *, $P < 0.05$; **, $P < 0.01$. (C) C57BL/6 mice were infected orally with a 1:1 mixture of wild-type bacteria and the indicated bacterial mutant strain (5×10^8 cells of each *S*. Typhimurium strain). Feces were collected at day 1 or 2 whereas cecum and colon tissues were harvested at day 3 post-infection and plated on *Salmonella* selective medium. The data represent the competitive index (CI) value for the CFU mutant/ wild-type bacteria in the feces at days 1 or 2. Bars represent the geometric mean CI value for each group of mice. Statistical significance is shown based on the ANOVA with a Dunnett's posttest compared to the corresponding CI values of the *Δhcp2* mutant: NS, not significant; **, $P < 0.01$; ****, $P < 0.0001$.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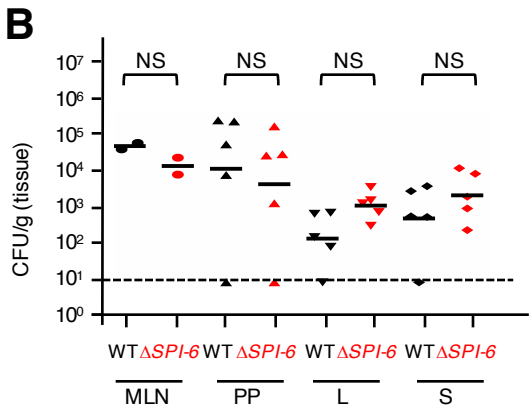
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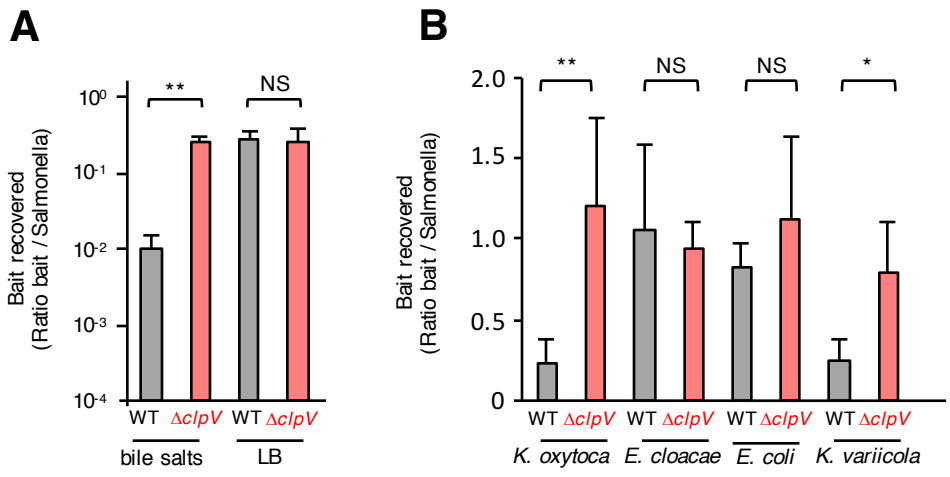
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(Ratio bait / Salmonella)
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10^-1
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**
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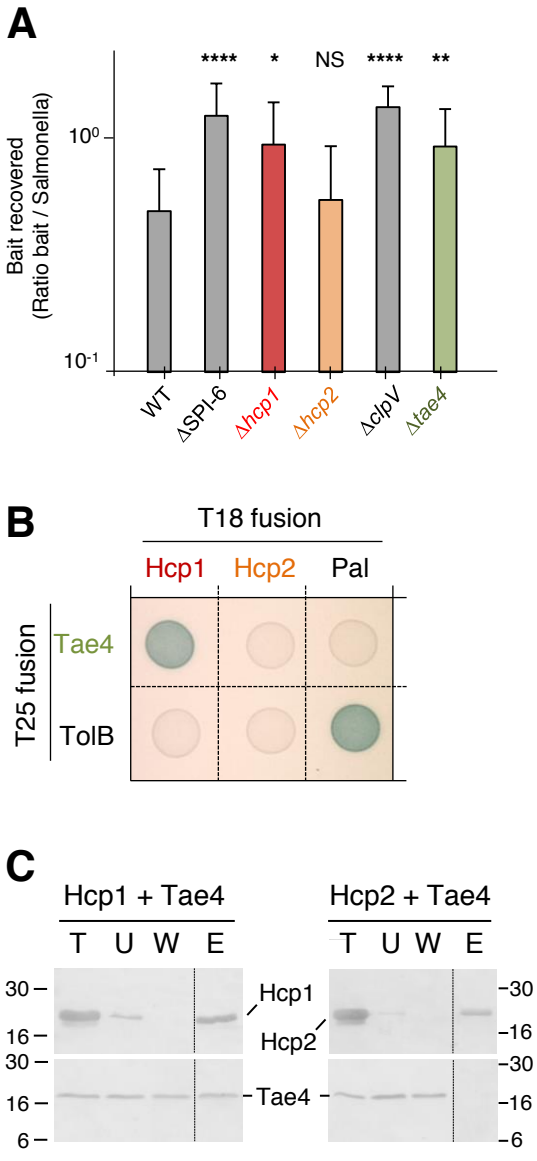
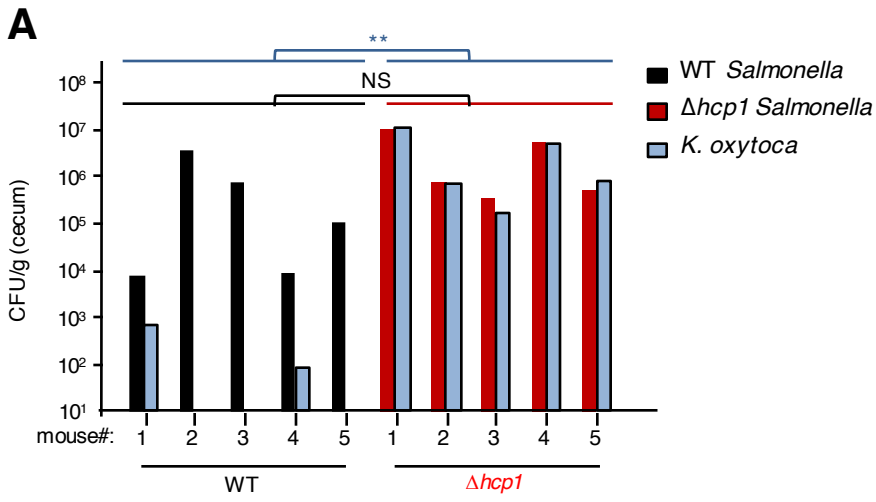
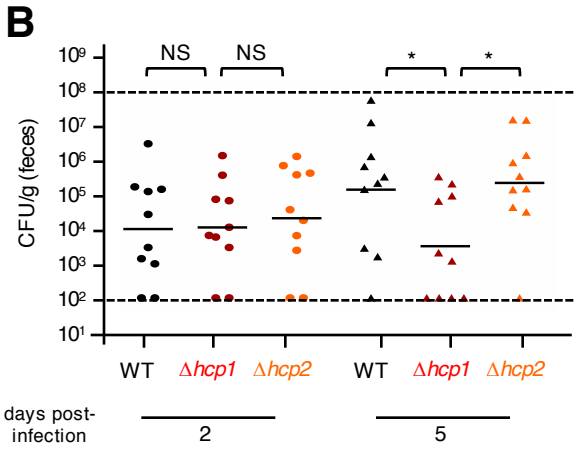
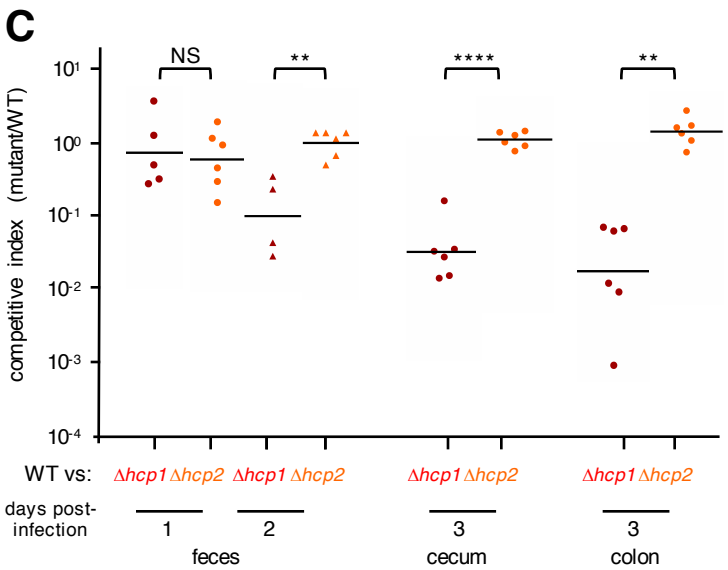
WT
ΔclpV
bile salts
LB
2.0
1.5
1.0
0.5
0
*
K. oxytoca
E. cloacae
E. coli
K. variicola



