Review

Evolutionary Stability of *Salmonella* Competition with the Gut Microbiota: How the Environment Fosters Heterogeneity in Exploitative and Interference Competition

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Abstract

Following ingestion, gastrointestinal pathogens compete against the gastrointestinal microbiota and overcome host immune defenses in order to cause infections. Besides employing direct killing mechanisms, the commensal microbiota occupies metabolic niches to outcompete invading pathogens. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) uses several strategies to successfully colonize the gut and establish infection, of which an increasing number is based on phenotypic heterogeneity within the *S.* Typhimurium population. The utilization of *myo*-inositol (MI) and the production of colicin confer a selective advantage over the microbiota in terms of exploitative and interference competition, respectively. In this review, we summarize the genetic basis underlying bistability of MI catabolism and colicin production. As demonstrated by single-cell analyses, a stochastic switch in the expression of the genes responsible for colicin production and MI degradation constitutes the heterogeneity of the two phenotypes. Both genetic systems are tightly regulated to avoid their expression under non-appropriate conditions and possible detrimental effects on bacterial fitness. Moreover, evolutionary mechanisms underlying formation and stability of these phenotypes in *S.* Typhimurium are discussed. We propose that both MI catabolism and colicin production create a bet-hedging strategy, which provides an adaptive benefit for *S.* Typhimurium in the fluctuating environment of the mammalian gut.

Heterogeneity in *Salmonella*: Competition, Cooperation and Evolution

Salmonella infection and colonization resistance

Salmonella enterica comprises more than 2500 serovars and is a major cause of food poisoning worldwide. It is closely related to *Escherichia coli* and has a broad host range as it has co-evolved with cold-blooded host species and then expanded its host range to warm-blooded animals [1]. In animal and human hosts, *S. enterica* raises enteric fever, gastroenteritis, bacteremia and systemic infection. *S. enterica* serovar Typhimurium (*S.* Typhimurium) evokes a disseminated infection in mice, which serves as a model for human typhoid fever. When the microbiota of mice is compromised, as, for example, upon antibiotic treatment, *S.* Typhimurium induces an acute gut inflammation, recapitulating many aspects of the human pathology [2]. *Salmonella* gastroenteritis proceeds in several distinct phases: the oral uptake of a sufficiently high infectious dose, pathogen colonization of the gastrointestinal tract, and epithelial tissue invasion followed by an inflammatory response of the host. Finally yet importantly, *S.* Typhimurium can survive and proliferate during Enterobacteriaceae blooms

within the inflamed gut [3]. Overall, successful infection requires the capacity of *S*. Typhimurium to overcome colonization resistance, a protective mechanism mediated by the host's commensal gut microbiota. Colonization resistance is mainly based on three pillars: production of antimicrobial molecules, restriction of nutrients required for the core metabolism of *Salmonella*, and microbiota-induced mucosal immune defenses [4].

To proliferate during the complex infection process, *S*. Typhimurium is therefore challenged not only to withstand the host's immune response but to compete for nutrients with the commensal microbiota that occupies the majority of metabolic niches. Furthermore, during the different phases of infection, the pathogen faces a vast number of metabolically different environments, with fluctuating substrate composition and nutrient access [5]. Consequently, growth on a single carbon source is likely the exception during infection [6]. While the gut offers a broad spectrum of potential substrates that could be exploited by *S.* Typhimurium like glycerol, host nucleosides, sialic acids, hexoses and pentoses, the competitive environment forces the invading population to acquire specific metabolic capacities. Those pathways include the utilization of MI, fucose, rhamnose, xylose, ethanolamine and 1,2-propanediol [7,8].

Besides nutrient availability being a limiting factor, a range of growth-inhibiting factors produced by the gut microbiota challenges the invading *S.* Typhimurium population [9]. Pathogenic, but also commensal Enterobacteriaceae (e.g., *E. coli*, *Salmonella* spp., *Citrobacter* spp.) can produce antimicrobial peptides as competition factors. For instance, commensal *E. coli* strains, isolated from the mammalian gut, produce group B colicins [10,11]. These toxins can kill enteric pathogen *E. coli* 0157:H7, but also several non-typhoidal *Salmonella* strains. Thus, colicin-producing commensal *E. coli* strains may limit infections [11,12]. Besides, *E. coli* Nissle produces microcin M and microcin H47, which have antibacterial activity against *Salmonella* under iron-limiting conditions. In the inflamed intestine, *E. coli* Nissle can efficiently compete with commensal *E. coli* strains or with enteric pathogens like non-typhoid *Salmonella* spp. [13]. On the other hand, *S.* Typhimurium SL1344 also harbors a colicin Ib (ColIb) plasmid, which can confer a growth benefit over competing colicin-sensitive commensal *E. coli* during infection [14,15].

In this review, the capacity of *S*. Typhimurium to utilize MI and produce colicin is addressed with respect to competition, cooperation and evolution.

Heterogeneity in *Salmonella* pathogenicity and social evolution

Bacteria can exhibit a heterogeneous behavior caused by the presence of at least two distinct phenotypes within an isogenic population. This heterogeneity is often based on stochasticity in gene expression at the single-cell level, a phenomenon termed bistability [16], which is considered to offer fitness benefits in fluctuating environments [17]. Bistable gene expression is controlled by a specific regulatory network architecture that often includes a regulatory feedback loop with a cooperative (non-linear) response to an activator [18–21]. In distinction to genetic phase variation, bistability is an epigenetic phenomenon that is reversible and not caused by mutation or recombination. Such a phenotypic variation was first described for lactose utilization by *E. coli* requiring the expression of the *lac* operon, which is negatively regulated by LacI [22].

An increasing body of literature documents that bistable gene expression contributes to successful *S.* Typhimurium infection. In *in vivo* processes, heterogeneity in gene expression presumably has an adaptive value for persistence, proliferation, invasion and transmission of this pathogen. Examples include the expression of the fimbrial genes *csgD* involved in biofilm formation [23], of *lpf* responsible for long-term carriage of *S*. Typhimurium in mice [24], and of the motility genes *fliC* [25]. The latter finding was supported by the results of a promoter screen that identified flagellar genes, especially *fliC*, with high levels of phenotypic noise [26]. Heterogeneity in gene expression also serves as a mechanism underlying bile resistance of *S*. Typhimurium [27].

Using a mouse colitis model for infection experiments [2], it was demonstrated that stochasticity in gene expression leads to the formation of a slow-growing and invasive subpopulation of salmonellae, and another subset of rapidly growing, but less virulent cells [28]. This heterogeneity is reflected on the single-cell level by the stochastic fluctuation of the promoters of *hilA* and *hilD*, both of which encode hierarchical regulators that control the expression of the invasion-associated type 3 secretion system (T3SS) of the *Salmonella* pathogenicity island 1 (SPI1) [29]. The invasive subpopulation with activated SPI1 genes ($SPI1^{ON}$) invades the mucosa by causing an inflammatory response, resulting in this subpopulation being killed by the immune system. In turn, the gut inflammation triggered by *S*. Typhimurium invasion alters the environmental conditions in the gut for the benefit of *Salmonella*, because it impairs the commensal microbiota and thus reduces the competition for nutrients and niche occupation. In other words, a self-destructive fraction of salmonellae increases the fitness of another, non-invasive subpopulation ($SPI1^{OFF}$) that benefits from the dying one, a population dynamic which is understood as division of labor [28].

