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Can laboratory reference strains mirror 'real-world' pathogenesis?

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The extraordinary plasticity of bacterial genomes raises concerns about the adequacy of laboratory-adapted reference strains for the study of 'real world' pathogenesis. Some laboratory strains have been sub-cultured for decades since their first isolation and might have lost important pathophysiological characteristics. Evidence is presented that bacteria rapidly adapt to *in vitro* conditions. Genomic differences between laboratory reference strains and corresponding low-passage clinical isolates are reviewed. It appears that no bacterial strain can truly represent its species. For DNA microarray and proteomic studies, this limitation might be overcome by the summation of individual genomes to produce a species-specific virtual supragenome.

Introduction

Bacterial growth conditions *in vitro* are strikingly different from *in vivo* ecosystems, including bacterial infections. Growth within multispecies communities in complex and changing environments of the 'real world' contrasts with the standardized and idealized conditions in laboratory monocultures (Figure 1). Despite these differences, microbial research is largely based on laboratory-adapted reference strains that are essentially non-pathogenic [1–3]. As outlined in Box 1, laboratory strains, such as K12 of *Escherichia coli*, PAO1 of *Pseudomonas aeruginosa* and COL of *Staphylococcus aureus*, have been sub-cultured for decades since their first isolation. A variety of genotypes have consequently evolved over time [4]. As a result, current PAO1 strains from various laboratories express notably different biofilm phenotypes under ostensibly identical growth conditions (P. Stoodley, unpublished).

The extraordinary plasticity of bacterial genomes, which might be best illustrated by the diversity in genome sizes within one species [5,6], is only now being fully appreciated. This high degree of genetic flexibility, however, raises concerns about the adequacy of laboratory-adapted reference strains for the study of 'real-world' pathogenesis. In the course of sequential *in vitro* passage, laboratory reference strains might have significantly differentiated from non-passaged clinical samples. Therefore, any research conducted on the basis of current laboratory strains and their genome sequence could miss important pathophysiological mechanisms that only are present in clinical strains. This limitation obviously affects DNA microarray studies, but it also influences proteomic studies that virtually reconstruct proteins on the basis of the sequenced genome of a laboratory

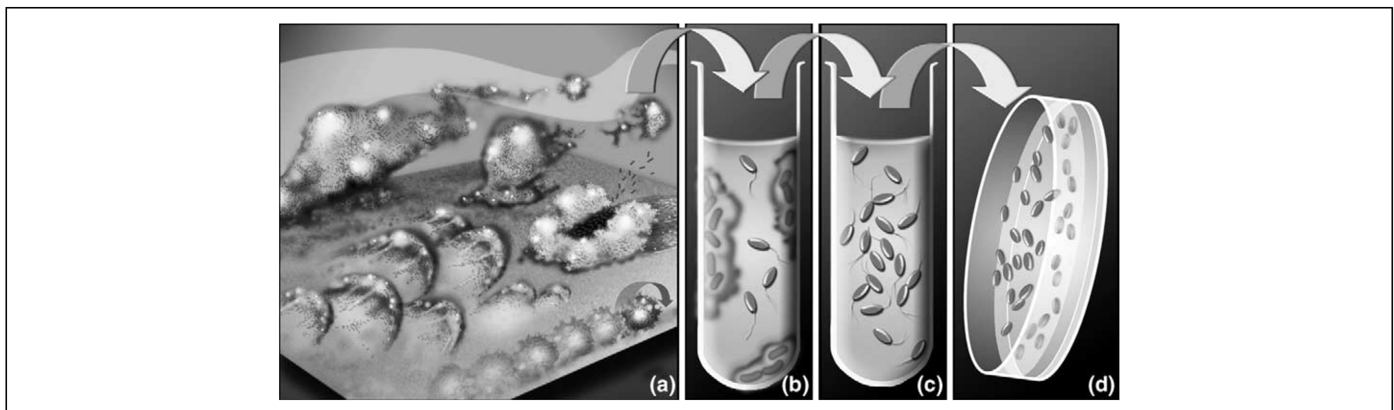

Figure 1. Conventional sampling and passage techniques enrich for planktonic populations that have adapted to idealized *in vitro* growth conditions. **(a)** *In vivo*, bacteria predominantly grow as biofilms – complex multispecies communities in a continually changing milieu. **(b)** The first selection for planktonic populations occurs during sampling, in which predominantly detached biofilm and planktonically grown cells are collected. **(c)** The sequential passage of broth cultures selects for planktonic cells, whereas surface-adherent biofilm phenotypes are left behind. **(d)** By the time bacteria are maintained in a surface-adherent manner on agar media, many of the biofilm phenotypes that enable survival in the 'real world' – and their specific genetic information - might have disappeared.

