**Abstract**

[GENERAL INTRODUCTION]

The gastrointestinal tract of humans and animals is populated by a dense community of diverse microorganisms. This intestinal microbiota ~~features intricate metabolic interactions involving the breakdown and reuse of host- and diet-derived nutrients. The competition for these~~ competes for resources, such as host- and diet-derived nutrients, which can limit pathogen growth. Nevertheless, some enteropathogenic bacteria can ~~invade this niche~~ overcome this limitation through mechanisms that remain largely unclear

[CONSEQUENCE]

1. , hampering the rational development of interventions for gastrointestinal infections. Here, we discovered that
2. . This lack of knowledge could limit our capability to effectively treat enteropathogenic infections in the intestine. To close this gap, we used a mouse model to discover that

initial growth of *Salmonella* Typhimurium (*S.* Tm) in the unperturbed gut is powered by *S.* Tm *hyb* hydrogenase. This enzyme facilitates the consumption of hydrogen (H2), a central intermediate of microbiota metabolism. In competitive infection experiments, a *hyb* mutant exhibited reduced growth early in infection compared to wild-type *S.* Tm, but these differences were lost upon antibiotic-mediated disruption of the gut microbiota. Introducing additional H2-consuming bacteria into the microbiota interfered with *hyb*-dependent *S.* Tm growth. Thus, our results show that H2 production by microbiota metabolism can be exploited by pathogens.

[CONCLUSION]

Interference with this metabolic exploitation might offer opportunities to develop new strategies to prevent infection.

**Introduction**

The mammalian intestine is densely colonized by microorganisms, collectively referred to as microbiota (Ley et al., 2008). The microbiota features a network of metabolic activities facilitating the efficient breakdown of complex diet- and host-derived carbohydrates to short-chain fatty acids (SCFAs), hydrogen (H2), and carbon dioxide (Fischbach and Sonnenburg, 2011; Flint et al., 2008). These fermentation products are subsequently consumed by cross-feeding secondary fermenters, absorbed by the host, or released into the environment (add reference). Gut ecosystem invasion is defined herein as the initial growth phase of a pathogen (or any other newcomer) in the host’s intestine. At this stage, the intestinal mucosa appears healthy, and the microbiota is (still) intact and limits nutrient availability. This prohibits growth of most newly arriving bacteria. Despite the scarce nutrient availability, enteropathogens can invade the gut ecosystem. Yet, the factors enabling ‘‘gut ecosystem invasion’’ by enteropathogens remain unclear.

The human food-borne pathogen *Salmonella* Typhimurium (*S.* Tm), a causative agent of diarrhea, can grow up in this nutrient-depleted environment to high numbers and cause disease. Animal experiments established that gut luminal pathogen densities must rise to 107–108 colony forming units (cfu) per gram of stool before enteropathy is elicited (Ackermann et al., 2008; Barthel et al., 2003). As inoculum sizes as low as 103–105 bacteria suffice for causing diarrheal disease in humans (Food and Agriculture Organization of the United Nations, 2002), we speculated that *S.* Tm can grow initially in the face of an intact microbiota and a healthy gut. The mechanisms fostering *S.* Tm growth in this densely colonized niche are still enigmatic. Such mechanisms can be studied using ‘‘low complex microbiota’’ (LCM) mice, which are permissive for gut luminal *S.* Tm growth (Figure S1A available online; Stecher et al., 2010). LCM mice are ex-germ-free mice which had originally been colonized with strains of the ~~‘‘~~Altered Schaedler Flora~~’~~’ (Experimental Procedures, Figures S1A and S1E) and permit gut luminal colonization by inoculum sizes as small as 200 cfus (Endt et al., 2010; Stecher et al., 2010). During the first 2 days, there are no signs of enteropathy, and the pathogen grows up to 106–108 cfu/g stool (gut ecosystem invasion). Mucosal inflammation is elicited at days 3–4 postinfection when the pathogen reaches a final density of 108–1010 cfu/g stool (Stecher and Hardt, 2011; Figure S1A). Thus, LCM mice should provide a unique model for analyzing all phases of host gut colonization, including gut ecosystem invasion.

[HYPOTHESIS/PROBLEM PARAGRAPH]

Methods

Analysis of polymyxin B resistance. The equivalent of 1 OD600 unit/ml of exponentially growing cultures from serovar Typhimurium wild-type strain SL1344 or the serovar Typhimurium Δ*wbaP* strain (SKI12) was spun down, resuspended in 150 μl cold sterile ~~1 ×~~ phosphate-buffered saline (PBS), and diluted 5 × 106-fold before use. For the assay, 45 μl of the diluted cultures was mixed with 5 μl of polymyxin B (final concentration, 1 μg/ml; Sigma) or 5 μl PBS and incubated for 1 h at 37°C under slight agitation. After addition of 80 μl LB agar, bacteria were plated on LB agar plates containing streptomycin. The survival efficiency was calculated by dividing the number of CFU of the peptide-treated culture by the number of CFU of untreated culture and multiplying by 100. The assay was performed in triplicate for two independent experiments, and data are shown as means ± standard deviations.