S. Typhimurium has therefore emerged as widely used model organism to study cooperative behavior of bacterial pathogens during infection. Central questions include how phenotypic heterogeneity resulting in cooperation and division of labor has

evolved and is stabilized under the pressure of evolutionary forces. Two aspects are of particular interest: the molecular mechanisms that allow a *Salmonella* population to exhibit phenotypic heterogeneity (the proximate causes), and the evolutionary mechanisms that lead to the development of this phenomenon (the ultimate causes) [30]. The molecular mechanisms underlying heterogeneity include stochasticity of single molecules involved in regulatory pathways [31]. Together with regulatory feedback loops and hysteresis, clearly defined subpopulations exhibit different phenotypes [19,32]. Possible mechanisms that are involved in the evolution of heterogeneity will be addressed in this review. For that purpose, relevant terms in this field are defined as follows: Social behaviors are those that have fitness consequences for the actor as well as for the recipient. While cooperation mainly leads to a fitness benefit for the recipient, altruism further decreases the fitness of the actor [33].

The above-mentioned mechanism of division of labor in an invading *Salmonella* population is an illustrative example for social behavior within a species, where phenotypic heterogeneity results in one subpopulation providing a fitness advantage for the population good. Phenotypic heterogeneity can exhibit cooperative [34], or, as in the case of the SPI1^{ON} subpopulation, altruistic properties. These interaction dynamics are prone to exploitation by cheaters that will benefit without carrying the costs. In a situation without assortment, cheaters are able to outcompete cooperating individuals [35]. Formation of cheaters is therefore prevented by mechanisms like assortment, kin discrimination, policing or punishment (negative frequency-dependent selection) [35,36]. Although it is difficult to experimentally prove that these mechanisms indeed stabilize cooperation, a number of experiments already confirm the theoretical predictions [35,37–40].

In the case of division of labor in the invasion process of *S*. Typhimurium, genetically avirulent (non-invasive) cells, so-called defector cells, could exploit inflammation without contributing to the heterogeneous response. When such cheaters were tested in infection experiments, *S*. Typhimurium was quickly outcompeted by the commensal microbiota and cleared from the gut [41]. Notably, cheaters (*hilD* mutants) emerged under infection conditions as they grow faster in the absence of SPI1 expression. In this context, Diard and coworkers demonstrated that bistable expression of the invasive phenotype in this pathogen is essential for the evolutionary stability of virulence, as it slows down the emergence of cheaters [41]. Thus, the heterogenic behavior of the wild-type salmonellae promotes the stability of the cooperative virulence by division of labor. A more recent study showed that a small number of SPI1^{ON} cells are sufficient to endow a *Salmonella* population with invasive capacities, which the SPI1^{OFF} cells may profit from [42]. Furthermore, it was shown that antibiotic treatment fosters this cooperative virulence of *S*. Typhimurium because persister cells that evade antimicrobial treatment were preferentially found at sites that the pathogens accesses upon the activity of virulence factors [43]. These data fit well to the observation that antibiotic killing correlates with single-cell division rates, promoting the survival of slow-growing subpopulations [44]. Such an *in vivo* tolerance possibly reflects stochastic fluctuations in the cells [45]. Therefore, phenotypic heterogeneity also plays a role in antimicrobial resistance of *S*. Typhimurium [46].

A closely related bacterial strategy based on bistability is bet-hedging [47]. It is defined as a risk-spreading strategy providing a fitness advantage in a time-varying, uncertain environment [48]. Under different environmental conditions such as high or low temperature and variation of nutrients or humidity, bacteria require different capabilities. As it often unpredictable to which environment a specific bacterial population will be exposed next, the population divides in subpopulations, each of which is optimally prepared for another environment. As described, *S*. Typhimurium faces highly variable environmental conditions *in vivo*, as the mammalian host's gut is spatio-temporally structured and provides a huge number of physiological niches [49]. As a consequence, *Salmonella* has acquired the metabolic capacities to utilize several nutrients including fucose, rhamnose, xylose, ethanolamine, 1,2-propanediol and MI [7,8]. We hypothesize that many of these substrates are present only from time to time or at different sites, and in an unpredictable concentration. In this challenging situation, *Salmonella* is expected to use bet-hedging in order to maximize its fitness.

Inositol Utilization by *Salmonella*: Example for Bistability in a Metabolic Pathway Used for Competition against the Microbiota

A specific metabolic pathway of salmonellae contributing to exploitative competition.

S. Typhimurium is among the few pathogenic bacteria that are able to utilize *myo*-inositol (MI) as the sole carbon and energy source. MI is a polyol abundant in soil and the mammalian bloodstream [50], and a building block for phosphatidylinositol and other membrane molecules of eukaryotes. Its phosphorylated form, phytate, is an important phosphorus storage of plants. Therefore, MI and its derivatives are present in the human gut following food uptake or epithelial cell shedding. The genes required for the MI utilization pathway are located on the 22.6-kb genomic island GEI4417/4436 and comprise *iol* genes encoding regulators, transporter and catabolic enzymes (Fig. 1A).

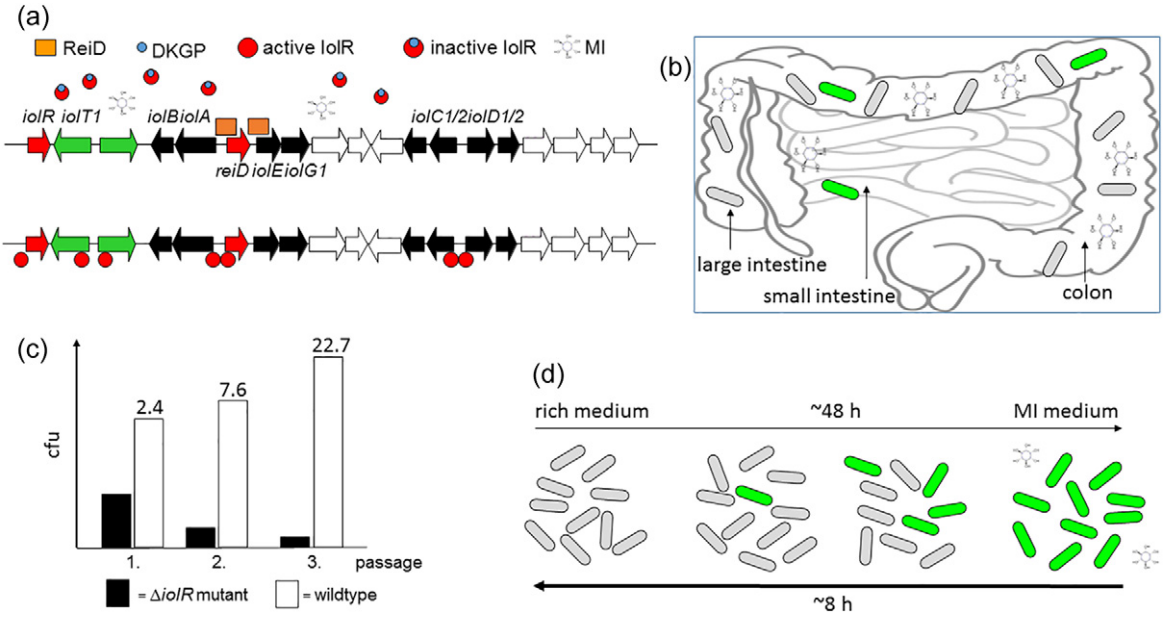

Fig. 1. MI utilization by *S.* Typhimurium. (A) Genetic organization and regulation of the *iol* genes. Regulatory genes are depicted in red, those encoding transporters in green, and genes essential for MI degradation in black. The metabolic island comprises a total of 19 genes. (B) Metabolic niche occupation in the gut by *S.* Typhimurium. The pathogen is assumed to encounter varying environments during gastrointestinal infection. (C) Fitness costs imposed by untimely expression of *iol* genes. In rich medium, the *S.* Typhimurium wild-type strain overgrows the *iolR* deletion mutant. The numbers above the columns indicate the ratio of colony forming units (cfu) of the wild type to those of the mutant. (D) Environmental switch from rich medium to MI medium, and vice versa. The metabolic change from rich medium to MI medium is a slow response in contrast to the adaptation from MI medium to rich medium. *S.* Typhimurium cells with transcriptionally activated *iol* genes are depicted in green, and those growing with other substrates in gray. See text for more details.

