Phenotypic Diversification of Microbial Pathogens—Cooperating and Preparing for the Future

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Abstract

Recent studies revealed an amazing phenotypic heterogeneity between genetically identical individual cells within populations of microbial pathogens. During the course of an infection, subpopulations occur, which differ in certain virulence-relevant factors, stress adaptation functions or physiological and metabolic abilities. The mechanisms driving this heterogeneity are divergent reactions of the pathogens to differences in host tissue microenvironments. In addition, certain genetic regulatory circuits with positive feedback loops and stochastic differences in gene expression can generate endogenous fluctuations in regulatory components leading to bistable expression of virulence-associated functions. Here, we focus on the occurrence of phenotypic heterogeneity in populations of well-studied examples of pathogens, which enables cooperative, social behavior where a subpopulation of producers shares fitness- and/or virulence-relevant goods and traits with non-producers. We further highlight that this strategy allows preadaptation of a subgroup of cells to recurrent and thus predictable changes of the environment that they encounter during the different stages of the infection. The diversity within bacterial communities has a significant influence on the survival of the pathogens within their hosts and the progression of the disease.

Introduction

Cooperation *versus* self-interest, defined as "regards for one's own interest and advantage with disregard of others" in dictionaries, is a fundamental, classical dilemma in the societies of any living being, even in the simplest form of life. How to get the individual to cooperate despite or in conjunction with their self-interest is crucial for the achievement of common goals in bacterial, plant, animal, and human populations. The results from self-interested behavior are immediate gains for the individual, whereas the long-term benefits from cooperation may be better fitness, survival, proliferation or selective advantages to the entire population [1–3]. In this respect, it is fascinating to see that molecular mechanisms that drive cooperative behavior have evolved already in the simplest form of an organism —in the prokaryotes [4–6]. One important feature of cooperation is *division of labor*, that is, separation of tasks, with each task performed by a separate cell or subpopulation of cells [3,7]. This is only possible if individual bacterial cells within a clonal population acquire different properties (e.g., by expressing different metabolic pathways, by inducing distinct stress adaptation traits or producing different virulence factors), and thus display new functionalities. This heterogeneity within populations of genetically identical cells (clonal populations) allows the microbe to share fitness-relevant goods and virulence-associated traits and enables them to preadjust their properties to sudden environmental changes.

Major questions in the context of phenotypic heterogeneity are (i) when and how heterogeneity is generated within a microbial population, (ii) which molecular mechanisms drive heterogeneity, (iii) which genetic systems/functions are differentially

