Short-sighted evolution of virulence for invasive gut microbes: from hypothesis to tests.

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Abstract

Why microbes harm their hosts is a fundamental question in evolutionary biology with broad relevance to our understanding of infectious diseases. Several hypotheses have been proposed to explain this "evolution of virulence." In this perspective, we re-examine one of these hypotheses in the specific context of the human gut microbiome, namely short-sighted evolution. According to the short-sighted evolution hypothesis, virulence is a product of niche expansion within a colonized host, whereby variants of commensal microbes establish populations in tissues and sites where the infection causes morbidity or mortality. This evolution is short-sighted in that the evolved variants that infect those tissues and sites are not transmitted to other hosts. The specific hypothesis that we propose is that some bacteria responsible for invasive infections and disease are the products of the short-sighted evolution of commensal bacteria residing in the gut microbiota. We present observations in support of this hypothesis and discuss the challenges inherent in assessing its general application to infections and diseases associated with specific members of the gut microbiota. We then describe how this hypothesis can be tested using genomic data and animal model experiments and outline how such studies will serve to provide fundamental information about both the evolution and genetic basis of virulence, and the bacteria of the intensively studied yet poorly understood habitats including the gut microbiomes of humans and other mammals.

Introduction.

The human gut abounds with a diversity of species and strains of bacteria, the vast majority of which are commensal. Still, some are opportunistic pathogens and responsible for potentially lethal invasive infections[1–5]. Why would bacteria that can maintain their populations without harming their host be responsible for the morbidity and mortality of those hosts, more metaphorically, 'why would a dog bite the hand that feeds it'? This "evolution of virulence" question has been a major source of interest to evolutionary biologists, and several hypotheses have been presented[6–9]. Here, we re-examine one of these hypotheses: short-sighted evolution and the virulence of pathogenic microorganisms[10]. According to this hypothesis, virulence is a product of niche expansion within the colonized host, whereby variants of commensal microbes establish populations in tissues and sites where the infection causes morbidity or mortality. This evolution is short-sighted in that the microbial variants that have evolved to infect those tissues and sites may not be transmitted to other hosts (except in animal cases of close contact with infected dead bodies or necrophagy[11,12]). In many respects, this phenomenon is similar to cancers, which, from an evolutionary standpoint, can be conceptualized as selfish rogue populations of cells that evolve through mutation and selection within the human body[13,14].

In this perspective the specific hypothesis that we are examining is that some bacteria responsible for invasive infections and disease are the products of short-sighted evolution originating from commensal bacteria in the gut microbiota. Whilst we present arguments and observations supporting this hypothesis, we also outline some of the difficulties in assessing its general application to invasive infections and disease associated with the gut microbiota. Lastly, we describe how this hypothesis can be tested with genomic data and animal model experiments.

Bacteria responsible for invasive infections are commonly derived from single cells.

Central to the short-sighted evolution of virulence hypothesis are the results reported from two animal infection studies showcasing the monoclonal nature of pathogenicity. In the first study, isogenic marked strains of equal mixtures of streptomycin sensitive (Str^s) and resistant (Str^r) *Hemophilus influenzae* type B were inoculated into the nasal cavity of neonatal rats. Among these 240 rats inoculated, bacteria were recovered from the blood of 60 of these experimental

infections. Of these 60 bacteremia's, 58 (96.7%) were pure Str^s or Str^r rather than mixed. Single rather than mixed isolate infections provided compelling evidence for the *de novo* evolution of virulence within hosts. This 1978 study by Richard Moxon and Patrick Murphy motivated further experiments performed by Margolis and Levin[8] that also used the *H. influenzae* and rat nasal infection model. However, in addition to confirming that bloodstream infections were derived from single rather than mixed isolate infections, they also demonstrated that one of six blood isolates tested for invasiveness in the animal model showed significant increases in invasiveness relative to its ancestor.

