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Current Opinion in Microbiology

Within-host evolution of bacterial pathogens during persistent infection of humans

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Many bacterial pathogens can form persistent infections, providing an infectious reservoir, which allows for infection of new hosts. Currently, the molecular mechanisms and evolutionary dynamics driving persistence are still not well-understood. High-throughput sequencing methods have enabled the study of within-host evolution of persistent bacterial pathogens, revealing common trends among bacterial species in how they adapt to persist. We will focus on trends emerging from longitudinal human-cohort studies, including i) genome-size reduction, ii) metabolic adaptation to the host, iii) antimicrobial resistance, iv) changes in virulence and the bacterial cell surface, and v) hypermutation, and comment on where the field should focus going forward.

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Current Opinion in Microbiology 2022, **70**:102197

This review comes from a themed issue on **Microbiota**

Edited by **Lindsay Hall** and **Melanie Schirmerl**

For complete overview of the section, please refer to the article collection, "Microbiota"

Available online 2nd September 2022

https://doi.org/10.1016/j.mib.2022.102197

Introduction

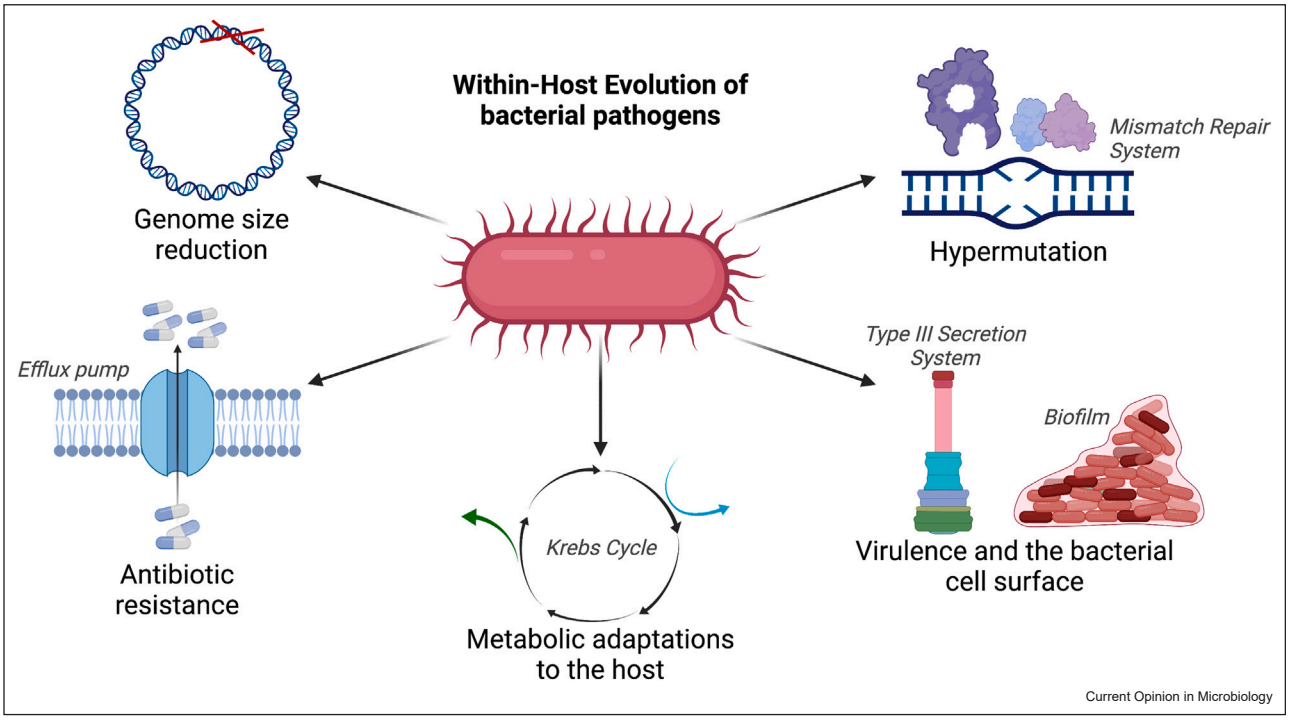
Some bacterial pathogens can form persistent infections, which can evade the action of antibiotics or the host immune system, possibly by entering into a non-replicating or metabolically altered state or protected niche [1]. Lasting for years or even a lifetime, persistent infections create infectious reservoirs of disease, which can lead to recrudescence or onward transmission to new hosts and are associated with antibiotic resistance and other adverse outcomes such as chronic inflammation and cancer [2,3]. Large gaps remain in our understanding of these persistent infections, which hinder efforts to recognize and treat them. However, advances in sequencing technologies have enabled detailed investigations into the adaptive strategies required for persistence. This review will focus on what these technologies are revealing about the mechanisms and evolution of persistence from sequencing and comparative analysis of bacterial pathogens from human-cohort studies. While much has been learned about within-host diversity, site-specific adaptations, and evolutionary rates from multi-body-site studies [4–6], for the sake of conciseness, we will focus on longitudinal studies that trace the evolution of pathogens over the course of persistent infection. We summarize convergent trends across individual studies, species, and bacterial genera, and comment on where the field should focus going forward to inform improved diagnosis and treatment of persistent infections (Figure 1).