**Results**

**Hydrogen consumption by *S*. Tm is only required during the Initial phase of gut ecosystem invasion**

In order to verify the role of hydrogenases during gut infection, we constructed site-directed mutants (Figure S2B; Supplemental Experimental Procedures). In competitive infections, the *hyb* mutant (*S.* Tmhyb; hybBCAhypO, which lacks all structural genes of the hyb hydrogenase) displayed a pronounced growth defect compared to the isogenic wild-type strain (~100-fold; p < 0.05; Figure 2). This was corrobated by *hyb* expression in the gut lumen (Figure S2D). Interestingly, the growth defect of *S.* Tmhyb was restricted to the first day of the experiment when pathogen loads were still low (≤ 108 cfu/g stool) and no signs of mucosal inflammation were observed (Figures 2B–2D). Thereafter, the competitive index did not drop any further (Figure 2A). These data indicate that *S.* Tm requires *hyb* only in the initial phase of gut ecosystem invasion, ~~but not at later~~

~~stages of the infection,~~ and that this initial stage (days 0–1) is mechanistically distinct.

Further experiments excluded major contributions of two alternative H2-consuming hydrogenases encoded in the *S.* Tm genome (Figure S2B; Supplemental Experimental Procedures). Disrupting the two alternative hydrogenases yielded no defects in gut ecosystem invasion, and the hydrogenase triple mutant (*S.* Tmhyd3) displayed the same *in vivo* growth defect as did *S.* Tmhyb (Figures S3A and S3B). Thus, while *hyb* is necessary for robust pathogen growth in the host’s intestine, the other two hydrogenases contribute little. This was further supported by complementation (Figure S3B). Furthermore, the gut ecosystem invasion defect of *S.* Tmhyb was independent of the inoculum size and also observed upon gavage of 5 x 103 cfu (data not shown; standard inoculum size = 5 x 107 cfu; Experimental Procedures). Finally, in vitro experiments in anaerobic broth culture verified that the growth defect of *S*. Tmhyd3 was only observed in the presence of H2~~,~~ ~~but not in its absence~~ (Figures S4A and S4B). In conclusion, these data confirmed the pivotal importance of *hyb* for H2-dependent *S.* Tm growth. Our initial data suggested that the *hyb* hydrogenase may fuel pathogen growth during gut ecosystem invasion, i.e., the first 24 hr p.i. (Figure 2A). At this stage the pathogen grows in the face of the resident microbiota (which presumably still produces H2) and overt inflammation is not yet triggered (Figures S1A and 2B–2D). To further substantiate the need for hydrogenases in the noninflamed gut, we performed competition experiments in the avirulent strain background. The isogenic *S.* Tm mutant (*S.* Tmavir; DinvGDsseD; Supplemental Experimental Procedures) colonizes the gut but remains ‘‘locked’’ in gut ecosystem invasion phase of the infection, as it lacks two key virulence factors and therefore cannot elicit overt mucosal inflammation (Hapfelmeier et al., 2005; Stecher et al., 2007). To this end, we constructed a hydrogenase-deficient mutant in the *S.* Tmavir background (*S.* Tmavir hyd3). First, we tested this strain’s capacity to grow up in the gut of LCM mice. In competitive infections, *S.* Tmavir hyd3 displayed a pronounced colonization defect on day 1 p.i. but no further decrease from day 1 to day 4 p.i. (Figure 3). These results were strikingly similar to those obtained in the wild-type *S.* Tm strain background (compare Figure 2A and Figure 3A) and verified that hydrogenases are indeed only required during gut ecosystem invasion, whether inflammation is triggered or not. Accordingly, intravenous infection experiments confirmed that hydrogenases are not needed for growth at systemic sites (Figure S3C). This further supported the notion that gut ecosystem invasion is a distinct step in host intestinal colonization, which prepares the ground for subsequent stages of the infection.

**Discussion**

Our findings establish gut ecosystem invasion as a critical step of the orogastric *S.* Tm infection. During this initial phase of the infection, pathogen growth in the gut relies at least in part on metabolites provided by the microbiota. This differs markedly from the interactions observed later (i.e., during expansion/maintenance), when the host’s mucosal immune response fuels pathogen growth and suppresses the microbiota (Kaiser et al., 2012; Winter et al., 2013). Thus, colonization of the host’s gut comprises different phases featuring distinct sets of positive and negative interactions. The interactions between the pathogen, the microbiota, and the host are clearly more complex than previously anticipated.

Gut ecosystem invasion by S. Tm relies on the availability of H2. This is true for mice harboring two different microbiotas of reduced complexity (LCM mice used throughout most of this study; VLCM mice used in Figures S4C and S4D) or animals with a normal SPF microbiota, alike (Figures 4D–4F and 5B). In contrast, intravenous infections did not yield any evidence for H2-dependent pathogen growth at systemic sites (Figure S3C). At first sight, this

Our finding that H2 promotes STm to invade the gut ecosystem in mice, seems to be in conflict with earlier work in the oral infection model for typhoid fever (Maier et al., 2004). Our results showing that *S.* Tmhyb failed to colonize the liver and spleen may suggest that this attenuation was attributable at least in part to defective growth in the gut, before the bacteria had actually disseminated to systemic sites. This hypothesis would be in line with hydrogenase expression of ATCC14028 in the murine ileum (Zbell et al., 2008). However, we cannot formally exclude that ATCC14028 differs from the SL1344 strain used in our study in being capable of utilizing H2 in liver and spleen. Such strain-specific differences may affect the adaptation to new hosts. Clearly, S. Tm SL1344 requires H2 only for gut colonization, but not at systemic sites (Figure S3C). This provides a striking example for a central intermediate of microbiota metabolism fueling pathogen growth at a site occupied by a dense commensal community. Due to the conserved nature of the metabolic network of the gut microbiota, this metabolite will likely be available in any host animal as well as in humans. Thus, H2 could be regarded as an ‘‘Achilles’ heel’’ of microbiota metabolism which can be exploited by S. Tm for gut ecosystem invasion, etc.