Sana et al., Figure 3


A
**
NS
WT Salmonella
Δhcp1 Salmonella
K. oxytoca
CFU/g (cecum)
mouse#:
WT
Δhcp1


B
NS
NS
*
*
CFU/g (feces)
WT
Δhcp1
Δhcp2
days post-infection
2
5


C
NS
**
****
**
competitive index (mutant/WT)
WT vs:
Δhcp1 Δhcp2
days post-infection
feces
cecum
colon

Supplementary Figures Legend

Figure S1. Representation of the SPI-6 Type VI secretion system. Representation of the SPI-6 genetic locus starting at STM0266 and ending at STM0292 (A) and schematic representation of the apparatus. Genes encoding the sheath components (TssB/TssC), the ClpV ATPase, the VgrG spike protein, the membrane and baseplate complexes and the Hcp proteins are colored in light blue, orange, light green, dark green, pink and grey, respectively as indicated and within the T6SS architecture scheme. *hcp1*, *hcp2*, and *tae4* are highlighted respectively in red, orange and green, a color code that is conserved all along the figures of this manuscript. Cyto, cytoplasm; IM, inner membrane; PG, peptidoglycan layer; OM, outer membrane.

Figure S2. *Salmonella* does not compete in a T6SS-dependent manner with *B. fragilis*, *B. longum*, *P. distatonis*, *P. copri*. WT and mutant *S.* Typhimurium strains were incubated with commensal strains to assess T6SS-dependent killing. The indicated *S.* Typhimurium attacker strain was mixed with respective commensal bacteria prey at a 1:1 ratio and incubated for 96 h on Blood agar plate placed in anaerobic chamber at 37°C. Recovered mixtures were plated onto selective media. Statistical significance is shown based a Student's *t* test corresponding to the values of the WT strain: NS, non significant; **, $P < 0.01$.

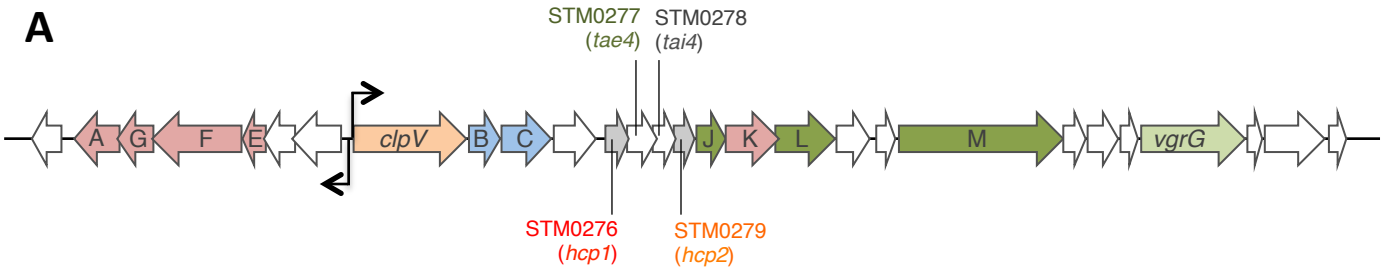
Figure S3. Complementation of hcp1 and tae4 mutations. WT and mutant *S.* Typhimurium strains were incubated with *K. oxytoca* at a 1:1 ratio and incubated for 48 h on LB supplemented with 0.05% bile salts at 37°C, to assess T6SS-dependent killing. Recovered mixtures were plated onto selective media. pDiGc is the control empty plasmid, and *hcp1* and *tae4* are encoded under arabinose inducible promoter, which is repressed by glucose. Glucose and arabinose were added at a final concentration of 0.5% and 0.05% respectively, where indicated. Statistical significance is shown based on Student's *t* test corresponding to the values of the WT strain: NS, non significant; **, $P < 0.01$; ***, $P < 0.001$.