The majority of sequence-based longitudinal studies of persistent bacterial pathogens focus on lung infections, often in the context of patients with cystic fibrosis (CF) [7]. Multiple opportunistic lung pathogens can persistently infect CF patients, including *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, and other members of the *Burkholderia cepacia* complex, including *Burkholderia dolosa* and *pseudomallei*; *Stenotrophomonas maltophilia*, and *Achromobacter sp.* (including *ruhlandii*, *xylosoxidans*, and *insuavis*). Though fewer in number, longitudinal human-cohort studies have also focused on *Mycobacterium tuberculosis* persistence in the context of tuberculosis (TB) and of lung-associated nontuberculous mycobacterium, such as *Mycobacterium abscessus*. Outside the context of the lung, longitudinal human-cohort studies have focused on *Salmonella enterica*, the causative agent of salmonellosis and typhoid fever, and on several commensal bacteria that can cause disease, often in immunocompromised patients, including *Enterococcus faecium* and *faecalis*, *Staphylococcus aureus*, and *Neisseria meningitidis*. Trends in functional convergence during persistence across species are summarized in Table 1.

Genome-size reduction

It is well-documented that bacterial genomes can become streamlined during speciation, including as bacteria transition into niches where they no longer need to produce specific compounds to support their growth [8]. Longitudinal human-cohort studies have shown that genome-size reduction can also occur over relatively short timescales in which bacteria adapt to a host during persistent infection. For example, a study of 45 *P. aeruginosa* isolates from 16 CF patients collected over 35 years found extensive genome-size reduction across all patients, including 27 reductive events ranging from

Figure 1



Major trends found across bacterial pathogens in studies of within-host evolution using longitudinal cohort studies. Figure created with BioRender.

Table 1

Summary of studies supporting trends in functional convergence from longitudinal human- cohort studies during persistent infections of humans.

Genus	Species	Genome-size reduction	Metabolic adaptation	Antibiotic resistance	Virulence	Hypermutation
Pseudomonas	*aeruginosa*	9, 10	9, 10, 14, 15, 16, 17, 18	13, 14, 15, 17, 27, 28, 29	14, 27, 28	19, 17, 27, 28, 29
Burkholderia	*pseudomallei*	11		11	11	11
	multivorans	20	20	20		20
	dolosa			38	38	
Mycobacterium	*tuberculosis*		19	24, 25, 37	45, 46	
	abscessus		26	26	26	26
	cenocepacia	12			12	
Enterococcus	*faecium*		23	23, 38, 41	23	
Staphylococcus	*aureus*			32, 33, 34, 35	32, 36, 50	33
Neisseria	*meningitis*				51, 52	52
Salmonella	*enterica*			47	47, 48, 49	
Achromobacter	*xylosoxidans*		21, 22	21, 22, 30, 31	21, 22, 44	21, 22, 30, 31, 44