These experiments strongly support the idea that monoclonality observed for bloodstream infections could be attributed, at least in part, to invasive virulent mutants that arose from the ancestral inoculum and are consistent with the short-sighted evolution of virulence hypothesis. However, the methodologies used did not allow for a complete test of this hypothesis as they could not unequivocally demonstrate that the bacteria responsible for symptoms were genetically different from the ancestral bacteria from whence they were derived, much less determine the nature of the genetic difference. Unfortunately, low-cost whole genome sequencing methods that could have been employed to test for any potential genetic difference(s) and determine the nature of the difference(s) were not available at the time[16]. This level of genomic resolution is required to fully evaluate the hypothesis as invasive infections do not necessarily require a specific mechanism of bacterial virulence and invasion as translocation from the gut to other sites can also be linked to other factors including to the physiological status of the host *e.g.* the age and underlying clinical status, as well as the size of the intestinal bacterial population[17–20].

In fact, many bacterial species, including non-pathogenic ones have the potential to translocate and be detected in blood cultures or other body sites, and there is evidence to potentially support both "spontaneous" translocation and the within-host evolution of virulence within a single study[21]. For instance, the results of a comparative genomic study that investigated the consequences of oral administration of the probiotic bacteria *Lactobacillus rhamnosus* GG (LGG) on LGG associated bacteremia's in an ICU cohort reported single nucleotide polymorphism (SNP) level variation between blood isolates obtained from different individuals[21]. Even though some of the minute genetic variation observed between isolates mirrored genetic heterogeneity found in the probiotic product pre-administration, five different mutations were exclusively associated with LGG blood isolates. The presence of these

mutations in blood-only isolates highlight the potential for *de novo* evolution and selection within hosts, and are in principle consistent in part with what one would expect to observe to support the short-sighted evolution of virulence hypothesis from a genomics perspective. However, if we are to assume that all or at least most of mutations reported in this study underpin an invasive phenotype then the genomic data, taken as a whole, suggests that there may be multiple independent translocation events associated with different genetic variants of LGG with only some, as outlined, possibly due to within-host short-sighted evolution for invasion. Alternatively, we must consider the possibility that none of the minute genetic variation observed in blood isolates that were present probiotic pre-administration or owing to within-host evolution played a role in translocation and that other factors such as the physiological status of the host and population size of the bacteria played a role. Whilst it is not possible to discount this null hypothesis based on the data presented, it is worth noting that even though cohort in the study were a critically ill patient population, the authors pointed out that none of the patients that developed LGG bacteraemia were severely immunocompromised or had compromised bowel integrity, which are typical host related physiological risk factors associated with *Lactobacillus* bacteraemia.

More generally, this important study not only serves as a cautionary tale but also as a prompt reminder as to how little we know about the specific factors that shape the evolutionary trajectories and phenotypic outcomes of bacterial populations in individual hosts colonized by commensal bacteria. Even if the role of natural selection in shaping the evolution of bacterial populations in different host niches is increasingly recognised[22–24] it is clear that our understanding of how local adaptation to specific abiotic and biotic factors within individual gut microbiomes shapes the virulence of resident intestinal microbial populations to play causal roles in disease pathogenesis is greatly limited. In the following section, we assess the specific assumptions of the short-sighted evolution of virulence hypothesis in the context of diseases in which some members of the gut microbiota are implicated.

Criteria for short-sighted evolution of bacterial invasiveness and virulence

The classic short-sighted evolution of virulence hypothesis has three assumptions[10]: 1- The microbes responsible for causing harm to the host are a genetically distinct subpopulation that arises by *de novo* evolution within the host during colonization; 2- These subpopulations

become established because have a local advantage within their host relative to the population from which they are derived; and 3- The selective advantage of the evolved virulent subpopulation is short-sighted because it is uniquely local and advantageous at the level of the individual host, and disadvantageous with respect to transmission and colonization of new hosts.