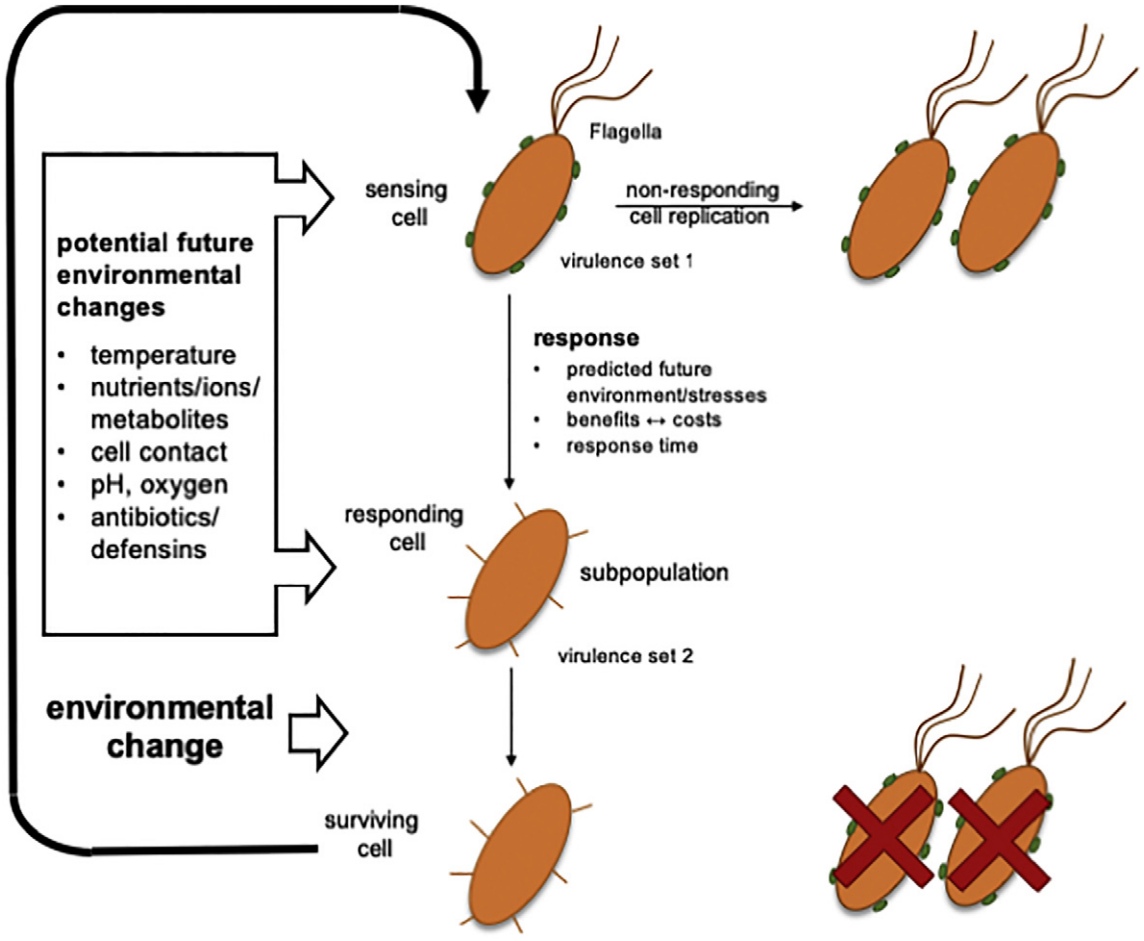

Fig. 1. Environmental signals and host factors influencing response of sensing cells and selection of developed subpopulation. A microbial pathogen senses environmental parameters and changes in its host environment and has to decide how to respond. Based on the signals, they have sensed that they can make a decision to respond to the current conditions and/or predictable future conditions. The overall outcome of this response will depend on the expected cost and benefits of the reactions, cooperative or competitive actions of other surrounding microbial cells, and the response time.

expressed in distinct subpopulations, and (iv) which of them come at a fitness cost and/or offer additional benefits. One possible benefit would be "*preadaptation*" to recurrent environmental changes/stresses experienced in host niches, which will be colonized by the pathogen in the course of the infection. This can be the expression of certain structures, physiological processes, properties, or the behavior by a subpopulation of cells that are highly suited to the new, recurrent host habitat. Up to date, several examples of phenotypic heterogeneity in populations of microbial pathogens have been discovered and were further characterized [8–17]. Based on these examples, we present the different regulatory strategies and underlying mechanisms promoting cooperative behaviors and discuss their impact on the biological fitness and pathogenicity of the pathogen. A special focus is set on enteric pathogens, in particular *Yersinia pseudotuberculosis*, a mainly extracellular growing intestinal pathogen studied in our laboratory, which gained attention with respect to this topic.

Developing Preadaptation Strategies

Advantage of preadaptation strategies in fluctuating environments

Although heterogeneity itself is not genetically determined, the driving mechanisms are selectable, beneficial traits, which are under evolutionary pressure [4,18,19]. During the infection process, microbial pathogens are constantly exposed to sudden and often detrimental changes in their host niches, and this process promoted the evolution of preadaptation strategies [20–22]. For instance, enteric pathogens such as enteropathogenic *Escherichia coli*, *Yersinia*, and *Salmonella* species