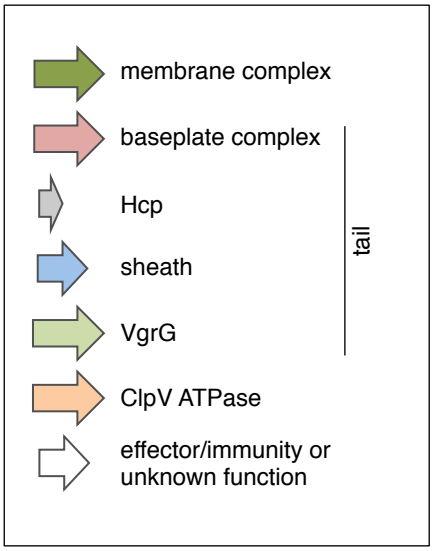
Figure S4. SPI-6 antibacterial activity is active in colons. (A) 129X1/SvJ mice feces were plated on MacConkey agar pre- (i), post- (ii) antibiotics treatment, or post colonization with *K. oxytoca* (iii). (B) 129X1/SvJ mice were treated with antibiotics for two weeks prior to colonization with *K. oxytoca*. The day after, mice were infected orally with 10^8 WT or Δ*hcp1* mutant *S.* Typhimurium cells. Colons were harvested and plated at day three post infection. The bar represents the geometric mean. Statistical significance is shown based on Mann Whitney U test: NS, non significant; *, $P < 0.05$; ***, $P<0.001$; ****, $P<0.0001$.

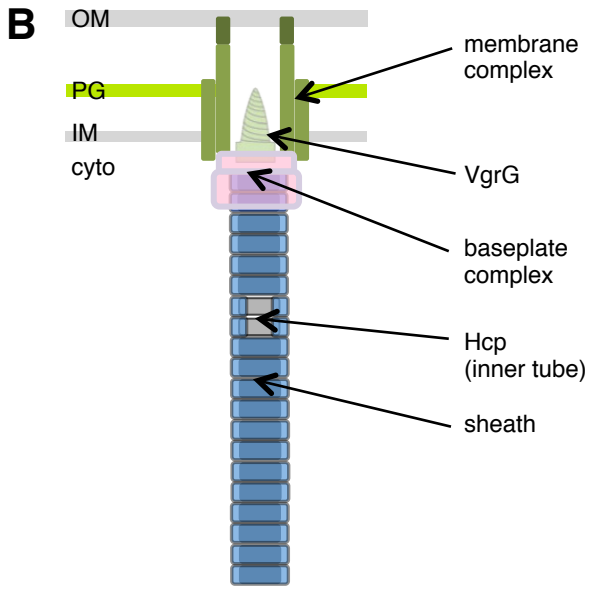
Figure S5. SPI-6 antibacterial activity is not necessary to establish in the host gut of mice treated with antibiotics. 129X1/SvJ mice were orally treated with a cocktail of antibiotics for 2 weeks and then orally infected with 10^8 cells of the indicated *S.* Typhimurium strain. Feces were collected at day 1 and 2 and plated over time on *Salmonella* selective medium. Every data point represents one mouse, and the line represents the geometric mean. Statistical significance is shown based on Mann Whitney U test: NS, non significant.

Figure S6. Comparison of the Hcp1 and Hcp2 sequences and structures. (A) Sequence alignment of Hcp1 and Hcp2. Identical and different residues are indicated in red and black respectively. (B) Molecular modeling of the Hcp1 and Hcp2 hexameric structures. All conserved residues are shown in ribbon

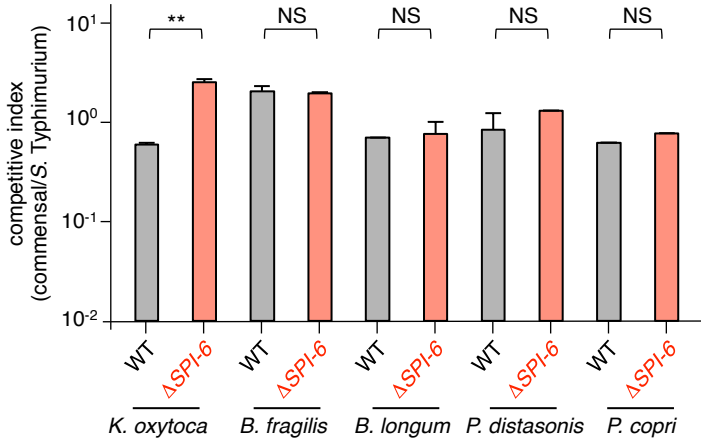
whereas the variant residues are shown in balls. Two of the four variant residues face the lumen (positions 124 and 125) whereas two (positions 124 and 125) are located at the hexamer-hexamer interface within the tube.