Many conceivable traits could facilitate local advantage and confer a capacity to invade in evolved subpopulations of bacteria originating from the human gut microbiome. Such characteristics include the ability to evade host defences and exploit niches and resources, including host cells and tissues inaccessible to the ancestral population. Of note, recent population biology studies indicate that the possibility for such evolution may be asymmetrical among the phylogroups, clones and even subclones of a given commensal bacterial species given the predominance of certain phylogroups and ST types over others in invasive infections. For instance, long-term observational studies of bacteraemic strains reveal that in the case of *E. coli*, the predominant ones belong to phylogroups B2 and D, particularly B2, subgroup 1, and clone STc131[25]. These strains are frequently harmless inhabitants of the gut, but abundance in the microbiota has increased because of its association with antibiotic resistance, mostly to 3rd generation cephalosporins and fluoroquinolones, particularly in the subclone STc131-C. Similar phenomena occur in Gram positives, such as *Enterococcus faecium*, where the ampicillin-resistant clones STc17/18 prevail in bacteraemic isolates[26]. These invasive clones can disseminate epidemically both in the hospital and in the community. Therefore, assumption 1) should be conceived as the evolution of a genetically distinct population from the ancestral clone(s) endowed with particular traits facilitating colonization and invasion. That is, we can propose a "double step" process: selection of a "pre-invasive" or "primary invasive" clone (generally a good colonizer) where further variation gives rise to a more efficient invader, a "secondary invasive" variant. With respect to assumption 2), this secondary efficient invader should have local advantage within the tissues of the host (not necessarily in the intestine) relative to the population from which they are derived. For instance, some populations colonizing the intestine may produce urinary tract infections, and the bloodstream invasion occurs from the urinary tract (particularly in pyelonephritis) and not from the previous intestinal niche. Considering assumption 3), this secondary invader is "short-sighted" as, within host, the transmission to new hosts is reduced; however, the pre-invasive clone will persist in the intestine and be transmitted, giving rise to epidemic/endemic bursts of bloodstream infections by the continuous emergence of efficient invaders with different genetic

changes. In fact, sequencing of blood-stream isolates belonging to the same clone has revealed SNP level variation[25].

Another case for the short-sighted evolution of virulence can be made for the particularly invasive strains of *E. coli*, such as those carrying the K1 antigen (the K1 capsule is a sialic acid polysaccharide that likely mimics carbohydrates structures associated with host tissues and facilitates evasion of phagocytosis[27,28]) like *E. coli* O18:K1:H7, that also belong to phylogroup B2. These *E. coli* K1 strains are of clinical interest given that they are among the most prominent Gram-negative bacteria responsible for meningitis, particularly in new-borns[29]. Although these bacteria are present in gut microbiomes and are transmitted by an oral-fecal route, why, then, would these *E. coli* strains cross the blood-brain barrier and establish populations in sites where they will not be transmitted? Is it coincidental (non-inherited) and not the product of within-host evolution? This is conceivable given that they are resident in the gut, encode key virulence determinants such as the K1 antigen and translocation is often contingent on bacterial population size and the physiological status of the host, as outlined earlier. However, mutations do exist as in the gene ybdO, (a transcriptional regulator) that promotes *E. coli* K1 gene expression to increase K1 capsule synthesis[30]. Other experimental studies have also highlighted the importance of mutations in transcriptional regulators such as those in RpoS that facilitate *E. coli* K1 strain invasion of brain microvascular endothelial cells *in vitro*[31]. However, the most compelling data to support the within-host short-sighted evolution of virulence for K1 strains (and the hypothesis more generally) comes from a relatively recent study that explicitly demonstrated that *de novo* mutation and natural selection within the host conferred increased invasiveness and mortality in the K1 strain *E. coli* A192[32]. This study built on previous observations that showcased considerable variation in the invasiveness and mortality of this strain in *in vivo* models. In this more recent study, however, the researchers found that two rounds of passage of the K1 isolate *E. coli* A192 in susceptible neonatal rat pups selected for a mutant of the passaged strain (named *E. coli* A192PP) that, upon administration, led to bacteraemia and mortality in all colonized susceptible pups (compared to 23% bacteraemic infections observed for the ancestral A192 isolate in the same study). Whole genome sequence analysis of the evolved mutant demonstrated that these two rounds of within-host passage selected for SNPs in four genes linked to bacterial metabolism that were not associated with any previously known virulence determinants implicated in translocation or otherwise. These mutations conferred a growth rate advantage to *E. coli* A192PP over its ancestor *E. coli* A192 resulting in a ten- to one-hundredfold increase in