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Box 1. The 'family history' of three laboratory reference strains

***Escherichia coli* K12**

Originally isolated from the stool of a convalescent diphtheria patient in 1922, K12 was sequentially passaged for 50 years in the bacteriology department at Stanford University before various derivative strains were typed and categorized [44]. In 1997 the genome of MG1655 was sequenced, a derivative of K12 that had been cured of the temperate lambda phage by ultraviolet irradiation and the F plasmid by acridine orange [45].

***Pseudomonas aeruginosa* PAO1**

The strain was originally isolated from a burn wound in 1955 by B.W. Holloway in Melbourne, Australia [46]. Subcultures have been passaged on laboratory media for more than three decades and shared among microbiological laboratories all over the world. The actual ATCC strain (BAA-47) represents a descendent of the original strain that had been passaged in the laboratories of V.N. Krylov and H.W. Ackermann. The PAO1 genome sequenced in 2000 was derived from a strain of B.W. Holloway's collection, which had been maintained in the laboratory of P. Phibbs, University of Georgia [4].

***Staphylococcus aureus* COL**

Isolated from the air of an operating theatre in England and cultured by the Central Public Health Laboratory in Colindale (i.e. COL), the strain was first described in 1966 [47]. Later on, it was transferred to L. Sabath's laboratory and maintained by serial passage on agar medium, providing the basis for a publication in 1972 [48]. In 1976, the strain was sent to B. Wilkinson's laboratory, where stock solutions were frozen down. The COL genome was sequenced based on cultures from this stock (TIGR databases).

reference strain. Sequential passage in planktonic cultures might be particularly fatal for biofilm-forming subpopulations, which will be left behind adherent to the vessel wall when planktonic cells are removed for subculture (Figure 1). As a result, genes controlling the spatial and metabolic interactions within complex biofilms could be lost. Importantly, major laboratory reference strains show impaired biofilm formation compared with clinical representatives of the same species [6].

To substantiate our concerns about using laboratory reference strains, we present evidence that (i) bacterial genomes evolve during serial *in vitro* passage and (ii) that these alterations are not random, but the result of specific growth conditions. Important genomic differences between three laboratory reference strains and individual clinical isolates of the same species are reviewed. We emphasize the fact that no clinical strain, let alone a laboratory reference strain, can truly represent a species. Rather, the complete species-genome is dispersed throughout the population, as detailed in the distributed genome hypothesis developed by Garth Ehrlich and colleagues [7].

Learning from Pasteur

The history of the Bacille Calmette–Guerin (BCG) vaccine provides an illustrative example for the evolution of bacterial strains *in vitro*; repetitive passage has transformed virulent mycobacteria into an attenuated live vaccine [8]. Recognizing from the work of Louis Pasteur that laboratory adaptation of bacteria is associated with attenuation of virulence towards the host, Calmette performed 230 passages before first exposing a human to the evolved strain in 1921. Attenuation of virulence had already been reported after 15 passages, but continued over 1173 passages until the lyophilisation of BCG in 1961 [8]. Until this time the vaccine strain had been distributed all over the world and passaged further in individual laboratories. Behr *et al.* [8] established a genealogy of BCG vaccine strains by comparing genetic alterations over time and between different strains. The observed deletions, single nucleotide polymorphisms and duplications are thought to account for the phenotypic differences observed between current vaccine strains [8].