In vivo screening approaches suggest that *iol* genes play a role in *S.* Typhimurium proliferation in mice and pigs [51–55]. A TraDIS-based approach systematically testing a transposon mutant library in chicken, pigs and calves revealed a strong attenuation of several *iol* gene mutants in enteritis models of these animals [56]. Bicarbonate, which is secreted from the pancreas for stomach acid neutralization, serves as a trigger of this pathway [57], again suggesting that MI degradation might play a role for replication of *S.* Typhimurium especially in the gut, where *S.* Typhimurium encounters an altered or limited availability of nutrients. Thus, the capability to degrade MI is a specific adaptation that provides a fitness advantage by allowing *S.* Typhimurium to overcome the nutrient limitation in the intestine [58]. This pathway therefore contributes to the survival, colonization, proliferation and persistence of *S.* Typhimurium in its host organisms (Fig. 1B).

Regulatory mechanisms controlling the MI degradation pathway

Heterogeneous colony sizes and an extraordinarily long lag phase are hallmarks of *S.* Typhimurium cells growing with MI as sole carbon and energy source [59]. The heterogeneity in growth is abolished following adaptation to MI-containing medium, by adding 0.1% hydrogen carbonate, or by deleting the negative regulator gene *iolR* [59]. Reversibility of all these effects and the inducible switch from one metabolic state into the other point to a mutation-independent mechanism underlying the heterogeneity of *S.* Typhimurium growth with MI. It is assumed that the heterogeneous phenotype on solid medium mirrors the growth in liquid MI medium with the highly variable long lag phase of approximately 2 days.

Detailed genetic studies revealed a complex, multilevel regulation of MI utilization that includes the repressor IolR, the global silencing protein H-NS, the cAMP receptor protein, the degradation intermediate 2-deoxy-5-keto-D-gluconic acid 6-phosphate (DKGP) [60] acting as an inducer of IolR, the novel *Salmonella*-specific activator ReiD, and the global virulence regulator of this pathogen, SsrB [57,61,62]. Recently, a novel non-coding sRNA termed RssR, which is induced by SsrB, was identified to contribute to the stability of *reiD* and thus to the degradation of MI by salmonellae [63]. The repressor IolR

revealed to play a pivotal role in the control of genes responsible for MI utilization by *S.* Typhimurium. This control is accomplished by direct binding of IolR to all promoters that drive the transcription of the essential *iol* genes [57] (Fig. 1A). Surface plasmon resonance spectroscopy revealed that IolR oligomers bind with high affinity to their specific binding sites in *iol* gene promoters. To elucidate why the *iol* genes are so tightly repressed, we investigated whether or not the untimely induction of MI utilization results in increased fitness costs for the pathogen. For this purpose, we performed competitive growth assays with a 1:1 mixture of a wild-type *S.* Typhimurium strain and its *iolR* deletion mutant, in which the *iol* genes are highly expressed even in the absence of MI. Following three passages of such a mixture, during each of which the cultures were allowed to grow until stationary phase and then re-inoculated into fresh rich medium, the cell number of the parental strain exceeded those of the *iolR* mutant by a factor of approximately 23 (Fig. 1C). These data showed that cells with *iol* promoters in the ON state in the presence of rich medium have a high growth disadvantage of ~15% over those with silenced *iol* genes, but only in the absence of MI as major substrate. Therefore, we propose that the very tight control of the *iol* genes by IolR is the result of selection processes due to fitness disadvantages of untimely induction of the MI degradation pathway.

Feedback loops in the expression of *iol* genes

As discussed above, bistability is fundamental for decision-making processes in population dynamics in fluctuating environments, and bacteria use feedback loops as regulatory mechanism to create bistability. In a positive feedback loop, a deviation in the controlled quantity is further amplified by the control system, whereas a negative feedback occurs if a deviation of that quantity is counterbalanced by the control element(s) [20]. Given this definition, two positive and one negative feedback are found in the control systems of MI utilization by *S.* Typhiumurium (Fig. 1A). The number of MI molecules in the cell is critical for the switch from the OFF to the ON modus with respect to MI utilization. In case that this number exceeds a certain threshold value, the catabolic intermediate DKPG is produced that interacts with the repressor IolR, resulting in IolR release and in an increase of MI import and degradation, until all IolR molecules with DNA-binding activity are titrated. A second positive feedback loop is constituted by the IolR-controlled activator ReiD that positively regulates its own expression and transcriptionally activates the two gene *iolE* and *iolG*, which encode proteins responsible for the two initial steps of MI degradation [62]. In contrast, if MI as the controlled quantity is reduced in the medium, less MI is catabolized in the cell, and the DKGP molecule numbers decrease, enhancing copy numbers of IolR that autoregulates its own gene and, upon its DNA-binding activity, represses *iol* gene activity in a negative feedback loop (Fig. 1A).

Bistability of *iol* gene transcription

As described above, a clonal population of *S.* Typhimurium shows a visible and high degree of heterogeneity of colony growth on solid medium in the presence of MI as sole carbon and energy source. Using fusions of the *gfp* reporter gene with *iol* promoters, fluorescence analysis demonstrated transcriptional bistability of most *iol* genes and identified two distinct metabolic states with one subpopulation in the ON mode and another in the OFF mode regarding utilization [59,64]. For example, while only 3% of *S.* Typhimurium cells expressed *gfp* at an OD_{600} of 0.037 during lag phase, the fraction with cells in the ON state rapidly increased during growth in MI-containing medium to 70% at OD_{600} at the beginning of the exponential phase to a maximum of 97% cells during the middle and late exponential phase [59] (Fig. 1D). Bistability of gene expression, however, was abolished in the presence of hydrogen carbonate or the absence of IolR.

Cellular processes contributing to bistability

In contrast to evolutionary mechanisms delineated in the next chapter, the molecular intracellular processes influencing stochasticity on the level of gene expression are not well investigated. Kotte and colleagues [65] proposed that fluctuations in intracellular molecule abundance are responsible for distinct phenotypes that coexist within an isogenic population. Considering MI utilization, the following model might be hypothesized: Little amounts of the MI transporter IolT1 are produced in rich medium. Upon switch to medium containing MI, uptake of the first MI molecules is therefore slow. The transcriptional expression level of *iol* genes encoding catabolic enzymes is measurable, but not far below that in cells adapted to MI [57]. In few cells and at different time points during incubation, the threshold of the copy numbers of enzymes is exceeded, and the intermediate DKPG as well as the regulatory activities of IolR and ReiD trigger the positive feedback loops involved in MI utilization. This is in line with the assumption that due to the inherent stochasticity of chemical reactions at low concentrations [6,66], the number of metabolically active cells within a *Salmonella* population is highly variable. Such a stochasticity or noise explains the heterogeneity of colony growth and the fluctuations in the length of the long lag phase when *S.* Typhimurium switches from rich medium to MI medium. In contrast, when *S.* Typhimurium is fully