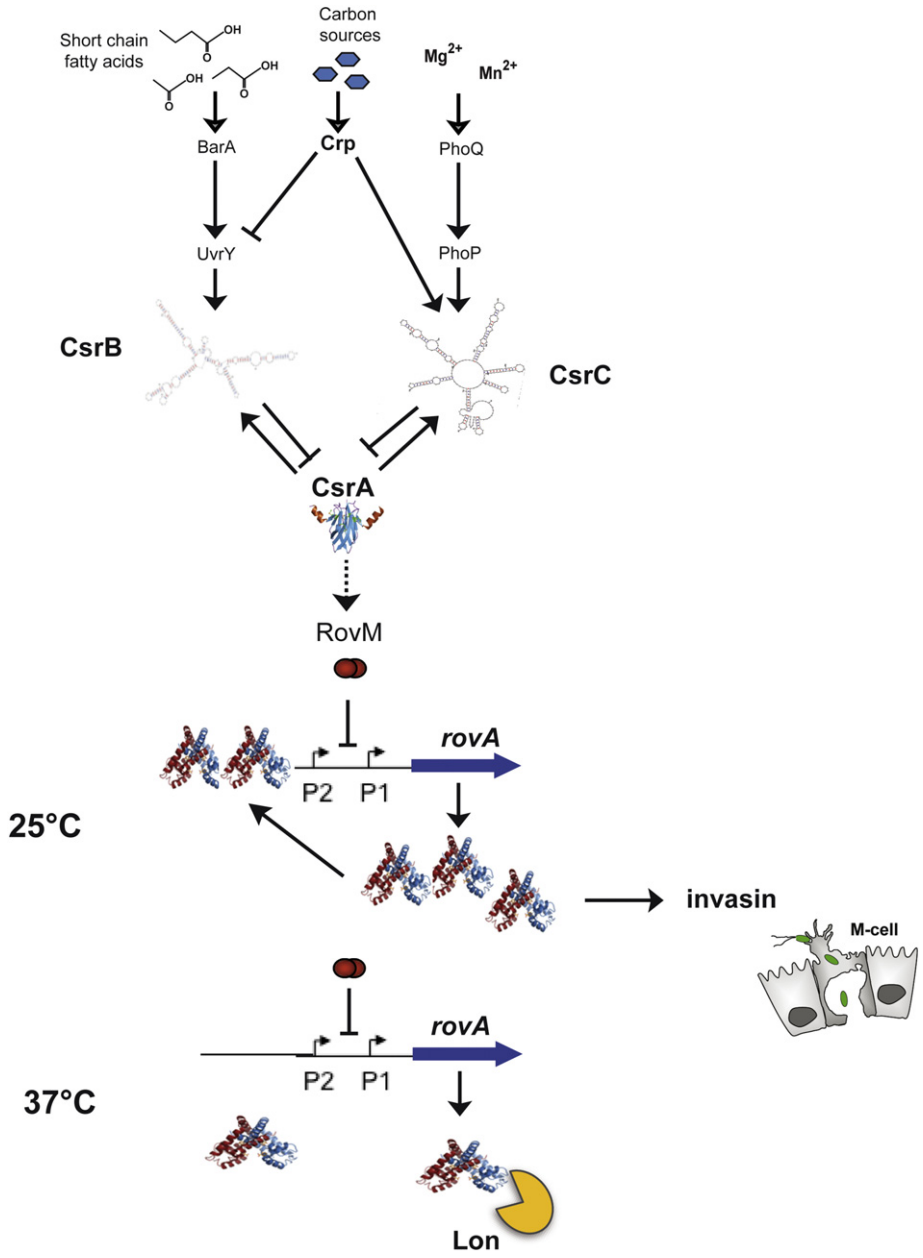

Fig. 2. Regulatory network controlling bistable expression of RovA and invasin (InvA). The transcriptional activator RovA senses temperature changes with an internal implant. A thermal upshift from moderate to body temperature induces a conformational change that reduces its DNA-binding activity and renders the regulator more susceptible to degradation by the Lon protease. Consequently, less RovA is present to activate its own and *invA* expression, leading to less-invasive bacteria. The activation level can be further modulated by a complex regulatory cascade, which is triggered by the two two-component system BarA/UvrY and PhoP/PhoQ through the post-transcriptional CsrABC system and the LysR-regulator RovM. Arrows display direct activation of gene expression or protein synthesis, whereas dashed arrows label indirect regulation. T illustrates the repression of gene expression.

experience an increase of temperature and a drastic drop in pH values when they pass the esophagus into the stomach. Upon entry into the intestine, they face a reduction in oxygen and limitations in the nutrient availability due to the microbiota. When they have the ability to cross the intestinal barrier and to invade the subepithelial lymphoid tissues, they are confronted with a strong depletion of essential ions (in particular Fe^{2+}, Mg^{2+}, and Zn^{2+}), the presence of antimicrobial compounds, and attacks by immune cells [23,24]. Microbial pathogens sense these parameters and make a decision on how to respond

to the current conditions (Fig. 1). As the course of infection and opposed environmental changes/stresses follow a recurrent pattern, a preadaptative heterogeneity can be selected and evolved, in which a subpopulation expresses fitness- and virulence-relevant factors, which are not useful in the current, but in the following host niche(s) (Fig. 1). In this respect, a pathogen can use environmental cues from the current niche to prepare and preadapt a subgroup for the next stages of the infection process. The overall outcome will depend on the expected costs and benefits of the reactions, cooperative or competitive actions of other surrounding microbial cells, and the response time.