Several studies have documented that genetic alterations during serial passage are not random. In planktonic cultures, selection for rapidly growing bacteria occurs at the expense of genes that are unnecessary *in vitro* [9–11]. Edwards *et al.* [9] randomly inactivated individual genes in *E. coli* before growing planktonic cultures for 60 to 90 generations. Out of a total of 40 gene inactivations that provided fitness advantages, 17 were found in operons required for flagella production. Flagellar loss is believed to provide substantial savings in energy and amino acid requirements, but it reduces surface adhesion and biofilm formation. Cooper *et al.* [10] analyzed the gene expression of 12 *E. coli* populations after 20 000 generations in planktonic culture. Populations had adapted to a rich-medium environment and exhibited substantial gains in competitive fitness relative to their ancestors. Eight out of twelve populations showed mutations that resulted in a downregulation of flagella and guanosine tetraphosphate (ppGpp) concentrations. The latter is believed to stimulate growth by increasing tRNA and rRNA transcription rates. In *S. aureus*, sequential *in vitro* cultures selected for mutations within the *agr* operon, thereby decreasing the production of virulence factors and increasing the growth yield [11].

It is crucial to recognize that standard laboratory growth conditions might fail to reproduce adaptive processes found *in vivo*. Point mutations that convert non-mucoid *P. aeruginosa* into an alginate-producing mucoid phenotype prevail in the lungs of patients with cystic fibrosis (CF), but are rare under standard laboratory conditions [12]. To induce the *in vitro* generation of mucoid phenotypes and antimicrobial-resistant small-colony variants, bacteria must be challenged with sublethal concentrations of antimicrobials, a phage or surfactants [13,14]. The fact that these phenotypic switches are closely related to improved biofilm formation [13,15] again underlines the selective disadvantage of conventional laboratory growth conditions for biofilm-forming populations. Two recent publications correlate biofilm formation with the expression of cytotoxic (type III secretion) phenotypes, thereby illustrating the impact of the bacterial mode-of-growth on the expression of virulence factors [16,17].

Analogies of laboratory reference strains with obligate intracellular bacteria might help us to explain adaptive processes in the former (Box 2).

Are clinical strains different?

Improvements in genome sequencing, genomic subtraction techniques and constructing DNA arrays have

Box 2. Analogies to obligate intracellular bacteria

Both laboratory reference strains and obligate intracellular bacteria, such as chlamydia, rickettsia and mycoplasma, exclusively grow in a highly specific environment. Growth conditions are characterized by an abundance of nutrients and the restricted exposure to foreign bacterial DNA, abolishing the acquisition of new genetic material. These features could explain the reduced genome size of obligatory intracellular and laboratory-adapted bacteria compared with free-living organisms, which contain considerably more mobile genetic elements and repeated sequences [49]. The unselectively rich milieu in eukaryotic cells and standard growth media renders the biosynthesis of many molecules optional [49]. Assuming that bacterial genomes are prone to the deletion of non-essential DNA [21], their coding genes might subsequently degrade. Both rickettsia and chlamydia rely on host cells for their supply of nucleoside monophosphates and appear to have discarded all genes involved in their synthesis [50]. Up to 25% of non-coding DNA in intracellular bacteria has been suggested to represent such ancient genes in a state of degradation [49,50]. By contrast, free-living bacteria have a low amount of non-coding DNA [51], which presumably reflects a faster elimination rate to keep up with the high rates of DNA influx by horizontal gene transfer.

facilitated the genetic comparison of individual strains within one species. Important genetic differences between laboratory reference strains and their clinical counterparts have been described for *E. coli*, *P. aeruginosa* and *S. aureus*. The greater virulence of clinical isolates has not only been related to the presence of individual genes [18] or gene clusters (referred to as pathogenicity islands) [19,20] but also to the inactivation of individual genes [21].