1Kb to > 500Kb [9]. Isolates from five patients lost the little-studied homoprotochatechuate (*hpc*) metabolic pathway, suggesting it is a selective target for deletion [9]. In addition to its association with CF, *P. aeruginosa* causes up to 10% of healthcare-associated urinary tract infections (UTIs), which are frequently persistent and difficult to treat [10•]. Genome-size reduction was also seen in this context. A study of 108 persistent *P. aeruginosa* UTIs from seven patients over 48–488 days [10•], revealed deletions of 1–5% of the pathogen genome in three patients, with two exhibiting convergent deletion of 70 genes [10•], including those encoding transcriptional regulators, two-component systems, antimicrobial resistance, and carbon-compound catabolism.

Genome-size reduction has also been observed in persistent *Burkholderia sp.* infections and in hospital-acquired infections caused by *E. faecium*, a common gut commensal.

In a study of *B. pseudomallei* sampled longitudinally from seven CF patients over 4–55 months, strains from two patients experienced large deletions over time [11]. In one, a 35-kb deletion affected the *Nar*IJHG respiratory nitrate reductase system likely required for growth under hypoxic conditions, and in the other, a 45-kb deletion encompassed the gene encoding the mismatch DNA repair protein MutS, likely leading to the development of a hypermutator strain [11]. Additionally, a study of 215 isolates of *B. cenocepacia* isolates from 16 CF patients observed recurrent gene loss, including the complete loss of chromosome III [12]. In a study of 96 *E. faecium* isolated from five asymptomatically colonized patients over 1–6.5 years, a cluster of carbohydrate-metabolism genes in the early isolates of a patient was lost in all subsequent isolates [13].

Metabolic adaptations to the host

In addition to genome-size reduction, changes in metabolic capacity can occur through mutations accruing in retained genes (or intergenic regions) that control expression of key metabolic functions. Mutation-induced reductions in metabolic capacity were found among 26 *P. aeruginosa* isolates from four CF patients over 19 years; mutations accruing in genes encoding the CbrAB two-component system involved in carbon and nitrogen utilization, and amino acid biosynthesis and central carbon-utilization pathways, were hypothesized to reduce metabolic capacity in isolates from two patients [14]. Similarly, a study of within-host evolution of intergenic regions of 534 *P. aeruginosa* isolates from seven studies identified convergent mutations that changed expression of their downstream genes involved in metabolism of aromatic amino acids (*hmgA*), sphingolipids (*cerN*), and zinc (*zrmA*), some of which were previously implicated in virulence [15•]. On a similar note, several studies have shown that *in vivo* populations compared with *in vitro* cultures of *P. aeruginosa* have lower expression of genes involved in transport and/or metabolism of carbon and purines, amino acids, lipids, and secondary metabolites [16–18].

Though in single patients, this trend was echoed in persistent infections caused by *M. tuberculosis* and *B. multivorans*. A study following a single TB patient during a 21-year-long *M. tuberculosis* infection identified mutations in genes encoding putative exporter proteins, BacA, Rv1819c, Rv2326c, and MshA, which the authors speculate may have led to a decreased metabolic state and allowed for chronic infection [19]. In a study of 22 *B. multivorans* isolates from a single CF patient over 20 years, genes involved in lipid metabolism, amino acid metabolism and transport, and cell division were found to be under selection and associated with increased doubling time and reduced biomass *in vitro* [20]. In another related *Achromobacter sp.* study, the majority of genes varying between same-patient isolates were metabolism-related [21].

