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Experimental approaches to phenotypic diversity in infection

Saskia Kreibich and Wolf-Dietrich Hardt

Microbial infections are burdening human health, even after the advent of antibiotics, vaccines and hygiene. Thus, infection biology has aimed at the molecular understanding of the pathogen–host interaction. This has revealed key virulence factors, host cell signaling pathways and immune responses. However, our understanding of the infection process is still incomplete. Recent evidence suggests that phenotypic diversity can have important consequences for the infection process. Diversity arises from the formation of distinct subpopulations of pathogen cells (with distinct virulence factor expression patterns) and host cells (with distinct response capacities). For technical reasons, such phenotypic diversity has often been overlooked. We are highlighting several striking examples and discuss the experimental approaches available for analyzing the different subpopulations. Single cell reporters and approaches from systems biology do hold much promise.

Address
Institute of Microbiology, ETH Zürich, CH-8093 Zürich, Switzerland

Corresponding author: Hardt, Wolf-Dietrich (hardt@micro.biol.ethz.ch)

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Introduction

Bacterial infections result from the interaction between a microbial pathogen and the host [1]. This interaction is typically quite dynamic and comprises multiple stages. 'Cellular microbiology', infection immunology, clinical studies and animal models have provided a wealth of information on the molecular and the cellular events with particular emphasis on the pathogen's virulence factors (Box 1; [2,3]) (reviewed in [4]). Numerous bacterial toxins, adhesins, protein secretion machines and host responses have been discovered, including target structures for powerful vaccines, for example, the A-B toxins of *Corynebacterium diphtheriae* [5] or *Clostridium tetani* [6]. The individual combination of virulence factors is thought to explain the disease caused by a given pathogen, each virulence factor fostering a particular step of the pathogen–host interaction. However, for many pathogenic bacteria this approach has not (yet) yielded applicable therapeutics or vaccines. This may reflect our incomplete understanding of the pathogen–host interaction. Recent data suggest that phenotypic diversification of both, the pathogen and the host's response, might be a key aspect that had been missed (Figure 1a). Here, we will provide a few examples for diversity at the cellular level and review the methods available for studying such phenotypic heterogeneity.

Phenotypic diversity

Phenotypic diversity (or heterogeneity) arises from differences in gene expression between different cells of the pathogen (or host; Figure 1a). In an infection, the different phenotypes (e.g. virulence factor profiles, metabolism, innate immune defences, among others) determine the outcome of a pathogen–host cell encounter, the basic step of the infection process. Thereby, the phenotypic heterogeneity could explain why some tissue culture cells remain uninfected even at high multiplicities of infection [7], or why typically just very few pathogen cells do successfully colonize a host or disseminate to other body sites [8•,9••,10•,11••,12–17]. In other words, it is the particular phenotypes of the incoming pathogen and of the interacting host cell which permit (or terminate) disease progression. To understand disease (or resistance to infection), we do therefore need to assess the phenotypes of the respective bacterial and host–cellular phenotypes.

Heterogeneous gene expression can arise from stochastic processes or from differences in the local micro-environment. Stochasticity roots in the stochastic nature of gene expression, which can cause significant cell-to-cell differences in protein concentrations [18] (Figure 1b; Box 2). In combination with certain genetic circuits, this can even lead to the formation of two (or more) distinct phenotypes by genetically identical cells in the same environment. Virulence factor expression by the enteropathogenic bacterium *Salmonella* Typhimurium (*S.*Tm) is one striking example (Figure 1c). This pathogen encodes two type III secretion systems: T1 is a key virulence factor needed for host cell invasion and the elicitation of gut inflammation while T2 is expressed within host cells and mediates survival of the pathogen within macrophages [19]. Strikingly, T1 is expressed by just 30% of the bacterial population in broth culture and in the host's gut lumen [20–23]. Both, the T1-expressing and the non-expressing