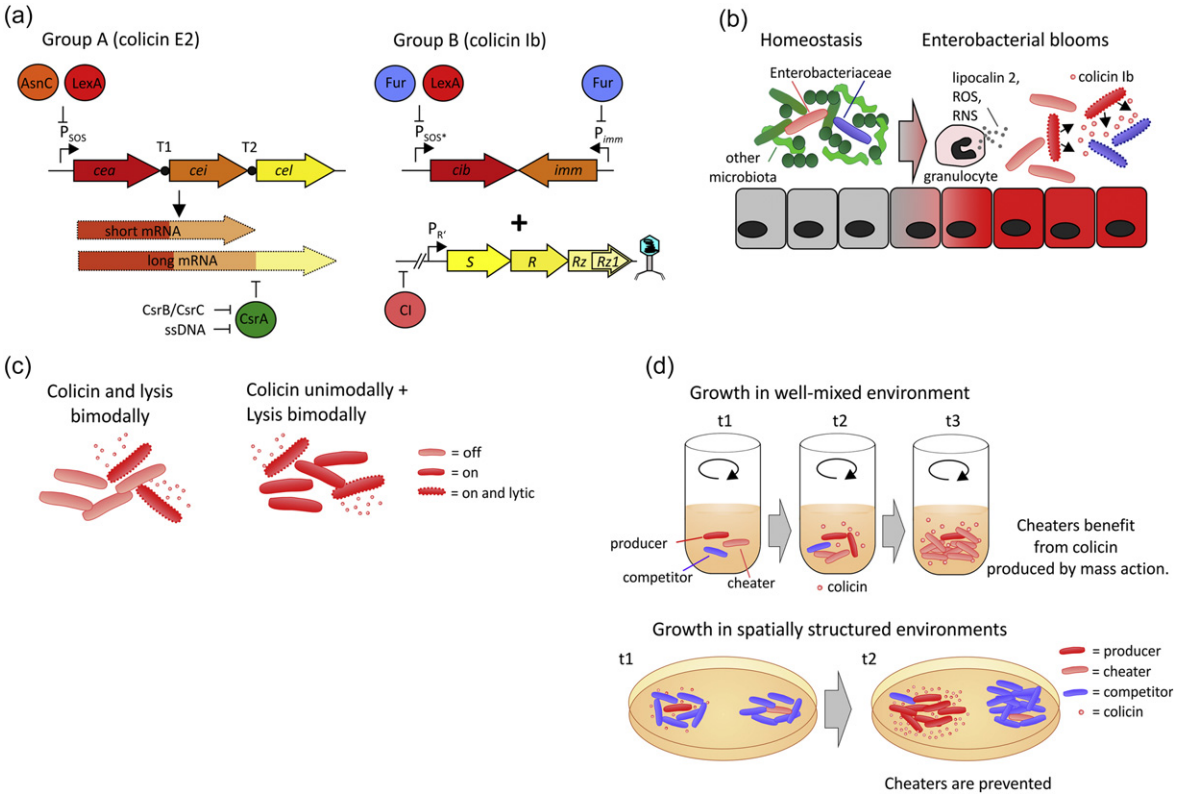

Fig. 2. Strategies to evolutionary stabilize colicin production. (A) Tight regulation of colicin production and release. Left: regulation of group A ColE2 activity gene (*cea*), immunity (*cei*) and lysis gene expression. Adapted from Refs. [81,82]. Right: regulation of group B Collb activity (*cib*) and its immunity gene (*imm*) expression, and of temperate phage lysis gene (*S*: holin, *R*: endolysin, *Rz*: i-spanin, *Rz1*: o-spanin) expression used for Collb release. In contrast to the ColE2 operon, *cib* and *imm* are located at the same locus but on opposite DNA strands. (B) Colicins are expressed in Enterobacteriaceae blooms. In the healthy intestine, the resident microbiota (green) are present in high numbers, which keep Enterobacteriaceae (light red and blue) at low numbers. Upon inflammation, for example, induced by *S.* Typhimurium infection, the host immune response leads to environmental changes in the gut, which favor high loads of Enterobacteriaceae. Upon such environmental cues, Collb is produced and released by *S.* Typhimurium (red), and provides a competitive advantage over colicin-sensitive commensal *E. coli* (blue) in enterobacterial blooms [14]. (C) Bimodal gene expression can be used to evolutionary stabilize colicin production. Either a small fraction of a population produces and releases colicin or, if the entire population produces colicin, only a small fraction releases the toxin. (D) Prevention of the emergence of cheaters. In a well-mixed environment (e.g., growth in liquid culture), the coexistence of a colicin producer (red), a sensitive competitor (blue) and a cheater (light red) (e.g., resistant to colicin but not able to lyse and release colicin) is not favored (t1). As in this scenario, colicin is released globally and the producer outcompetes the sensitive strain (t2). In return, it is outcompeted by the cheater, as this strain does not bear the cost for colicin release (t3). Hence, the cheater can benefit from colicins produced by mass action. In contrast, growth in spatially structured environments enables local colicin production and multilevel evolution. In the presence of a sensitive competitor strain, local colicin production by the producer evolutionary stabilizes the producer population despite high costs.

adapted to growth with MI and then inoculated into a medium with glucose, a fast repression of MI degradation takes place, possibly due to the synergistic and well-balanced activity of the regulatory steps described above.

Furthermore, the dilution of enzymes due to cell division and partitioning into daughter cells or due to the lifetime of proteins involved in MI degradation might play a role in the heterogeneous phenotype [67]. We therefore speculated that hysteresis, for example, the state of a biological system that is determined by past rather than present conditions [68], might be another facet of the MI catabolism in *S.* Typhimurium. Indeed, such a memory effect, which is a common feature of bistability [48], is observed when *S.* Typhimurium is adapted to MI-containing medium, exposed to rich (LB) medium for several hours, and re-inoculated into MI medium. The longer the cells resided in rich medium, the longer it takes to restart growth in MI medium. After 10 h of adaptation in rich medium, the memory of *S.* Typhimurium is completely lost [64], and the lag phase of these cells in MI corresponds to that of cells taken from an overnight culture. Obviously, a subpopulation, which

shrinks with increasing exposure time in rich medium, still remembers the previous state to possibly switch back to the former metabolic condition and thus gain a fitness advantage. These data also demonstrate that a *S.* Typhimurium culture responds asymmetrically when (i) cells are deprived of rich medium and exposed to MI medium, or when (ii) cells adapted to MI utilization are shifted into medium with a better carbon and energy source. While the reaction time in exposure to the first setting is very low and heterogeneous, it is fast and homogeneous in the second setting.

Heterogeneity in Colicin Production and Release

Bacteriocins as bacterial competition factors in *Salmonella* and *E. coli*

Production of bacteriocins is a widespread example of interference competition in complex bacterial ecosystems. Bacteriocins in Enterobacteriaceae are classified according to their molecular weight as colicins (25–80 kDa) or microcins (>10 kDa) [69–71]. Once released by the producer, colicins and microcins kill susceptible closely related bacteria, typically of the same species, whereas the producer population is protected by the expression of a cognate immunity protein [69,71,72]. Colicins are further categorized, according to the pathway to enter the target bacterium, in group A (Tol-dependent; e.g., colicin A, E1–2, K) and B (TonB-dependent; e.g., colicin B, Ia, Ib) [73]. Microcins are grouped in class I and II, based on their molecular weight, the presence of disulfide bonds and posttranslational modifications [74]. Notably, colicin- and microcin-producing bacteria are abundant in the mammalian intestine [13,75,76]. Importantly and in contrast to microcins, colicin production is a very costly trait, as bacteria do not possess a specific colicin export machinery [69,71]. Instead, colicins are released by cell lysis, killing those individuals that produced the public good. Several mechanisms were identified to evolutionary stabilize colicin production, as outlined below.

Tight regulation of colicin production and release

One strategy to evolutionary stabilize colicin production is to ensure that a sufficient amount of colicin molecules is produced within the bacteria prior to cell lysis. Colicin production is under negative control by the LexA-repressor and is therefore induced by the bacterial SOS-response following DNA damage for instance by UV-irradiation, antibiotics or metabolic by-products like reactive oxygen species (ROS) [77] [78]. In addition, some colicins are also under control of nutrient responsive regulators such as ferric uptake regulator Fur (colicin Ib) [14], transcription regulator AsnC (colicin E2/6/8) [79] or IscR (colicin E1, K, N) [80]. This confers a tight repression of colicin production until colicin release really pays off for the population (Fig. 2A).