This strategy of adaptive prediction [21,22] may reduce the overall biological fitness of the preadapted microorganisms in their typical/current conditions. However, it will, if the pathogen preadapted appropriately, increase the overall biological fitness, resulting in more offsprings and improving the chances of survival of this subpopulation under subsequent selection pressures. Thus, it is not surprising that the benefits of this "*bet-hedging*" concept are most evident in microbial pathogens, which live basically anywhere from soil to mammals, where they experience rapid and broad fluctuations in the microenvironments inside and outside their hosts. However, the pay-off and overall success of this strategy will depend on the ratio of bacteria within the preadaptive state, the response time and the fitness costs of the response (Fig. 1). Obviously, a small population of preadjusted bacteria would be advantageous, if the optimal phenotype for subsequent changes comes with a very high fitness cost. However, transfer of epithelial barriers is often achieved by a very small number of bacteria. Thus, if the preadjusted subset of bacteria is too small, the probability that an optimally preadapted microbe will reach the subepithelial niche is also very low. Consequently, often very distinct and highly specialized regulatory systems have been evolved to drive phenotypic heterogeneity of fitness- and virulence-relevant traits (described in detail in recent reviews [25,26]). Accordingly, the signaling pathways and regulatory networks driving heterogeneity of distinct functions can differ significantly. Moreover, species and even sub-species differences were observed, for example, when host-specific colonization strategies demand different measures.

A bistable switch used as preadaptation strategy

One striking example for the evolution of a professional preadaptation strategy implicating phenotypic heterogeneity and cooperative behavior has been identified in *Y. pseudotuberculosis* [13]. This microbe is extremely responsive to changes of temperature. About 25% of its genes are down- or upregulated in response to a temperature shift from moderate temperature (20–25 °C) to body temperature (37 °C) [27]. This includes the gene of the major cell invasion factor invasin (*invA*), which is predominantly expressed at environmental temperatures in the majority of bacterial cells in a culture [28,29]. The reason for this apparently nonsensical expression pattern can lie in the microbe's ability to promote rapid and efficient transcytosis of the epithelial layer after ingestion. This guarantees host tissue colonization before the host defense systems are activated. Immediate shut-down of *invA* expression upon an increase of temperature also makes sense in light of subsequent colonization of the specialized underlying lymphoid organs (Peyer's patches). There, presentation of immunogenic surface-exposed factors such as invasin is no longer required, but it could trigger activation of a deleterious immune response [29,30].

This strategy immediately raises the following questions: Does invasin expression impact fitness and how is its production regulated to balance costs and benefits? Comparative growth analysis in standard laboratory media *in vitro* did not reveal a significant growth defect between a wild-type, and an *invA* mutant strain at moderate temperatures [29]. This indicated that invasin synthesis at environmental temperatures does not come with a large fitness cost *in vitro*. However, this can be different during infection, when expression of invasin can lead to recognition and elimination by neutrophils and other recruited professional phagocytes, as it was shown to be immunogenic [30].

Expression of this preadaptive function is controlled by the regulator of virulence A (RovA), a dimeric, MarR-type transcriptional activator, which forms a temperature-responsive bistable switch. This leads to high-invasive (RovA-ON) and low-invasive (RovA-OFF) subpopulations within a narrow temperature range [13] (Fig. 2). That type of bistable expression is promoted by a highly precise thermoresponsive control element within RovA. This thermosensing implement, a certain region of the RovA dimerization domain, triggers a conformational change upon a shift from 25 °C to 37 °C that reduces the DNA binding activity and increases the susceptibility to the protease Lon [31]. This feature is combined with a positive feedback control loop of *rovA* expression, which is sensitive to stochastic fluctuations and noise amplification, similar to other positive autoregulatory systems [2,13,32] (Fig. 2). As a consequence, a bistability exists with respect to *rovA* expression in a way that individual cells can spontaneously switch from one state (e.g., RovA-ON) to the other (e.g., RovA-OFF). This reversible switching process is not only influenced by the temperature-sensitive implant; it is further manipulated by a tightly coordinated, multi-level regulatory cascade composed of the ion- and metabolite-