the numbers of colonizing bacteria within the host. It was postulated that the observed increase in invasiveness and mortality was due to these novel mutations evolved within the host, that facilitated population expansion above a critical threshold in numbers required for translocation in the model[32]. Taken together, this growing body of data on K1 *E. coli* isolates suggest that minute genetic differences observed between isolates could facilitate translocation for individual cells or a minority of the population to cross the intestinal barrier and establish populations in potentially dead-end sites in a manner consistent with the short-sighted evolution of virulence. It is also worth reiterating here that the severity of bloodstream infections derives not only from the host innate immunological reaction, but probably also from the total population size in tissues of the challenging bacteria. As indicated by this study such high bacterial density may derive from within-host acquired short-sighted mutations, but more generally high bacterial densities within host tissues may have additional negative consequences including facilitating the emergence of antibiotic resistant mutants or reducing the efficacy of antibiotics owing to higher populations.

As alluded to previously, the gut is also a reservoir for many bacterial species (e.g., *E. coli, Klebsiella, Proteus*) responsible for urinary tract infections (UTIs)[33,34,35]. Although genetic analysis of strains sampled from faeces and urine highlights the clonal nature of many infections and provides evidence for within-host evolution, the epidemiological analysis also indicates that many of the pathogen species and strains responsible for UTIs are transmissible[34] and therefore, some cases may not meet the third assumption of short-sighted of virulence hypothesis. However, most of these transmissible clones (such as *E. coli* ST73, ST95, ST127, and ST131[34]) also belong to the phylogroup B2, a good gut colonizer and thus with the ability to be transmitted between hosts or from the intestine to the perineal area. These clones might evolve into efficient invaders (either in the gut or in the upper urinary tract) and even if the ancestor is not short-sighted, the derived efficient invader may be, and as such, specific cases of morbidity associated with complications of UTIs, such as urosepsis, might meet all three assumptions of the hypothesis (mutation, niche expansion, and local adaptation at the individual level). In support of this, sequence analysis of isolates from urine and blood from single individuals with urosepsis confirmed the monoclonal nature of infection with a small number of SNPs differentiating urine and blood isolates in most cases[36].

Whilst we have thus far focused on studies that support the short-sighted evolution of virulence of gut microbes associated with extra-intestinal infections, this hypothesis also has potential implications for our understanding of disease pathogenesis that is localised to the gut. For example, another *E. coli* phenotype that has the potential to fit the assumptions of the short-sighted hypothesis is the the Adherent-Invasive *E. coli* (AIEC) phenotype which has been implicated in pathogenesis for several different intestinal diseases, including Crohn's Disease (CD), ulcerative colitis (UC), and colorectal cancer (CRC)[37,38]. This phenotype is characterized by an enhanced ability to adhere to and invade human tissues and to escape phagocytosis within macrophages[38–41] and has been demonstrated to play causal roles in disease pathogenesis *in vivo* models[4,40]. There are many aspects of the AIEC phenotype that seem compatible with the short-sighted evolution of virulence hypothesis. Firstly, this phenotype is linked to SNP level variation and can evolve from different phylogenetic backgrounds[42]. However, it should be noted here that although not all AIEC belong to phylogroup B2 some AIEC genes are primarily associated with the pre-invasive B2 and/or D phylogroups mentioned above[43], supporting the hypothesis that efficient invaders can evolve from primary phylogenetic branches prone to invasion. Secondly, the AIEC phenotype enables access to niches such as host tissues and immune cells that are more accessible/available under disease conditions. Not only is this consistent with niche expansion, virulence, and the capacity to drive host inflammation[4], but these traits are likely maladaptive in the context of a healthy gut environment where the potential niches afforded by the disease environment are not available and thus limit transmission of such strains to healthy hosts. Moreover, *in vivo* studies have also demonstrated that adaptation of AIEC to the mouse gut selects for novel genotypes, including hypermotility, that facilitate invasion and establishment in the mucosal niche[44].