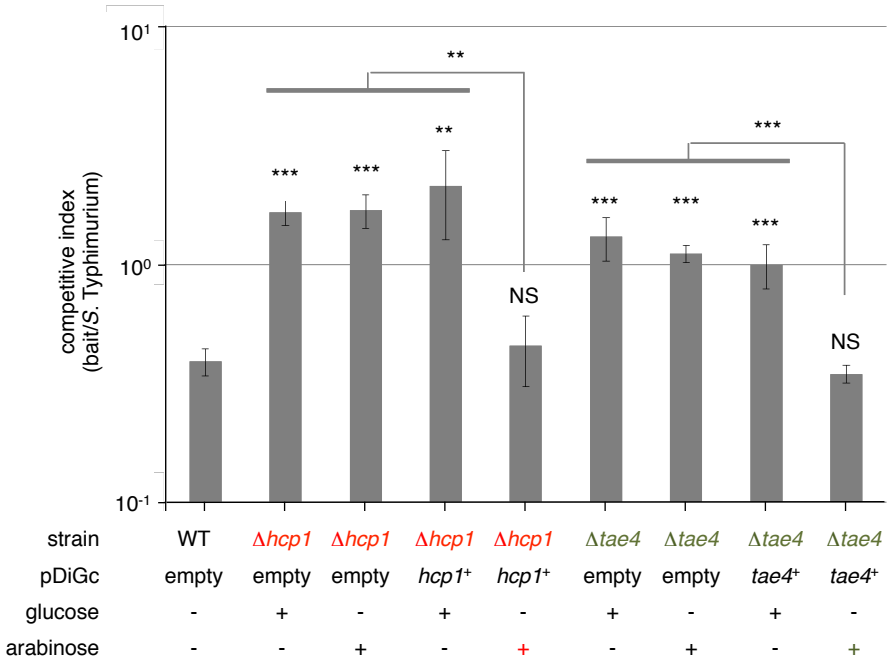




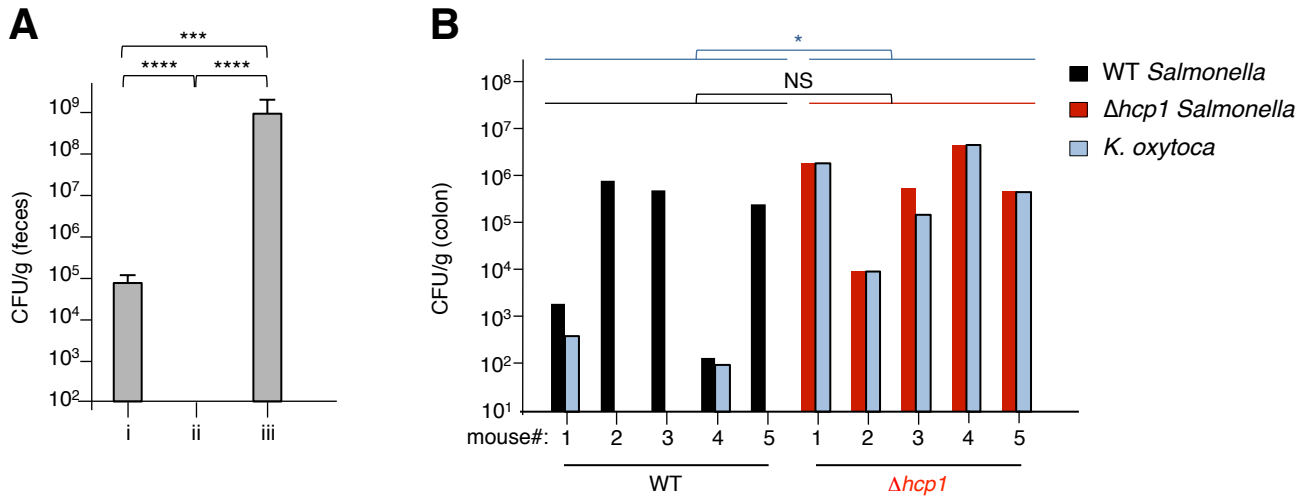
Sana et al., Figure S1



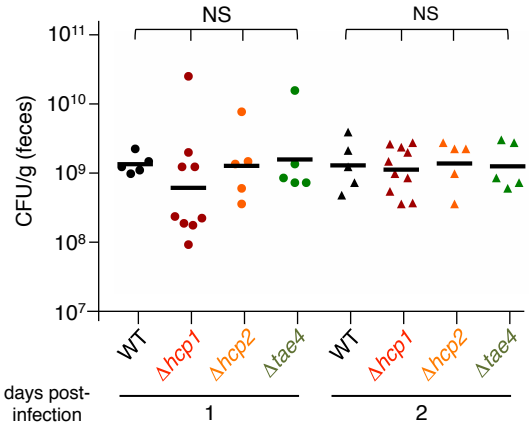
Sana et al., Figure S2



Sana et al., Figure S3



Sana et al., Figure S4



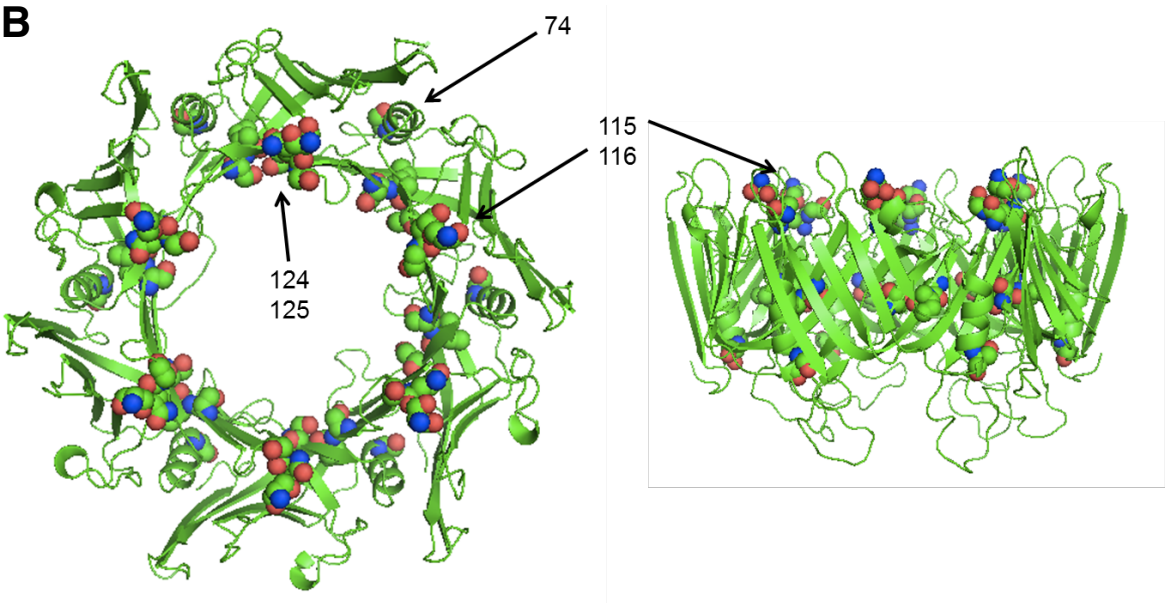
Sana et al., Figure S5

A

```
                10        20        30        40        50        60        70        80
                |         |         |         |         |         |         |         |
Hcp1   MAYDIFLKIDGIDGESMDDKHKNEIEVLSWRWNIHQESTMHAGSGLGSGKVSVTNLDFDHYIDRASPNLFKYCASGKHIPQA
Hcp2   MSYDIFLKIDGIDGESMDDKHKNEIEVLSWRWNIHQESTMHAGSGLGSGKVSVTNLSFEHYIDRASPNLFKYCSSGKHIPQA
       *.**************************************************.*:**************.********

                90        100       110       120       130       140       150       160
                |         |         |         |         |         |         |         |
Hcp1   ILVMRKAGGNPLEYLKYTFTDLIVAVVSPSGSHDGEIASRETVELSFSTVKQEYVVQNQQGGSGGTITAGYDFKANKEI
Hcp2   ILVMRKAGGNPLEYLKYTFTDLIIAMVSPSGSQGGEIASRESIELSFSTVKQEYVVQNQQGGSGGTITAGYDFKANKEI
       ***********************:*.******.  *******::************************************
```