Genome comparisons between uropathogenic, enteropathogenic and the avirulent laboratory reference strain K12 of *E. coli* revealed that only 39% of their combined set of proteins is common to all strains [22]. Genome comparisons between the enterohemorrhagic *E. coli* O157:H7 and K12 strains revealed a total of 1632 O157:H7-specific open reading frames [19]. Both strains contained a conserved (98.4% nucleotide sequence identity) backbone genome of 4.1 megabases (Mb) interrupted by strain-specific islands [19,23]. Strain-specific islands measured 1.4 Mb for O157:H7 and 0.5 Mb for K12, respectively. Differences in G+C content and the codon usage between the backbone genome and genes on the islands suggest that strain-specific regions have been imported from other bacteria by horizontal gene transfer. Prophages and prophage-like elements occupy more than half of the O157:H7-specific sequences [19]. The higher rate of defective DNA mismatch repair systems in O157:H7 [24] (and many other pathogenic bacteria) might enable more frequent mutations and horizontal gene transfer to take place, which further promotes pathogenicity [25,26]. Taken together, the laboratory reference strain K12 is characterized by a smaller genome with fewer pathogenicity islands and possibly impaired genetic plasticity compared with the clinical strain.

To elucidate the evolution of *E. coli* strains, Reid *et al.* [24] built phylogenetic trees by comparing gene sequences. The laboratory strain K12 differed from pathogenic strains by a surprisingly high number of nucleotide changes [24]. On the basis of this discrepancy, the genetic separation of K12 and O157:H7 was set back to as long as 4.5 million years ago. However, because K12 is the product of innumerable *in vitro* passages, an alternative explanation is possible: the limited selection pressure of idealized growth conditions *in vitro* might have tolerated the accumulation of as many genetic alterations within decades as hostile natural environments would have in thousands of years.

Several clinical and environmental isolates of *P. aeruginosa* have been compared with the laboratory reference strain PAO1 [6,20,27,28]. Similar to *E. coli*, a highly conserved backbone genome contrasted with significant differences in hypervariable regions. Within hypervariable regions, between 0.4% and 19% of the genomes of non-laboratory strains are missing in PAO1 [6,20,27]. It has been hypothesized that the predecessor of the current PAO1 possessed many of the genes found throughout *P. aeruginosa* isolates, but that whatever was not advantageous in a laboratory environment has been progressively deleted [6]. By contrast, three PAO1-specific islands were detected [27]. Inserted strain-specific regions include the *P. aeruginosa* genomic islands (PAGI); their codon-usage pattern differs significantly from the PAO1 genome [20]. The selective advantage of these pathogenicity islands has been attributed to the protection against oxidative damage (PAGI-1), to additional metabolic functions and transporters (PAGI-2 and 3), to altered flagellin-glycosylation or to unspecified transcriptional regulations [20,29,30]. For example, PAGI-1 has been found in 85% of isolates from sepsis and urinary tract infections and appears to confer virulence traits [20]. The genome island pKLC102 is a hybrid of plasmid and phage origin [31]. Its coexistence as a plasmid and a genome island suggests its intraclonal evolution from a mobile ancestor via a reversibly integrated state to irreversible incorporation and dissipation in the chromosome.

Phenotypic comparisons between strains derived from lungs of CF patients and PAO1 revealed that adaptive responses to one environment attenuate the performance in another setting. The majority of CF-isolates expressed reduced planktonic growth *in vitro* and decreased lung colonization in a mouse model, but showed more efficient biofilm formation [6].

In addition to a genetically stable backbone, the *S. aureus* genome includes roughly 22% of strain-specific sequences, which are organized into 18 large hypervariable regions containing prophages, plasmids, transposons and insertion sequences [32]. A maximum of 12% of open reading frames were absent in any clinical strain compared with the laboratory strain COL [32]. Clusters of strain-specific genes (the pathogenicity islands) encode antibiotic resistance, virulence factors and an extraordinary heterogeneity of superantigens [33,34]. Allelic variations within these islands further increase genetic variability among *S. aureus* strains [35].

Genomic diversity among clinical strains

In vivo, bacteria have developed elaborate strategies to cope with particular habitats that result in significant genetic diversity within one species. Mechanisms of this genomic plasticity are summarized in Box 3.

The specific assembly of pathogenicity islands in enterobacteria has resulted in disease-associated clones [22]. For

Box 3. Mechanisms of genomic plasticity

Genomic plasticity depends on point mutations, gene rearrangements, deletions and horizontal gene transfer [35]. The latter includes recombination as well as the insertion of mobile genetic elements (bacterial phages, conjugative plasmids, transposons and insertion elements). The localization of many virulence-associated genes within such mobile genetic elements underscores the importance of horizontal gene transfer for the evolution of pathogenicity [52]. The rate of horizontal transfer correlates with the content of mobile and repeated elements within a genome [53]. Interactions between mobile elements are therefore thought to accelerate the successive acquisition of virulence factors. For example, in *Vibrio cholerae* pili formed by one phage are indispensable for host colonization, but also as a receptor for a second phage carrying the cholera toxin gene [54].