Some metabolic adaptations found to occur within a host are associated with adaptation to certain nutrient limitations or availability of novel metabolites in the host. In a study of *Achromobacter sp.* infections in the context of CF, all persistent isolates from five patients over four years accumulated nonsynonymous single nucleotide polymorphisms (SNPs) in metabolic pathways [22]. Two of the five genes exhibiting convergent evolution across patients were *kdpD*, encoding a potassium channel histidine kinase, and a gene encoding the terminal oxidase of the electron-transport chain in aerobic conditions, both of which were speculated to be important for survival in a potassium- or oxygen-limited host environment [22]. In a study of 110 *E. faecium* isolates from gastrointestinal and blood cultures of 24 pediatric patients over 10 years, variants arising during infection were enriched in genes involved in carbohydrate metabolism, including a mutation in the gene encoding the sorbitol operon transcriptional regulator GutR that was associated with increased bacterial growth in the presence of sorbitol [23•].

Antimicrobial resistance

Given that antibiotics are widely used to treat persistent infections, often without much success, it is unsurprising that emergence of antibiotic resistance is a major theme across most persistent species. A number of studies have documented the accrual of mutations in known resistance determinants, including for extensively drug-resistant (XDR) TB [24,25]; *M. abscessus* lung infections [26•]; *P. aeruginosa* [14,15•,27–29], *Achromobacter* [21,22,30,31•], *Burkholderia sp.* [20], and *S. aureus* [32,33] infections in the CF context [21,22,30,31•]; vancomycin and daptomycin resistance in *S. aureus* infections in the context of endocarditis [34], bloodstream infections [35], and diabetic foot infections [32,33,36]; *Burkholderia* melioidosis [11]; and linezolid and daptomycin resistance in vancomycin-resistant *E. faecium* infections [23•].

In addition, longitudinal studies have shed light on the evolutionary dynamics of how resistance arises *in vivo*. For example, in a study of 12 TB patients sampled for up to eight weeks comparing different drug regimens under effective antibiotic treatment, purifying selection constrained the evolution of resistance, but there was continuous turnover of minor variants [37]. The authors speculated that, under lessening drug pressure, these minor variants could give rise to resistance [37]. This idea of transient mutations contributing to

microheterogeneity was also observed by Xu and colleagues in a study of 16 isolates from four pre-XDR TB patients [25]. In a GWAS study of 112 *B. dolosa* isolates collected from 14 individuals over 16 years, independent acquisition of only one of two known resistance-conferring mutations in *gyrA* was observed among isolates from six patients, illustrating the strong selective pressure of fluoroquinolones and suggesting relatively few paths to resistance *in vivo* [38].

Sometimes, changes in antibiotic resistance can be traced to the gain or loss of plasmids within a host. For *E. faecium*, multiple longitudinal studies have shown that there is a dynamic balance between vancomycin-susceptible and -resistant subpopulations within a patient, where plasmids and genes encoding vancomycin resistance (*van* genes) can be highly mobile [39,40]. In a study of 693 stool samples from 45 nursing home residents over six months, two individuals carried vancomycin-susceptible and -resistant strains that were highly similar [41]. One patient's isolates carried identical *vanA*-carrying transposons in distinct *E. faecium* strains, suggesting that *vanA* was being mobilized, likely on plasmids, across isolates within a patient [41]. In another study of 180 *E. faecium* isolates from the stool and blood of four immunocompromised patients who developed bloodstream infections during longitudinal surveillance, both stool and bloodstream *E. faecium* populations shifted to become resistant under vancomycin treatment. However, 36 days after treatment, susceptible populations were detectable again [39].

While not discussed here, the evolution of antibiotic tolerance, where bacteria are able to survive exposure to antibiotics without an increase in minimum inhibitory concentration, is undoubtedly another important trend in within-host evolution [42,43].