Most importantly, *E. coli* represents only one bacterial group that could evolve virulence *de novo* in the gut environment. Many clinically relevant members of other bacterial genera are associated with specific gut diseases, including inflammatory bowel diseases (IBDs) and CRC[45–47]. Of note, a comparative phenotypic analysis of *Fusobacterium nucleatum* strains recovered from inflamed biopsy tissue taken from IBD patients were significantly more invasive *in vitro* invasion assays than strains isolated from healthy tissue from either IBD patients or controls[48]. These data highlight the local adaptation of this species to a specific niche within the disease gut environment (i.e., inflamed tissues) but also underscore how local adaptation may limit the colonization of healthy individuals. Once again, it is worth noting that *F. nucleatum* is genetically heterogeneous, with several subspecies and recombinant variants

evident based on recent comparative genomic analysis[49–51]. Elsewhere, a recent *in vivo* model highlighted the evolution of virulence in *Enterococcus gallinarum* as a pleiotropic consequence of adaptation to the mucosa[52]. Here, evolved virulent variants of *E. gallinarum* were more able to evade immune detection and clearance compared to their ancestor and could induce increased intestinal and hepatic inflammation, the latter following translocation to the liver[52]. Moreover, even though we primarily focus on invasive phenotypes in this perspective it is also worth considering that short-sighted evolution of virulence need not be restricted considering invasive phenotypes only, and within-host evolution and local adaptation could select for virulence-related traits linked to bacterial metabolism and the production of toxic compounds that can drive inflammation and disease processes[53,54].

Collectively, our perspective on the short-sighted evolution of virulence hypothesis in the context of the gut microbiota implies that different members (subspecies, clones, subclones) of a particular species can have different adaptations to local niches, and some of them may have a greater potential to evolve into a "short-sighted" invasive phenotype. In addition, nothing precludes the possibility of considering the evolution of pathogenic phenotypes as the result of a path of consecutive "short-sighted" mutations, an idea that is consistent with the ecological niche specialization theory, proposing that the niche of a population is the result of the niches occupied by all its individual variants[55].

Hypotheses should be questioned and tested, not championed.

Although the studies and data cited support the hypothesis of the short-sighted evolution of virulence, they are insufficient in that they were not specifically designed to test that the evolution of virulence of the pathogens responsible for the morbidity or mortality of humans or animals is the product of short-sighted evolution within that host. To formally test and support this hypothesis in the context of the gut microbiota will require the explicit demonstration that the genetic change(s) responsible for the virulence and invasiveness of bacteria evolved *de novo* within that host are derived from the commensal bacteria in the gut microbiome of that same host. This is a difficult task. Hypothetically, it is necessary to demonstrate that save for the genetic changes responsible for the virulence of bacteria, the pathogenic variant of that bacteria is identical to the gut bacteria from whence it was derived. Such a demonstration will require matching the genomes of intestinal clones with those isolated from bloodstream, or other sites of infection. Whilst the detection of clones in the intestine using metagenomic and other techniques is currently in development[56,57] this comparison could

306 be readily performed with whole genome sequencing of cultivated isolates. This is possible if
307 the focal bacteria of interest is readily culturable, and importantly culturation affords the ability
308 for detailed phenotyping and direct experimentation with potential ancestral and evolved
309 strains that is required for testing the hypothesis. Intuitively the short- sighted evolution of
310 virulence is likely mediated by single mutational events (e.g. single SNP or HGT event) or a
311 small number of genetic changes within a monophyletic lineage. However, it is also important
312 to avoid over-simplifying the short-sighted evolution hypothesis as a "single event of
313 translocation" only. It could be possible that translocation of bacteria from the gut to other
314 tissues or blood owing to short-sighted evolution is due to independent or simultaneous
315 translocation of different polymorphic bacterial cells from the same population in the intestine,
316 as may be the case in the study of Yelin and co-workers[21]. This point is important for evaluating
317 the hypothesis as evidence for polymorphic population genetic structure in the tissue or blood
318 samples should not necessarily discard the short-sighted evolution, which may occur for each
319 of these independent introductions. Moreover, fundamental questions with respect to how
320 different factors such as bacterial population size and mutation rate[58–60] may facilitate the
321 potential evolution of short-sight virulent phenotypes within different host niches will need to
322 be addressed.