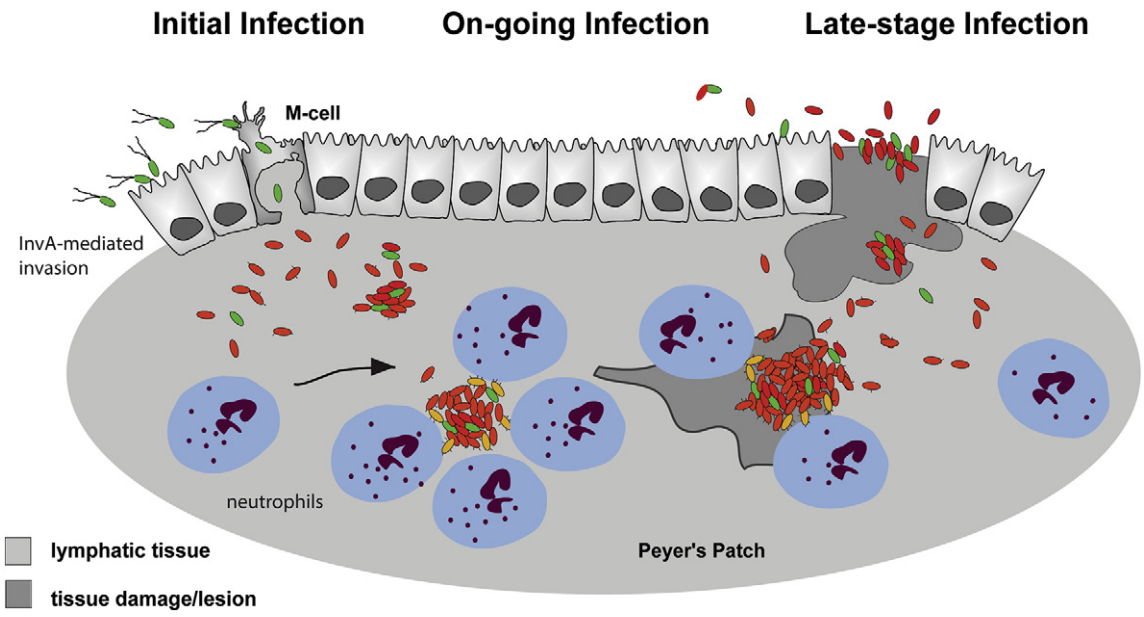

Fig. 3. Overview of heterogeneous *rovA, invA* and Ysc/Yop T3SS expression in *Yersinia* during infection. After oral ingestion of *Y. pseudotuberculosis* by contaminated food and water from the environment in which they express the virulence regulator RovA and RovA-activated invasion factor InvA (green cells), the bacteria reach the intestinal lumen. Sensing 37 °C, the bacteria start to switch off *rovA* expression. In the ileum, InvA promotes binding of the bacteria to the surface of M-cells located in the intestinal epithelial layer. After invasion and transcytosis, the bacteria reach the underlying lymphoid tissues (Peyer's patches). In this environment, most cells should have switched off *rovA* expression, which prevents new InvA synthesis (red cells), and have repressed flagella synthesis, thereby limiting their recognition by immune cells. However, individual cells, which still express RovA, are found in microcolonies formed in the infected tissue. In parallel, expression of the Ysc/Yop T3SS (black needle structures) is induced at body temperature, and its synthesis is further enhanced upon contact with recruited neutrophils and macrophages. Moreover, cells in the periphery that are in close contact with the immune cells produce NO detoxifying enzymes (yellow cells). This is likely to reduce their growth rate and makes them more resistant against immune cell-derived stresses, including nitrosative and oxidative stress, the release of antimicrobial peptides, and inflammation. Initiated tissue damage at a later stage of the infection will allow the bacteria to re-enter the intestinal lumen where RovA- and thus InvA-expressing bacteria have an advantage reinfecting the host and are better prepared for their environmental lifestyle and host-to-host spreading.

responsive two-component systems PhoPQ and UvrY/BarY, and the nutrient-responsive global carbon storage system (Csr), the cAMP-binding repressor protein Crp, and the LysR-type repressor RovM [33–36] (Fig. 2). Integrating this information has the potential to modulate the ratio of RovA-expressing cells and fine-tune the expression of virulence-relevant traits in different tissues and during different stages of the infection [13]. As the analysis of the RovA regulon uncovered different metabolic and stress adaptation genes and programs (e.g., *aceFE*, *icdA*, *sucCBD*, *acnA*, *glt*A, *ibpA*, *uspA*, *cspB*, *cspC*) for the wild-type and the *rovA* mutant [36], this may endow the bacteria with a better fitness for distinct host niches—the intestine or the lymphatic tissues. In fact, expression of alternative virulence programs by the RovA-ON and RovA-OFF population is important for virulence as a strain expressing an unstable RovA variant was strongly attenuated [13]. The continuous integration of multiple signals and parameters by the RovA regulatory network can further improve inference of the current environmental state and enables anticipation of future changes. The analysis of infected gut-associated lymphoid tissue demonstrated that a small number of bacteria are RovA-ON, whereas the majority of bacterial cells are RovA-OFF. Having inferred from an increase in temperature that they are in a host, *Yersinia* reduces the amount of RovA-ON cells to avoid mounting a protective immune response. However, the bacteria also predict that they may re-enter the intestinal tract after expulsion from damaged intestinal tissue and maintain a small number of RovA-ON cells to be prepared for potential opportunities to re-infect the host (Fig. 3).