Virulence and the bacterial cell surface

There is growing evidence that bacteria accumulate mutations during persistence in genes regulating virulence and other pathways at the bacterial cell surface, potentially allowing better host-immune evasion. Several studies point to changes in global regulators playing a key role in persistence. In *M. abscessus*, longitudinal cohort studies identified nonsynonymous mutations in genes encoding the global regulators PhoR, Crp/Fnr, EngA, and IdeR [26•]. Similarly, in a study of 55 *A. xylosoxidans* isolates from six patients with CF-associated chronic airway infections, genes encoding the global regulators PhoQ, BigR, SpoT, and CpxA showed convergent evolution, which aided their persistence [44]. Among other lung pathogens, changes in regulators linked to virulence-related phenotypes, including increased motility and extracellular protease activity, were also observed in *P. aeruginosa* (in the two-component system GacAS [14,27], and the global regulator *mucA* [25]), and increased infection efficiency in recurrent TB (in the virulence operon regulator *mce3R*), shown using competitive co-infection assays in mice [45,46].

In gut-related pathogens, SNPs were acquired in global virulence regulatory genes in studies of persistent *S.* typhimurium infections, including *dksA*, *rpoS*, *hilD*, *melR*, *rfc*, and *barA* [47], and *flhC*, encoding a master regulator of flagellar biosynthesis [48], though none of these changes were found to be convergent across multiple patients. A recent study of 639 isolates from 256 patients with persistent infection found regulatory variants accruing in isolates from 46% of patients showing variation, with mutations in the SirA/BarA two-component system that regulates the *Salmonella* pathogenicity island 1 (SPI-1) occurring most frequently [49•]. RNA-Seq experiments showed accrued variation in SirA/BarA led to decreased SPI-1 expression and decreased virulence in an *in vivo* mouse model [49•].

Global regulators have also been implicated in the evolution of persistent bacteria, which can transition from asymptomatic carriage to invasive disease, such as *S. aureus*, *N. meningitidis*, and *Enterococcus sp.* Because *S. aureus* is frequently carried asymptomatically (27% of adults), but can cause bacteremias (80% matching the carried strain), longitudinal changes can shed light on a strain's transition from asymptomatic to pathogenic. Studies of persistent *S. aureus* revealed premature stop codons in the AraC-family transcriptional regulator implicated in pathogenicity in a patient who developed a bloodstream infection [50] in SsrA/SsrB and VraS/VraR, two-component systems that control pathogenicity, in patients with diabetic foot infections [32]; as well as signatures of positive selection in the transcriptional regulators AgrA and RsbU, implicated in virulence, in children with chronic CF [36].

In addition to regulatory adaptations, several studies also point to the importance of outer-surface protein adaptations during persistence. In *Burkholderia sp.* infections of the CF lung, several studies observed changes in genes that control the number of O-antigen repeats in the lipopolysaccharide (LPS) of the bacterial outer membrane, required for serum resistance and virulence. Some studies identified mutations that changed the phenotype from rough, characterized by lack of O-antigen repeats, to smooth, due to removal of a stop codon [38], whereas other studies in *B. multivorans* and *B. pseudomallei* found that the majority of late isolates changed to the rough phenotype [11,20], predicted to impair LPS biosynthesis and lead to lowered virulence. In *E. faecium* isolates from pediatric patients, changes in the putative *E. faecium* capsular polysaccharide (*cps*) biosynthetic locus [23•] were correlated with an increase in biofilm-formation capability in later isolates. In *N.*

meningitidis, a commensal organism that can colonize the human upper respiratory tract but can also become invasive, causing meningitis and septicemia, studies found pervasive changes in phase-variable cell-surface genes such as *pilE*, the gene responsible for pilus-phase variation, suggesting this might be a mechanism of *in vivo* adaptation [51,52].