323 Nonetheless, for human hosts, even if it is possible to show that the bacteria responsible for
324 symptoms of a specific host is, save for the mutations, genes or accessory elements responsible
325 for the virulence, is genetically identical to that species and specific strain of non-pathogenic
326 bacteria isolated from the gut microbiome of that host it would not be ethical to test the
327 hypothesis that the pathogenic isolate would generate the same symptoms responsible for its
328 isolation upon infection of a new human host. However, one could test this hypothesis with an
329 appropriate animal host to determine if the pathogenic variant, unlike its ancestor, would
330 generate symptoms similar to those of the original host. A positive result in that experiment
331 would provide compelling evidence that the pathogenic variant of those bacteria evolved in the
332 original human host and with experimental animals, but not humans, it would be possible to
333 test that evolved virulence is short-sighted, i.e., does not increase the likelihood of colonization
334 and infection.

335 Another interesting and related approach, requiring experimental animals, is to use "dead-end"
336 (evolved) bacteria that have invaded and killed a host to challenge another (genetically
337 identical) host. A higher pathogenicity in the second host in comparison with the common

bacterial ancestor will be indicative of the within-host acquisition of pathogenic short-sighted mutations. Even though the paper did not set out to test the short-sighted evolution of virulence, the aforementioned study that tested variation in virulence between an ancestral and evolved *E. coli* K1 strain presents data that is wholly consistent with the short-sighted hypothesis but also consistent with an appropriate experimental design given that the phenotypic variation for invasiveness and mortality in the *in vivo* model could be linked to mutations evolved *de novo* within the same host. However, to evaluate the final assumption of the hypothesis and that this evolution is short-sighted, it will be necessary to demonstrate that the pathogenic variant is no more likely to transmit to a new host than its avirulent ancestor.

Finally, it is also important to point out that in testing the short-sighted evolution of virulence hypothesis the experiments described will also serve to enhance our understanding of the links between specific polymorphisms and invasive infection. This is of crucial importance given that we currently lack a comprehensive catalogue of mutations that enhance virulence and invasiveness. To date, most studies on "virulence mutations" have been focused on loss of virulence by mutation, not on a gain in pathogenicity, and known virulence effectors in well-studied species are assessed based on the relative presence or absence of different virulence genes located in genetic elements such as plasmids, bacteriophages, transposons and pathogenicity islands, as opposed to polymorphisms within those genes[61]. Of note, studies have shown that some of the more invasive clones are missing many of the so-called pathogenic genes as well as genes that are part of the core genome[62]. Crucially, however, polymorphic variation in single genes can underpin specific clinically relevant phenotypes and single, or a small numbers of SNPs, in genes not linked to previously recognised virulence effectors can be responsible for invasiveness and mortality as evidenced in the aforementioned study of *E. coli* strain A192PP[32]. Such detailed bioinformatic comparisons between whole genomes of invasive and commensal strains of the same phylogenetic origin will help to establish a catalogue of mutations that are associated with virulence, and which might be acquired during the short-sighted evolutionary process thus shedding further light on the validity of the short-sighted evolution of virulence hypothesis.

Conclusions

Although the intuitive logic of the short-sighted evolution of virulence hypothesis may be appealing, and observational data from different studies of pathogenic microbes are consistent with its assumptions, it has yet to be adequately tested. Whilst it may not be possible to fully

test the hypothesis of short-sighted evolution of virulence in the context of the gut microbiota in humans, it could be formally tested with experimental and domestic animals. More generally, these experiments have the added benefit of providing key information about the evolution of virulence in bacterial populations. This is important as we know a great deal more about the mechanisms and genetic basis of bacterial virulence[63–65] but not very much about the evolution of that virulence. As noted earlier, the evolution of virulence is a long-standing subject of considerable interest to evolutionary biologists[6,7], for which there are hypotheses with strong advocates and little evidence.

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