As colicin release by cell lysis can be detrimental for the producing population, release mechanisms need to be tightly regulated as well. All group A colicin operons encode, in addition to the colicin activity and immunity gene, a cognate lysis protein. As the lysis gene is generally located at the end of the operon, it is co-transcribed with the colicin, yielding high amounts of short mRNA transcripts (only colicin or colicin and immunity gene) and low amounts of long mRNA transcripts (including all genes of the operon) [69]. Furthermore, it is known that colicins are released into the environment approximately 60–90 min after induction [81,83–85]. This suggests that the synthesis of colicin lysis genes is, in addition, regulated on the post-transcriptional level to ensure that sufficient amounts of colicin are present in the cell prior to lysis. Such a mechanism has indeed been shown for colicin E2 and E7, which are regulated by the carbon storage regulator CsrA (colicin E2 and E7), conferring a delay between transcription and translation of the lysis gene [81,86,87]. In case of ColE2, CsrA by itself was shown to be regulated by the small RNAs CsrB/CsrC and by ssDNA [81]. In addition, colicin cognate lysis proteins are small lipoproteins, produced first as precursors and thus underlying lipid-modification events, which also delays colicin release until a sufficient amount of toxin molecules are produced [69].

In contrast to group A colicins, a majority of group B colicin operons do not encode for specific lysis genes [69]. This is also true for colicin Ib (ColIb) produced by *S.* Typhimurium strain SL1344 [15]. Thus, the release mechanism of group B colicins has been unclear for a long time. Recently, we showed that ColIb relies on temperate phage-mediated cell lysis process for its release [84].

Phages propagate inside of the host cell mainly by the lytic or lysogenic life cycle. Virulent phages directly start to produce phage particles after host cell infection and thereafter follow the lytic cycle. In contrast, temperate phages integrate into the host's chromosome and replicate as prophages vertically together with the host cell (lysogenic life cycle) [88]. Upon environmental changes, which lead to the induction of the bacterial SOS-response, for example, ROS production upon inflammation, prophages can excise from the bacterial chromosome and start the lytic cycle to transmit to other hosts [78,89,90]. ColIb-producing strain *S.* Typhimurium SL1344 encodes four different prophages: SopEΦ, Gifsy-1/-2 and ST64B [91–94]. ColIb is mainly released by the ST64B mediated lysis, but prophages Gifsy-1 and Gifsy-2 also contribute to the release [84]. The temperate phage-mediated colicin

release mechanism is not restricted to *Salmonella* and is operational in *E. coli* strains carrying functional prophages.

In line with group A colicin release, it was shown for group B ColIb, in bulk assays as well as at single cell level, that its release occurs with a delay of ~60 min relative to colicin expression [84,85]. This can be explained by complex regulation of prophage activation. In contrast to colicins, the prophage-derived CI repressor of lambdoid phages regulates the SOS-dependent activation of prophages. Its cleavage, needed for de-repression, is less efficient compared to LexA. Thus, prophages are only induced upon a severe and permanent DNA damage [95]. In addition, prophage late genes, which including the lysis genes, were shown to be expressed with a delay, namely after the genetic switch to the lytic cycle [96]. The phenomenon of delayed host lysis as compared to group A and B colicin production might ensure the production of a sufficient amount of the toxin prior to lysis. For instance, in case of a regressive SOS signal and in some colicin systems, bacteria can switch from the colicin producing state back to a non-producing state before the irreversible host cell lysis starts [97]. As proteases are also known to be synthesized upon activation of the SOS response, colicins might be degraded.

Colicins are expressed under environmental conditions when it pays off

S. Typhimurium SL1344 synthesizes ColIb, which is encoded on a large conjugative colicinogenic plasmid (pColIB9), upon intestinal inflammation [15,98]. Colicin plays an important role as competition factor for *S.* Typhimurium under inflamed conditions against commensal Enterobacteriaceae [14]. Typically, in the healthy mammalian intestine, the resident microbiota harbor mainly obligate anaerobic bacteria, which suppress colonization of commensal and pathogenic Enterobacteriaceae and mediate their low abundance in the gut [99]. When environmental conditions change in response to intestinal inflammation [3], new substrates such as the electron acceptors O_2 and NO_3^- are generated that favor the growth of facultative anaerobic Enterobacteriaceae (Enterobacteriaceae blooms) [100]. Under these conditions, Enterobacteriaceae in the gut compete with each other for limited resources. With increasing abundance of colicin-susceptible relatives, populations producing colicin gain higher competitive advantage. Colib expression is induced by environmental signals found in the inflamed gut (Fig. 2B). Here, reactive nitrogen and oxygen species (RNS, ROS) [101,102] are generated, which are activators of the bacterial SOS response. Furthermore, neutrophils also produce lipocalin-2, which sequesters bacterial siderophores and profoundly limits iron [103]. As colicin Ib is also negatively regulated by the ferric uptake regulator (Fur), this iron-limited inflammatory environment promotes ColIb production [14]. Using ColIb as competition factor, *Salmonella* can outgrow ColIb-sensitive commensal *E. coli* [14,100]. On the other hand, also *E. coli* strains can compete with pathogens like non-typhoid *Salmonella* spp. by microcin-dependent killing in the inflamed intestine [13]. Hence, also microcin-dependent killing is enhanced under this condition [12].

Colicin systems rely on stochasticity in gene expression to confer a competitive advantage

As successful colicinogenic competition is inevitably coupled to colicin release by cell lysis, it is generally assumed that only a fraction of a population altruistically expresses the respective colicin system. Thus, division of labor at the level of colicin production allows to compete with colicin-sensitive strains and stabilizes the altruistic phenotype. Specifically, stochastic expression of colicins has been shown for group A colicins (colicin A, N, K, E1, E7) during growth in the stationary phase [86,104–106]. For ColIb, it was shown that stochastic expression is restricted to growth in stationary phase or low exogenous stress levels and is abolished when bacteria are growing under colicin-inducing conditions [107]. However, single-cell analysis showed that under colicin-inducing conditions, ColIb production is uniformly induced in the entire *S.* Typhimurium population [107]. Lately, this was also demonstrated for group A colicin E2 in *E. coli* [81,82]. The production of both colicin types seems to be a signal-intensity dependent process. The more the bacteria are affected by environmental stress, the faster and more uniformly the population responds with colicin production. The question arises how this reaction influences the evolutionary stabilization of the phenotype. Furthermore, how does the population evolutionary benefit from using this altruistic phenotype in competition with colicin-sensitive strains, when a high fraction of the population produces colicin and eventually lyses to release it?

In a follow-up study on ColIb production and its release mechanisms in strain SL1344, we demonstrated by single-cell analysis that ColIb is released by temperate phage mediated cell lysis in a fraction of the ColIb-producing population [85]. Thus, in this system, the strategy of division of labor is used at the level of temperate phage mediated cell lysis, resulting in heterogeneous colicin release, and not at the level of colicin production. In case of group A colicin E2, lysis (and colicin), gene expression is only stochastic under low exogenous stress levels [82]. Recently, it was shown that post-transcriptional regulation of CsrA by the small RNAs CsrB/C and ssDNA controls the time-point of ColE2 release [81]. This regulation of CsrA leads to a delay in lysis, which may evolutionary stabilize the phenotype of colicin production.