Hypermutation

Changes in the replication fidelity of the bacterial genome can accelerate within-host evolution. Hypermutation, or an increased spontaneous mutation rate *via*, for example, disruption of the DNA mismatch repair pathway, is a trend observed across many longitudinal studies of bacterial persistence. Mismatch repair machinery was lost during genome-size reduction in *B. pseudomallei*, leading to the development of hypermutators [11]. The study of CF-associated *A. xylosoxidans* found a hypermutator strain enriched in metabolism-related variants [21]. The *A. xylosoxidans* hypermutator strain was also the only strain in that study that evolved meropenem resistance [21]. Indeed, within-host evolution of hypermutation has been linked to the emergence of antibiotic resistance in many of the studies discussed here [11,17,20,22,26•,27,30,31•,33]. The study of *P. aeruginosa* that reported an acquired variant in the gene encoding the global regulator MucA also suspected this strain to be a hypermutator strain based on a frameshift mutation in *mutL* [28].

Where do we go from here?

Without question, the studies that have been performed to date and described herein have yielded incredible insights into the selective pressures bacterial pathogens face within an often antibiotic-treated, human host and the adaptive strategies these pathogens evolve to persist despite them. In our view, there are several key study-design tenets that will make future studies such as these particularly powerful in further dissecting within-host evolution of bacterial pathogens during persistent infection of humans: i) sampling infection diversity longitudinally — and as completely as possible — over the course of a persistent infection, including early points in infection, ii) using a large and representative human cohort to robustly capture convergent events that may be among the most overarching and clinically actionable, iii) incorporating transcriptomics, proteomics, and/or metabolomics to better capture pathogen-adaptive trends that converge at the pathway (or functional) level rather than the variant or gene level, and iv) validating genetic mechanisms *in vivo*, including assessing the effect of adaptations on the host's response.

While the studies discussed here captured evolution in a host during persistent infection, very few captured infection diversity beyond a few time points, and most relied upon single isolates that we now understand undersample the genetic diversity of a pathogen within a host at any given time. While it remains unclear what an appropriate sampling depth and frequency should be for any given persistent infection, understanding within-host evolution of persistent infections will require, not only an understanding of the genetic variants in individual pathogen genomes, but also an understanding of the full set of strains or sublineages within a host and their interplay. As sequencing becomes cheaper and more accurate, we are seeing an increase in the number of patients per study, which is revealing more convergent trends across patients, which many previous studies using 1–10 patients likely missed. The overwhelming focus of these studies has been in the CF context. CF patients are closely followed and sampled via sputum or bronchoalveolar lavage, making access to longitudinal persistent bacterial isolates relatively straightforward. Innovations aiding more frequent sampling in other persistent disease contexts would be helpful. Additionally, while the vast majority of studies rely on picking isolates retrospectively, the field requires more studies with prospective sampling such as Eklöf et al. in order to ensure standardized and systematically controlled sampling [29]. Similarly, the use of transcriptomic, proteomic, and metabolic profiling will allow us to find patterns of convergence at the pathway level, where multiple genetic variants can lead to similar responses. Last, many of the trends presented here have yet to be tested using *in vivo* models, and, therefore, remain speculative with respect to the actual mechanisms and the associated host response. Understanding the processes by which the variation accruing within patients allows for persistent infection will bring us one step closer to designing more effective clinical strategies to deal with persistent infections.

Conflict of interest statement

None.

Acknowledgements

We would like to thank Drs. Abigail Manson and Joshua Smith from the Broad's Bacterial Genomics Group, and Dr. Ohad Gal-Mor from Sheba Medical Center and Tel-Aviv University, Israel, for helpful discussions and feedback. ATG was supported by a postdoctoral fellowship from the Jane Coffin Childs Memorial Fund for Medical Research and by grant 2616/18 from the joint Israeli Science Foundation-Broad Institute program. AME was supported by Federal funds from the National Institute of Allergy and

Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Grant Number U19AI110818 and by grant 2616/18 from the joint ISF-Broad Institute program.

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