Box 1 Virulence factor

a) definition:
- a gene/factor present/expressed in pathogenic strains but not in apathogenic strains
- disruption of the virulence factor gene reduces the pathogenic potential of the pathogen
- re-introduction (complementation) of the virulence factor restores the pathogenic potential

b) classes of virulence factors:
- *non-specific virulence factors:* facilitate nutrient acquisition during infection
- *defensive virulence factors:* protect the pathogen from the host's innate or adaptive immune defense
- *offensive virulence factors:* allow host cell intoxication, adhesion or invasion

c) conceptual advances:
- virulence factor acquisition can explain evolution of pathogens [133–136]
- vaccines/drugs blocking virulence factor function should retard the infection

cells are required for eliciting disease, efficient growth in the gut lumen and sustained transmission [21,24••,25•,26]. Similarly, flagellar gene expression by *S*.Tm occurs in a bi-stable fashion resulting in flagellated and non-flagellated subpopulations *in vitro* and in the gut lumen and directs the flagella-expressing bacteria towards the gut epithelium [27–29]. Adaptation to micro-heterogeneities in environmental cues is a second source of phenotypic diversification (Figure 1d). Such micro-environmental heterogeneities can explain why *S*.Tm expresses T1, T2 or both during later stages after enterocyte invasion [30,31•], why *Yersinia pseudotuberculosis* lesions feature *hmp*-expressing cells at the rim and non-expressing cells at the center (Figure 1e [32••]), or why infectious lesions of *S*.Tm or *Mycobacterium tuberculosis* differ significantly with respect to bacterial defense gene expression and lesion-histopathology (reviewed in [11••,32••,33]). Clearly, phenotypic heterogeneity can affect pathogenesis. However, we do not know how common this really is. This is explained by the general use of bulk assays in microbiology which average the properties of millions of bacterial cells (e.g. Western blot, cfu plating, *lacZ*-reporter assays) and thereby systematically overlook phenotypic diversity. Here, we will discuss experimental approaches to detect and establish the full complement of features (RNA, protein, metabolites, among others) of the given phenotypes in order to establish their role in the infection process.

'Classical' microscopy/cell-biology based approaches

Fluorescent reporters and antibody staining can reveal phenotypic heterogeneity in host cellular and bacterial phenotypes. For example, green-fluorescent or red-fluorescent reporters for T1 expression by *S*.Tm have revealed pronounced phenotypic heterogeneity and established that only the T1-expressing subpopulation can actively invade into the gut epithelium to elicit diarrhea [20,21,34•]. Similarly, T2 expression reporters have been used to quantify *S*.Tm gut tissue invasion and revealed that some infected enterocytes permit intracellular pathogen replication, while others are sloughed off into the gut lumen, an inflammasome-dependent response reducing the mucosal pathogen burden [34•,35,36•]. On the other hand, antibody staining of lymph node cells, FACS sorting and plating established that dendritic monocytes lodge an important fraction of the slow-growing, 'persistent' *S*.Tm cells which survive antibiotic treatment [9••]. The use of fluorescence reporter dilution assays or TIMER-sensors, which rely on the time-dependent color-change of the fluorescent reporter, have come to similar conclusions [37,38••,39••]. Due to the ability to separate different subpopulations of the pathogen or of the infected cells, these techniques can be applied to isolate the cells of interest and study the underlying phenotypes in more detail, that is, by systems approaches which quantify a large number of parameters in parallel and integrate them into suitable models (see below).

Population dynamics approaches

Population dynamics approaches can detect 'bottlenecks', steps of the infection process mastered by only a small sub-fraction of the total pathogen population present. These approaches employ bar-coded but otherwise identical strains of a pathogen (e.g. 'wild type isogenic tagged strains', WITS; [13]). Comparing the barcodes in the inoculum, in different organs and determining how they change during the course of the infection can identify bottlenecks. Examples include the gut luminal bottleneck encountered by *S*.Tm at day 2 p.i. [10•], the colonization of the intestinal Peyer's patches [40•], spread to the mesenteric lymph nodes [8•,9••] and dissemination to different internal organs [13–15]. These bottlenecks can guide mechanistic follow-up experiments probing the phenotypic state of the pathogen and the infected host cell that facilitate disease progression.

Gene expression/transcriptomics

The transcriptional state of the pathogen or host cell can be analyzed in system-wide fashion. The initial microarray hybridization has been replaced by RNA-seq methods employing reverse transcription and high throughput sequencing (HTS; Figure 2a; Table 1) technologies to 'recognize' a certain transcript and quantify the mRNA levels by 'counting' [41–43]. In combination with enrichment techniques for the RNA species of interest ('differential RNA-seq'; [44,45]), this yields high-resolution maps of the expression status of the pathogen population [46,47] or the host [48]. Classical examples include the analysis of the primary transcriptome of *Helicobacter pylori* [45] and transcript maps of *S*.Tm revealing 140 small