Coupling colicin production to phage-mediated cell lysis may stabilize the ColIb system

Three different bacterial phenotypes exist with respect to colicinogenic competition: The producer itself, a colicin-sensitive and a colicin-resistant phenotype. Notably, only 10%–50% of the naturally occurring *E. coli* strains produce known colicins, while the majority of strains with 70% is resistant to at least one colicin. Furthermore, about 30% of the *E. coli* strains are even resistant to all investigated colicins, while the fraction of sensitive strains is small [72]. According to mathematical models, the mutual coexistence of these three different phenotypes is only possible in spatially structured environments such as biofilms and the gut, and is explained by a "non-transitive-interaction network" or the game of rock–paper–scissors [108]. In this scenario, which still lacks experimental confirmation, the producer outcompetes the sensitive strain due to colicin-dependent killing. The sensitive strain in turn can outcompete the resistant strain, as this strain bears the costs of resistance, for example, a defect of the import machinery that is also used for siderophores. However, the resistant strain benefits from the ongoing colicinogenic interaction but does not bear the cost for colicin synthesis and release (cheater), and thus can outcompete the colicin producer. This classical "non-transitive-interaction network" is not stable in a well-mixed environment (e.g., liquid culture), as the producer and the sensitive strain are outcompeted by the resistant strain, preventing coexistence of all species [108–110]. The gastrointestinal tract of colonized hosts is considered as a structured environment, in which all three types of colicin phenotypes can coexist. The fact that colicin production is highly prevalent in the mammalian gut asks for efficient additional means to evolutionary stabilize colicin production.

The existence of counteracting mechanisms such as transmission between different hosts, which promote group selection [28], might explain how the emergence and spread of cheaters (e.g., defective in lysis) is prevented. Another explanation could be the uncoupling of colicin release from production and immunity. The coupling of colicin release to the lifecycle of temperate phages seems to be an excellent evolutionary strategy for this purpose (Fig. 2C).

Modeling Evolution of Bet-Hedging in a Population that Experiences a Stochastically Switching Environment

Methods and approaches of evolutionary theory in a nutshell

In the next sections, we will discuss evolutionary mechanisms underlying formation and stability of MI degradation and colicin release in *S.* Typhimurium. First, we briefly introduce the most important concepts of evolutionary theory, which can also be applied to bacterial populations. The basis of all evolutionary theory is Fisher's fundamental theorem of evolutionary selection from 1930: The average fitness of a population is maximized. It is possible to decrease the fitness of a subpopulation (e.g., by division of labor or bet-hedging), as long as this decrease is (over)compensated by a consequential fitness increase of another subpopulation.

Already in 1932, Haldane understood that cooperation is stable if the recipient of a cooperative action is closely related to the actor. On this basis, Hamilton [111] developed his seminal theory about cooperation based on kin selection. At the same time, the importance of the group structure of populations was recognized, leading to a discussion of the consistency of these two theories. In contrast to higher animals, where individuality allows for reciprocal cooperation [112,113], it is generally accepted for bacterial communities that kin and group selection coincides, as bacterial colonies are most likely to consist of highly related individuals [36,38]. The unified concept is often addressed as multilevel evolution [114]. There are two options: In multi-level selection 1 (MLS1), groups are mainly addressed as the environment of individuals, while in multi-level selection 2 (MSL 2), the fact is emphasized that cells will create new colonies [115]. It is widely accepted that multilevel evolution is the most important mechanism that stabilizes bacterial cooperation, although further experimental evidence is still required. By chance, clonal communities can emerge that have a high fraction of cooperating individuals, for example, those that collaborate by division of labor. As the fitness of cooperating communities is above average, these subpopulations grow fast and spread the cooperative genotype sustaining cooperation. This mechanism is enhanced by a multitude of supporting mechanisms as kin recognition, punishment or privileged share [36,116,117].

In the 1970s, the combination of Fisher's fundamental theorem and game theory led to the notion of evolutionary stable strategies (ESS) [118]. An ESS cannot be invaded by other strategies and thus forms an endpoint of evolution. Around 1990, this concept has been extended by the investigation of non-ESS strategies' dynamics at the evolutionary time scale. Adaptive dynamics describes the fate of an isogenic resident population, in which from time to time, a rare mutant appears [119,120]. Importantly, the mutant faces an environment shaped by the resident. If the fitness of the mutant in this given environment is higher than that of the resident, the mutant will spread, out-compete the resident and become the new resident (Fig. 3). By analyzing this process, it

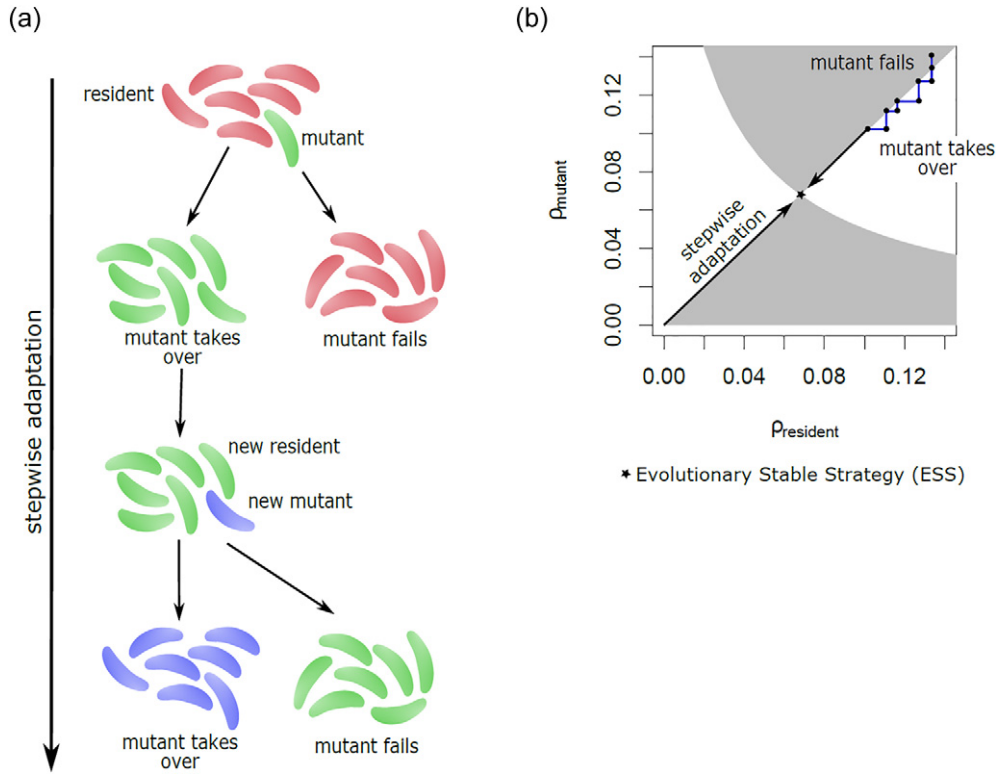

Fig. 3. Adaptive dynamics and pairwise invisibility plot (PIP). (A) Adaptive dynamics. Adaptive dynamics describes the fate of an isogenic resident population. From time to time, a rare mutant (green) appears with a different trait than the resident (red). This mutant either takes over or fails. If the mutant takes over, then it becomes the new resident. After some time, another mutant (blue) turns up that either takes over or fails. Thus, the bacterial population adapts a trait stepwise. (B) PIP. We investigate the probability ρ that a phage does not trigger the lytic pathway upon infection but becomes silent. The *x*-axis indicates the probability $\rho_{resident}$ for a resident population, the *y*-axis indicates the probability ρ_{mutant} of a mutant. If for a given value of $\rho_{resident}$ a mutant phage with value ρ_{mutant} can spread, the point ($\rho_{resident}$,ρ_{mutant}) is marked in white, else it is marked in gray. The gray/white region is computed on base of an ecological competition model for the two types of phages. This PIP allows to analyze the change of the parameter ρon the evolutionary time scale: The population starts with a given parameter $\rho_{resident}$ of the resident population. From time to time, a rare mutant appears. If the point ($\rho_{resident}$,ρ_{mutant}) is in the gray region, the mutant dies out and nothing happens. If the point ($\rho_{resident}$,ρ_{mutant}) is in the white region, the mutant out-competes the resident and becomes the new resident. In this, the parameter $\rho_{resident}$ changes. This mechanism yields a drift of the parameter until it reaches the ESS (indicated by *), where no mutant can invade any more. We find that the ESS is given by a positive, but small probability of the phage to become lysogenic instead of lytic.

is possible to follow the change of a population's trait at evolutionary time scale, for example, toward an ESS. This approach allows explaining the appearance of bet-hedging as a consequence of an environment that varies over time [121–123].