Within the genus, variations of the *rovA* expression pattern have been observed and they may also affect the biological functions of the entire population, which can be adaptive and lead to niche expansion or separation. A RovAP98S variant, which is less susceptible to proteolysis, was detected in *Y. enterocolitica* serotype O:3 strains, the dominant serotype found in pig/boar reservoirs and cases of human disease [37]. Expression of the

RovAP89S variant by *Y. pseudotuberculosis* increases the amount of RovA-ON cells in infected lymphatic tissue in mice. This amino acid exchange is important for the efficient colonization of the pig, but it reduces the virulence of *Y. pseudotuberculosis* in mice [38]. The divergence is considered to be indicative for a change in the lifestyle of *Y. pseudotuberculosis* and *Y. enterocolitica* O:3 [39]. Differences in the RovA activity represent the consequences of a transition from environmental ubiquity, including rodents and insect vectors, to a predominantly mammal-associated pathogen. It also includes the adaption to the change of the average body temperature of 37 °C–40 °C and the prolonged mean residence time in the more voluminous hosts. This clearly shows how strongly an evolved preadaptation strategy, involving the formation of differently adjusted subpopulations, can impact microbial fitness and pathogenicity.

Phenotypic heterogeneity of virulence genes with a high energetic burden

As many microbial pathogens colonize the intestinal, respiratory, or urogenital tract, and cross the epithelial layer to colonize deeper tissues with new conditions, similar modes of cooperativity can be found in other pathogenic bacteria, but also parasites and fungi [26,40]. Common environmental parameters, such as temperature increase [13], presence of certain metabolites such as bile in the intestine [41–43], or the composition or sequential abundance of nutrients, in particular sugars [21,44], are often used as a signal to regulate virulence-relevant traits. These traits are not linked to the actual signal but are advantageous at later stages of the infection (e.g., induction of iron acquisition genes, resistance to host-induced stresses or defense against the host immune system) or for transmission from host to host. The fungal pathogen *Candida albicans* undergoes a morphological yeast-to-hyphae switch, which is triggered upon contact with host cells at body temperature. This is accompanied by the expression of hypha-associated factors that are required for or after tissue invasion before this process is initiated [45]. Another example is *Plasmodium* spp., the causative agent of malaria. This parasite replicates asexually inside the blood stream of humans, whereby always a subset of them develop into the sexual stage/form—the gametocytes. They cannot replicate asexually, but only the gametocytes can later—after a mosquito bite—enter, differentiate, and mate within these insects [46].

As preadaptation comes with the risk that the predicted subsequent environment is not encountered, a microbial population must ensure, before it commits to a costly new phenotype, that (a) preadaptation relies on robust/reliable signals and associated fitness costs are low, or (b) that only a small subset of the population expresses the costly preadapted trait. A very costly trait is the expression of type III secretion systems (T3SSs). T3SSs are large protein complexes in the cell envelope, which are part of flagella promoting motility and the injectisome structures. The latter allow the bacteria to inject effector proteins, which manipulate host cells.

In *Yersinia*, the virulence plasmid-encoded Ysc-T3SS translocates multiple Yop effector proteins into neutrophils and macrophages to prevent phagocytosis [47]. Synthesis of the Ysc-T3SS is upregulated by temperature, and its expression is further induced in bacterial cells, which are in contact with host cells [48–51] (Fig. 3). This allows the cell-bound bacteria to block phagocytosis and the release of deleterious reactive oxygen (RO) and nitric oxide (NO) species [17] [52–54]. As a drawback, expression of the T3SS could increase recognition by the host immune system and leads to a strong reduction of the metabolic activity and to growth arrest [55,56], a phenomenon described as self-destructing cooperation [57]. The underlying mechanisms and overall fitness costs of this process still remain unclear. However, a sudden relief of the Yop secretion/translocation signal rapidly reverts the non-growing state and gives rise to phenotypically normal growing cells [55,56]. This suggests a dynamic, cooperative process. After the defense of the first attacks, faster-growing Ysc-T3SS variants, which are not in direct contact with host cells and repress T3SS expression (“cheaters”), could take over. They could outcompete Ysc-T3SS-expressing, neutrophil-inhibiting bacteria at the front row of defense (“martyrs”).