B



top view

side view

Sana et al., Figure S6

Table S1. Strains, Plasmids and oligonucleotides used in this study

Strains	Description	Source or reference
Commensal strains		
Klebsiella oxytoca TS1	Isolated from 129x1/SvJ mice feces	This work
Klebsiella varicola KL11	Isolated from C57BL/6 mice feces	This work
Escherichia coli JB2	Isolated from C57BL/6 mice feces	Behnsen *et al.*, 2014
Enterobacter cloacae KL1	Isolated from C57BL/6 mice feces	This work
Bacteriodes fragilis NCTC9343		J. Sonnenburg
Bifidobacterium longum NCC2705		J. Sonnenburg
Parabacteriodes distasonis ATCC 8503		J. Sonnenburg
Prevotella copri 18205		J. Sonnenburg
***S.* Typhimurium strains**		
LT2	LT2 WT	Laboratory collection
SL1344	SL1344 WT	Laboratory collection
SL1344 Δ*SPI-6*	SL1344 *SPI-6*::Km^R	This work
SL1344 Δ*clpV*	SL1344 *clpV*::Km^R	This work
SL1344 Δ*hcp1*	SL1344 *hcp1*::Km^R	This work
SL1344 Δ*hcp2*	SL1344 *hcp2*::Km^R	This work
SL1344 Δ*tae4*	SL1344 *tae4*::Km^R	This work
***E. coli* strains**		
DH5α	F-, *ΔargF-lac phoA supE44 ΔlacZ relA endA thi hsdR*	New England Biolabs
BTH101	F-, *cya araD galE galK rpsL hsdR mcrA mcrB*	Karimova et al., 1998
BL21(DE3)	*fhuA lon ompT gal (λ DE3) dcm ΔhsdS*	New England Biolabs

Plasmids	Description	Source or reference
pUT18	BACTH plasmid, ColE1, Amp^R, T18 domain of *B. pertussis* Cya	Karimova et al., 1998
pT18-Pal	*E. coli pal* gene cloned into pUT18	Gully *et al.*, 2006
pT18-Hcp1	*S.* Typhimurium STM0276 (*hcp1*) gene cloned into pUT18	This work
pT18-Hcp2	*S.* Typhimurium STM0279 (*hcp2*) gene cloned into pUT18	This work
pKT25	BACTH plasmid, pACYC, Kan^R, T25 domain of *B. pertussis* Cya	Karimova *et al.*, 1998
pKT25-TolB	*E. coli tolB* gene cloned into pKT25	Gully *et al.*, 2006
pKNT25	BACTH plasmid, pACYC, Kan^R, T25 domain of *B. pertussis* Cya	Karimova *et al.*, 1998
pKNT25-Tae4	*S.* Typhimurium STM0277 (*tae4*) gene cloned into pKNT25	This work
pRSF-1	Expression vector, RSF1030, Kan^R, T7 promoter, N-terminal 6×His	Addgene
pRSF-Hcp1	*S.* Typhimurium STM0276 (*hcp1*) gene cloned into pRSF-1	This work
pRSF-Hcp2	*S.* Typhimurium STM0279 (*hcp2*) gene cloned into pRSF-1	This work
pETG20A	Expression vector, Amp^R, T7 promoter, N-terminal TRX-6×His-TEV	Laboratory collection
pETG20A-Tae4	*S.* Typhimurium STM0277 (*tae4*) gene cloned into pETG20A	This work
pDiGc	Inducible expression of dsRed and constitutive expression of GFP	Helaine *et al.*, 2010
pDiGc-*hcp1*	Inducible expression of SL1344 hcp1 and constitutive expression of GFP	This work

pDiGc-*tae4*	Inducible expression of SL1344 tae4 and constitutive expression of GFP	This work