Heterogeneity of the gut environment favors bacterial cooperation

It is still unclear if cooperation or division of labor is generally widespread in intestinal bacterial populations. The experiments discussed in the present paper show that the enteric pathogen *S.* Typhimurium cooperates in form of division of labor to increase its virulence. In any case, the gut is a special environment with two particular properties that are well known to favor evolution of phenotypic heterogeneity: It is spatially heterogeneous and is heterogeneous in time, that is, subject to environmental fluctuations [124]. With a focus on the spatial structure of the gut, we note that bacterial populations in the gut do not form well-mixed communities. Instead, local bacterial communities establish in different regions of the gut, for example, the small and large intestine, the mucosa and the gut lumen (Fig. 1B). These clonal communities stably exist for certain time intervals. Multi-level evolution or group selection [115,125] is precisely based on this situation (Fig. 2D).

Heterogeneity in time—switching environment and bet-hedging

For the two examples in *S.* Typhimurium discussed above, the environmental fluctuation over time is even more interesting than the spatial

heterogeneity. *S.* Typhimurium cannot adapt to fixed environmental conditions, but needs to respond to a changing environment on a fast time scale. Changing conditions can be caused by variation of the diet and inflammatory immune responses eliciting Enterobacteriaceae blooms. It is known that a switching environment promotes phenotypic heterogeneity [122,123]. When considering the average fitness not only over all individuals but also over time, fitness is not maximized if individuals prepare for various environment conditions, as simultaneous expression of all corresponding pathways is energetically unfavorable. Instead, the population employs a bet-hedging strategy by which the majority will be optimally adapted to the current environment and the current environment only [47,48].

The bet-hedging strategy is very effective if the bacteria cannot directly sense the environment and thus not actively adapt their phenotype in response to a changed condition [122,123]. We are, however, interested in the less-well investigated case that bacteria are able to sense an environmental change and to respond to it, as exemplified here by MI utilization and Collb production. It is assumed that sensing a specific signal promotes a uniform response in a bacterial population [67]. We examined this situation in a toy model for MI utilization by *S.* Typhimurium. Here, we show that a varying environment may also led to a bet-hedging behavior.

MI utilization as a bet-hedging strategy in a switching environment

The activation of the pathway that allows *S.* Typhimurium to utilize MI as sole carbon and energy source comes along with a distinct metabolic burden as demonstrated recently [61]. Bacteria will only activate this metabolic pathway in the presence of MI and the absence of more favorable substrates. As discussed above, experimental results demonstrate that only a subset of the entire *S.* Typhimurium population activates this metabolic pathway. These experimental findings are now considered with respect to the resulting fitness consequences.

MI is repeatedly, but only temporally present in the gut environment (e.g., a dietary substrate). The amount of the resource is variable, and the bacteria cannot predict the time points at which the resource appears.

Access to the resource MI is costly as transporters and degrading enzymes have to be produced. That is, a cell that can access the resource has some benefit B and some costs C. Here, B is a random variable, as the benefit due to the resource (time interval during which it is available, amount of the resource) does change every time it appears. We define the background fitness of all bacteria within the population as β. We furthermore introduce an indicator variable θ that indicates if a given bacterium accesses the resource ($\theta = 1$) or not ($\theta = 0$). The fitness of a bacterium is thus

$$f = \beta + \theta\,(B - C).$$

The expected fraction of the population that access the resource is $p = E(\theta)$. Furthermore, $\overline{B} = E(B)$ denotes the expected amount of the resource. With these definitions, the average fitness of the population reads

$$\overline{f} = \beta + p\,(\overline{B} - C).$$

If the expected benefit outweighs the costs, $\overline{B} > C$, the average fitness is maximized by $p = 1$, such that all bacteria go for the resource, while $\overline{B} < C$ leads to the situation that no individual accesses the resource ($p = 0$). We get an all-or-nothing decision, and no phenotypic heterogeneity. Obviously, phenotypic heterogeneity (which is the case for $0 < p < 1$) is caused by an additional mechanism.

The main influencing factor is the capacity of the resource. The more bacteria access the resource, the smaller the benefit per bacterium. If we think about a fixed amount of nutrient, it is obvious that the more bacteria feed on this nutrient the less nutrient a single bacterium receives. A negative-frequency-dependent effect comes in. In that, we modify the fitness of a bacterium,

$$f = \beta + \theta\,(B(p) - C)\,.$$

where $B(p)$ is the benefit for one bacterium, given that the fraction p access the resource. It is a decreasing function. The average fitness now reads

$$\overline{f} = \beta + p\,\left(\overline{B}(p) - C\right)\,.$$

The function $p(\overline{B}(p) - C)$ is maximized and, due to the additional nonlinearity in $\overline{B}(p)$, phenotypic heterogeneity can appear. In an explicit example, we assume that each bacterium receives its fair share. Then, the benefit per individual is proportional to the total amount of the resource $\overline{B}$, divided by the size of the subpopulation that access the resource, $\overline{B}(p) = \overline{B}/p$, resulting in

$$p\left(\overline{B}(p) - C\right) = \overline{B} - pC.$$

This function is maximized if p is minimized. Only very few bacteria or one single bacterium should access the resource. This result can be understood intuitively as each bacterium pays the costs C to access the resource, while the benefit for the population is always $\overline{B}$, independently of the number of individuals that go for it. Therefore, it is optimal to have one single bacterium that pays the costs, but supplies the population with the complete benefit. In more realistic models, however, there is competition

with other species or loss of resources by dispersal, such that the shape of $\overline{B}(p)$ will be more complex. Another aspect not considered in our model is the symmetry of all bacteria: there is no way to exactly choose one single bacterium to synthesize all enzymes of the pathway, but there is a molecular mechanism that activates expression of the trait a small proportion of the population. This proportion needs to be large enough to ensure that the resource is effectively accessed, but not too large, such that the benefit per activated bacterium is still above the investment. Taken together, our model shows that a reliably sensed switching environment leads to phenotypic heterogeneity if the benefit per bacterium is negative frequency-dependent.

A model shows that the uncertainty about the time interval that the resource is available leads to bet-hedging [126]. Here, the author predicts that cells activate the pathway in a synchronized way, but only at stochastically distributed time points. In this way, only few bacteria will access the resource if it is present for only a short time, but an increasing number of bacteria go for the resource if it prevails. The picture developed based on evolutionary arguments agrees with empirical findings: *S.* Typhimurium activates the MI-pathway in response to MI, there is a long lag phase and a high stochasticity in timing the MI pathway activation, and only part of the population activates the pathway. To further test the validity of the evolutionary model, the quantification of costs and benefit and the quantification of the natural occurrence of MI can be used to identify the parameters of the model. It is then possible to verify if the parameters are within the range where phenotypical heterogeneity is predicted.