Similar to *Yersinia*, heterogeneity of T3SS expression is also found in other pathogens, including enteropathogenic *E. coli* (EPEC) and *Salmonella* Typhimurium, indicating that this is a conserved strategy of cooperative virulence. In EPEC, bimodal populations have been observed based on bistable expression of the *perABC* operon [15]. Per-ON cells with high bundle-forming pili and T3SS expression have the ability to rapidly attach to host cells and inject effectors to induce pedestal formation and the establishment of effacement and attachment lesions [15]. In contrast, Per-OFF cells are less immunogenic and thus, more suited for chronic infections. In *Salmonella* Typhimurium, the flagellar gene cluster and the T3SS gene cluster of the *Salmonella* pathogenicity island 1 (SPI-1) undergo bistable expression [58,59]. Flagellated bacteria are able to migrate efficiently to attractive nutrient-rich environments or escape repellent conditions. *Salmonellae* expressing the T3SS-1 injectisome of SPI-1 induce epithelial cell invasion and inflammation, which is required to outcompete the intestinal microbiota. The downside of this strategy is that expression of both

T3SSs is extremely costly [10,60]. Their expression does not only reduce the growth rate of the pathogen but also enhance the activation of the immune response through binding and activation of Toll-like pattern recognition receptors [61]. On the other hand, growth-arrested bacteria are more resistant against antibiotics and other stresses giving this subset of defending bacteria an advantage during antibiotic therapy [10,11]. This strategy has been brought to perfection by the formation of persister cells or small colony variants. These dormant or very slow-growing variants of regular bacterial cells have been detected in populations of many gram-negative and gram-positive pathogenic bacteria, and also fungi [14] [62–66]. The previously mentioned group of persisting cells could further be amended by bacterial endospores and the non-replicative sexual stages of parasites, for example, the gametocytes of *Plasmodium* [46]. These subpopulations are characterized by a reduced/arrested metabolic activity, a distinct expression pattern of virulence-relevant traits, and they allow for tolerance to different stresses, for example, antibiotics or immune system-mediated clearance [67–69]. Thus, it is not surprising that bacterial persisters often are associated with chronic infections and the formation of long-term reservoirs [70–72]. As the fitness cost is extremely high, it is also expected that the rate of persisters in a particular population is very low (often less than 0.1%). At this, particular stress can generate a very heterogenous set of persisters in which expression of individual genes promote tolerance to different antibiotics [64,73]. This cooperative action keeps the overall fitness costs at bay, while the entire preadaptation spectrum of the population is enlarged.

Sharing Goods and Labor

Another form of cooperative behavior during host–pathogen encounters is the *division of labor* [3,7]. This strategy is mostly used to separate the workload for a repertoire of fitness- and virulence-relevant functions. It allows the pathogen to provide and maintain a large diversity of traits and goods for the entire population by sharing, joint use or cross-feeding and reduces competition [20]. One frequently used division of labor strategy is the heterogeneous expression of bacterial toxins, in particular those encoded on lysogenic phages such as the Shiga toxins. Their production and release are generally induced only in a subset of the bacterial population by environmental stress signals (DNA damage, RO species) [74–76]. This does not only prevent the extinction of the total population but also reduce the costs and reap the benefits of toxin productions [77]. Moreover, the lysed cells could act as a source for nutrients or/and potential virulence-relevant factors.

One of the most important communities of work-dividing, heterogeneous populations are biofilms formed by various pathogens during acute and chronic infection stages. These structured communities consist of a population of cells embedded in a self-produced matrix of extracellular polymeric substances. Within these structures, energy costs for the synthesis of certain communal goods/metabolites and virulence traits of the population can be shared, similar to free-living bacteria [7,78]. In addition, these structures contain clonal cell subsets, which are adapted/resistant to different environmental stresses (e.g., low pH, RO species) and antimicrobial compounds. This increases the biological fitness of the individual cells and enhances the survival of the population [16,67,79]. One pitfall in the cooperation strategy "*division of labor*" is the appearance of "*selfish cheaters*." One example is *Pseudomonas aeruginosa* growing in biofilms within the lungs of cystic fibrosis patients where the iron concentration is generally very low. To scavenge Fe^{2+}/Fe^{3+} ions, *Pseudomonas* produces and uses energetically costly Fe^{2+}/Fe^{3+}-chelating siderophores to steal iron from host proteins [80]. However, single cells of the population switch off their production and import Fe^{2+}/Fe^{3+}-bound siderophores produced by their cooperating siblings [81]. Another type of cheaters has been characterized as *lasR* mutants, lacking a quorum sensing system [82]. The ratio between cheaters and cooperators will define the viability and survival of the entire population and is therefore also under evolutionary pressure and selection. It seems that a prospering population can always tolerate a coexisting small subset of cheaters. However, when the fitness of the cheaters is considerably higher than the fitness of the altruistic cooperators, the cooperators will likely be overgrown until the essential goods of the cooperator become scarce and halter reproduction of the cheaters.