Oligonucleotides	**Sequences**[a]	**Source or reference**
TSD5	5'-TATTTTTATGAATTTTTATGTCACAAGGCATAACACATGGTGTAGGCTGGAGCTGCTTC	
TSD6	5'-GCACTACGGACACTTCGAACGGCCGGTTTCAGCAAACGATCATATGAATATCCTCCTTAG	This work
TSD7	5'-GTCCCTGATCTGTATCATTG	This work
TSD8	5'-AGTCAACTGGTTGCCGCAAG	This work
TSD13	5'-AGGTTTATTTTAAGTAAAACTTAATAAGGATATAAAAATGGTGTAGGCTGGAGCTGCTTC	This work
TSD14	5'-CAGACATAACATCTGGCCGGAAAAACAGCCGTTAAATTTCCATATGAATATCCTCCTTAG	This work
TSD15	5'-AGTCCTCTGTTCTCCTGAAG	This work
TSD16	5'-GCATAGCTACCGCACATAAC	This work
TSD17	5'-TCAGGGCTTAATTTAGGTAGTTAAAAGGATAGTAGATATGGTGTAGGCTGGAGCTGCTTC	This work
TSD18	5'-CCAGATATAAATCTGGCCGGAAAAACAGCCGTTAAATTTCCATATGAATATCCTCCTTAG	This work
TSD19	5'-TACGTTCTCTTGCTCTGATG	This work
TSD20	5'-CATAGCTACCGCACATAACC	This work
TSD33	5'-TTTTTATACATCCTGTGAAGTAAAAAAAACCGTAGTGTAGGCTGGAGCTGCTTC	This work
TSD34	5'-ATGGCACATTAATTTGAAGCAGCTCTCATCCGGTCATATGAATATCCTCCTTAG	This work
TSD35	5'-CCGAAGTGTATCTGGCGATGA	This work
TSD83	5'-TTAATGACCTACACAGAATTTTTAGAGGTTAAGCAAAATGGTGTAGGCTGGAGCTGCTTC	This work
TSD84	5'-TATTAACCATTTCACGGCAGTATCCACAGTGTCCCAACTTCATATGAATATCCTCCTTAG	This work
TSD85	5'-GTCCATGAAGATACGTGTTG	This work
TSD86	5'-CATTCCACTTATGCTGAAAG	This work
TS107	5'-TCGGTGCGCTCGTACTGCTC	This work
KL1	5'-AAAAAGACGTCAGGAGGTAATTATGGCTTATGACATTTTTTTG	This work
KL2	5'-TTTTTACCAGGTTTAAATTTCTTTGTTGGCCTT	This work
KL3	5'-AAAAAGACGTCAGGAGGTAATTATGAACAGACCTTCATTCAAT	This work
KL4	5'-TTTTTCACGATGGTGTCACGGCAGTATCCACAGTG	This work
63F	5'-CAGGCCTAACACATGCAAGTC	This work
1387R	5'-CGGAACATGTGWGGCGGG	Marchesi *et al.*, 1998
ECO-2255	5'-CGCCACTGCAGGGATTATAAAGATGACGATGACAAGGCTTATGACATTTTTTTGAAAAT TGACGGCATTGAT	Marchesi *et al.*, 1998 This work
ECO-2256	5'-CGCCACTGCAGGGATTATAAAGATGACGATGACAAGTCTTATGACATTTTTCTGAAAAT TGACGGCATTGAC	This work
ECO-2257	5'-CGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCTAGTTAAATTTCTTTGTTGGCCTT GAAGTCGTAG	This work
ECO-2253	5'-CGGATAACAATTTCACACAGGAAACAGCTATGACCATGAACAGACCTTCATTCAATGAAGC	
ECO-2254	5'-GTTTGCGTAACCAGCCTGATGCGATTGCTGCGGCAGTATCCACAGTGTCCCAAC	This work
ECO-2217	5'-CATCATCACCACAGCCAGGATCCGGCTTATGACATTTTTTTGAAAATTGACGGCATTGAT	This work
ECO-2218	5'-CATCATCACCACAGCCAGGATCCGTCTTATGACATTTTTCTGAAAATTGACGGCATTGAC	This work
ECO-2219	5'-GGCGCGCCGAGCTCGAATTTTAAATTTCTTTGTTGGCCTTGAAGTCGTAG	This work
ECO-2220	5'-GCAGGCTTAGAAAACCTGTACTTCCAGGGTAACAGACCTTCATTCAATGAAGCGTGG	This work
ECO-2221	5'-GCTTTGATCTCGCCTGCCACCGGTAC*TCA*CGGCAGTATCCACAGTGTCCCAAC	This work This work

[a] Sequence annealing on the target plasmid underlined.