Colicins: cooperation is free-riding on a selfish trait

As CollB release is not directly controlled by the colicin system but is achieved by prophage-induced lysis, we first disregard colicin production and focus on the lysogenic phage that eventually mediates colicin release. After infection of a cell, a lysogenic phage has two different strategies: it takes either the lytic or the lysogenic pathway. In the lytic pathway, the phage replicates in the cell and immediately triggers bacterial lysis. In the lysogenic pathway, it integrates as a prophage into the genome and remains in a quiescent state. The prophage is reactivated from the quiescent state by signals triggering a SOS response. These two different lifestyles serve as a bet-hedging strategy [127,128]. The lytic pathway is beneficial for the phage primarily if an epidemic is going on, that is, if there are uninfected “prey” bacteria in the vicinity. If this is not the case, the virions released by the lytic pathway are—from the phage's perspective—a loss as it is better for the phage to remain lysogenic and to wait for new prey. A typical situation where prey bacteria are abundant is inflammation-induced Enterobacteriaceae blooms in the gut. Common environmental signals in blooms are iron deficiency and triggers of the SOS response. If the SOS signal reliably indicates the presence of susceptible bacteria, all prophages should switch to the lytic phase as soon as possible: the earlier a prophage triggers the release of virions, the more bacteria it can infect. If two phage strains compete, the faster strain will outcompete the slower strain. However, the assumption that an SOS signal reliably indicates the presence of susceptible bacteria is not true, as SOS signals may be triggered in the absence of blooms. If all prophages switch to the lytic pathway at a false signal, they are basically lost. Therefore, only part of the prophages will switch to the lytic pathway. This argument parallels the generic argument from above: The expected average fitness of lysogenic phages has a strong negative frequency-dependent characteristic. Only part of the phages triggers lysis in response to the SOS signal; the remaining part stays dormant. In the same way, only part of the phages that did infect a cell triggers lysis immediately; a certain fraction of the phages becomes prophages and stays quiescent (Fig. 3). Lysis, from the phage's perspective, is a selfish trait.

The release of CollB by cell lysis is, from the selfish gene's perspective, the ultimate altruistic trait as genes of a lysing cell will not have offspring. However, the colicin release improves the situation of clonal cells, and Hamilton's principle that requires assortment and multilevel evolution can explain this altruistic strategy. For colicin release systems of group A, which are independent of phages, recent experimental results are in line with this explanation [129]. For a group B colicin like *collb*, we recently showed that the combination with lysogenic phages makes colicin release an evolutionary stable system in the absence of a multilevel setting [85]. As we know that the phages induce the lysis of a subpopulation that will lyse in any case, the additional colicin release does not impose further costs, but rather provides an additional benefit for the remaining population. Any cooperation without costs is beneficial. That is, cooperation is evolutionary stable in this case, as the costs are not due to the cooperation but the population carries the costs anyway. The cooperative trait free-rides on the phage's selfish strategy. Metabolic prudence [130,131] is a similar concept to understand cooperation under certain situations. If the production of a public good only requires resources that are not growth limiting, the production basically does not cost anything. In this case, cooperation has only beneficial aspects and is assumed to be evolutionary stable.

Discussion

We investigated two *Salmonella*-specific strategies to compete with the challenges encountered during colonization of the gut, namely, the production of colicin to kill competitors and the utilization of MI to occupy a metabolic niche presumably not accessible for other commensal bacteria. ColIb production and MI utilization differ from each other in one aspect: production of a common good is not observed in the metabolic pathway. However, at last four mechanisms to evolutionary stabilize bistability that are common for colicin production and MI degradation were identified: (i) Factors involved in the production of ColIb or of catabolic enzymes are tightly regulated and controlled by specific environmental stimuli to avoid detrimental effects for the bacterial population. (ii) The regulation of gene expression strictly depends on the environmental conditions under which colicin production or MI degradation provide a selection advantage for the population, especially during infection and with respect to overcoming the colonization resistance. (iii) Heterogeneity in both cellular functions is based on stochastic gene expression. (iv) The stochastic switching on a single-cell level with regard to MI utilization or colicin release by temperate phages is a successful bet-hedging strategy of this pathogen in the sense that this risk-spreading strategy evolved in unpredictably changing environments as encountered in the gut [47].

With respect to the two strategies of *S.* Typhimurium to successfully colonize its host, several open aspects require further investigations. For example, experimental evidence is still lacking that confirms the advantage of bistability in MI utilization or colicin production under *in vivo* conditions. Furthermore, the molecular mechanisms underlying the heterogeneous gene expression are little investigated. It might also be interesting to address the fate of single *Salmonella* cells that switch from the OFF to the ON modus with respect to MI degradation or colicin production, but then encounter a switching environment that does not further require these capacities.

We hypothesized that coupling colicin production to the selfish phage lifestyle can evolutionary stabilize the phenotype of colicin production, as phages defective in lysis would be extinguished. Mathematical modeling with temperate ColIb-producing phages confirmed this finding even in the absence of spatial structure, for example, in the inflamed intestine (see modeling part below for more details) [85]. However, a number of questions regarding colicin production in *S.* Typhimurium still remain open. What are the *in vivo* cues leading to induction of colicin expression and release in the gut, and is colicin production induced in the entire bacterial population or in one subset only? Then, it is still unclear if temperate phage-mediated release also takes place during infection, in particular during intestinal inflammation. Inflammation activates prophages of *S.* Typhimurium and fosters the transfer of phages between bacterial populations [89], suggesting that ColIb may also be released by phage-mediated lysis in the inflamed intestine. Further questions arise whether this colicin release mechanism is also true for other colicins, which lack a cognate lysis gene. How did colicin systems evolve that uncouple colicin production from the colicin release process? It is also interesting to investigate differences in the ecology and lifestyle of bacterial strains producing group A and B colicins. A genome-wide survey provided first insights into differential distribution of colicin and phage lysis genes in specific *S. enterica* serovars [85].

In the classical theory of bet-hedging, individuals do not sense the environment. With respect to MI utilization, however, we observed a bet-hedging behavior of *S.* Typhimurium, which realizes the concentration of this substrate outside the cell. It is therefore of interest to understand why a switching environment may result either in a synchronized response or in a bet-hedging behavior. Can we find criteria that can be put to the test to tell these two behaviors apart? Evolutionary theory is challenged to identify general concepts that reliably predict if heterogeneity or a homogeneous response of the population is favored by evolution. Moreover, sensing of the environment still leaves uncertainty about the exact conditions, and their quantification would improve the analysis of conditions that select heterogeneity. We found that a main driving force that shaped the behavior of *Salmonella* is the uncertainty of switching environments during infection. Further modeling attempts are needed to derive theoretical predictions in this field that could then be confirmed by experimental analysis.

The (bio)geography of the intestine with its fluctuating environments is yet poorly characterized. It is assumed that spatio-temporal mechanisms impact the diversity of bacterial populations and thus the overall colonization of the intestine [124,132,133]. Therefore, it can be hypothesized that temporal fluctuations and spatial structures in the gut are driving forces of the evolution and stabilization of heterogeneity. However, not so much is known about the spatio-temporal distribution of nutrients. Approaches to model the metabolic interactions between the gut microbiota and the host [133–136] will help to gain a better understanding of heterogeneity in enteropathogens. Moreover, metabolic network modeling of microbial communities includes aspects like compartimentalization, non-transitivity, the game of rock–paper–scissors and the perturbation by dietary changes or antibiotic therapies [49,137,138].

To conclude, the pathogen *S.* Typhimurium is a well-suited model organism to further study the evolutionary processes stabilizing heterogeneity in exploitative and interference competition in the two strategies addressed in this review.

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Abbreviations used:
MI, *myo*-inositol; T3SS, type 3 secretion system; SPI1, *Salmonella* pathogenicity island 1; FC, flow cytometry; ESS, evolutionary stable state; Ctx, cholera toxin; ROS, reactive oxygen species; PIP, pairwise invisibility plot.

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