Some enteric pathogens, such as *Yersinia,* replicate mainly extracellularly within host tissue [83]. During the acute infection phase, they form replicating clusters of bacteria, so-called microcolonies, in the lymphatic tissue, and this helps them to prevent eradication by the immune system [17] (Fig. 3). These densely packed microcolonies consist of very heterogeneous siblings generated from one invaded bacterium, which are embedded into fibrin-rich matrices and surrounded by recruited and attacking neutrophils and macrophages [17,84]. Due to small local differences in the microenvironment of the bacteria within the microcolony, they show different expression patterns in response to host signals [17] (Fig. 3). The bacteria in the periphery of the colony sense and react to the attack of the immune cells, that is, to the release of NO species by upregulating the expression of NO-detoxifying genes [17]. Moreover, direct contact with the immune cell will trigger expression of the energetically costly Ysc/Yop T3SS [50,51]. This protects the bacteria in the microcolony.

Whether the additional fitness costs of these counter-measures reduce the overall bacterial fitness of the peripheral cells and result in a final stop of the colony growth or whether they are compensated by better access to nutrients at the periphery of the colony is still unclear.

Conclusion and Perspectives

Social behavior and cooperativity in communities can be classified according to the effects on the individual cell performing the act or the individual cell receiving it as mutually beneficial, altruistic, or selfish. It turns out that these interactions are pervasive across the living world including microbial pathogens, suggesting that this is a crucial virulence strategy and an evolutionary selectable trait. Among the first descriptions of cooperations of prokaryotes were the formation of bacterial biofilms or gliding formations of myxobacteria, in which bacteria, although genetically identical, develop different phenotypes and functions [85]. Other reports of phenotypic diversity resulted from antibiotic treatments, demonstrating that subpopulations with different susceptibilities to antibiotics exist in genetically identical populations [64,86]. Later studies identified phenotypic heterogeneity of pathogens, which are often exposed to environmental fluctuations [20]. It further became evident that a pool of heterogeneous individual cells allows preadaptation to predictable future stresses and growth conditions [21,22]. This permits the successful exploitation of a wide range of niches and promotes evasion of diverse immunological stresses. Over the past few years, many more examples of heterogeneous microbial populations have been reported, and the more we explore, the more examples we reveal. This indicates that this phenomenon could be rather the rule than the exception.

The existence of different subpopulations of microbial pathogens within host tissues causes major problems for the treatment of infections. The formation of antibiotic-tolerant persisters or growth-restricted variants and the development of preadaptated stress-resistant subsets of cells that are not sensitive to immune cell attacks and antimicrobials/defensins are highly problematic and demand new treatment strategies. One possibility would be to trigger the development of very costly, maladapted subpopulations in infected tissues, which would lead to a strong reduction or eradication of the population. Another strategy could be the induction of an awakening of the non-/slow-growing cells to improve antibiotic treatments. For the design of these novel strategies, a more detailed knowledge about the host microenvironments, the quality and quantity of the individual parameters, and the genetic expression programs that induce the formation of the heterogeneous populations is required. Moreover, we need to know the factors that influence the host–pathogen interaction outcome of phenotypic diversification *in vivo*: What are the costs and benefits of the production of certain virulence genes? What is the number of cheaters in microcolonies within the infected tissue and how do they influence the benefits for the whole population? How does a subpopulation change during the different stages of infection and within different infection niches? Hopefully, with the continued development of technologies such as single-cell imaging with a higher spatiotemporal resolution, single-cell labeling, and sorting, as well as system-level approaches, including single-cell transcriptomics, proteomics, and metabolomics and computational modeling to integrate the data [87,88], we will be able to identify, characterize and modulate phenotypic heterogeneity. This will not only help us to decipher the role of phenotypic heterogeneity in infectious diseases, but also allow us to develop strategies to target different subsets of cells in the microbial populations.

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Abbreviations used:
T3SS, type III secretion system; RO, reactive oxygen; NO, nitric oxide.

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