As the inflow of genetic material through horizontal gene transfer does not appear to have resulted in genome size expansions, reductive evolutionary processes must occur at similarly high frequencies [55]. The addition of a foreign gene might increase the probability of loss of some resident function of lower selective value [55].

Pathogenicity islands, clusters of strain-specific mobile genetic elements, might loose characteristic mobility genes over time and become integrated into the core genome. The existence of homologous genes on plasmids might then be the only indication that these genes represent chromosomal integrations of former mobile genetic elements [35]. Although most groups of Gram-negative and Gram-positive pathogens contain pathogenicity islands, none has been found in *Chlamydia spp.*, the spirochetes and most streptococcal species [35]. In chlamydia and spirochetes, this lack of pathogenicity islands correlates with a reduced genome size and the inability to replicate outside a host and might be due to their parasitic life style. By contrast, streptococci have a high degree of flexibility in their lifestyle despite the lack of pathogenicity islands. This presumable contradiction has been explained by the high rate of recombination in these bacteria. Newly-acquired mobile genetic elements would instantly be recombined, resulting in mosaic gene structures and counteracting the emergence of typical pathogenicity islands. The estimate that 10% of the *S. pyogenes* genome comprises bacteriophages and transposons [56] supports this hypothesis.

E. coli, distinct genotypes can be attributed to individual intestinal and extraintestinal pathologies. However, for *P. aeruginosa* a firm correlation could not be established between specific clones and habitats, arguing against a pronounced genetic specialization in this species [36].

In contrast to community-acquired methicillin-resistant *S. aureus* (MRSA), nosocomial strains contain a heterogeneity of superantigen gene clusters and a high number of transposons and insertion sequences [33,35]. This arsenal might enable them to resist a hostile hospital environment, but represents a burden for rapid growth [33].

Two recent studies on *S. aureus* provide good examples for the plasticity of bacterial genomes in response to environmental stresses. Robinson *et al.* [37] investigated the emergence of MRSA following the introduction of methicillin in 1959. According to their model, methicillin resistance has emerged at least 20 times following the acquisition of mobile genetic elements known as staphylococcal cassette chromosome mec (SCCmec), producing five major lineages of MRSA. A second study reported that genome alterations within one *S. aureus* strain were significantly more frequent during chronic lung infection in patients with CF than during nasal colonization in healthy individuals [38]. This suggests that the exposure to host defenses and sub-inhibitory antibiotic concentrations might promote genetic alterations. Out of 19 genetic differences between consecutive samples from individual patients, 8 could be related to phage mobilization, indicative of an enhanced potency of lateral gene transfer. Preliminary data further suggested that intrachromosomal inversions and duplications had occurred.

The observation that quorum-sensing, plasmids and phages are key players in both horizontal gene transfer and biofilm formation suggests that the capacities to form biofilms and to exchange genetic material are closely related. Notably, a particular quorum-sensing system in *Streptococcus mutans* promotes both genetic competence and biofilm formation [39]. Consequently, transformation frequencies were 10 to 600-fold higher in biofilm-grown bacteria than in planktonic cells. K12 dramatically improved biofilm formation after the acquisition of conjugative plasmids [26]. Thereby, cell-cell contacts through conjugative pili enhance biofilm formation, which provides a sufficient density of potential bacterial recipients to assure high transfer rates of plasmids [26]. Furthermore, biofilm formation mediates prophage expression in Gram-positive and Gram-negative organisms [40,41]. This upregulation not only favors horizontal gene transfer, but has recently been recognized to induce biofilm dispersal in *P. aeruginosa* [42].

The supragenome

The genetic plasticity of bacteria in response to the environment, fueled by high rates of horizontal gene transfer in biofilms, results in genetic diversity within a species that is currently difficult to quantify. Even non-passaged clinical strains will never be representative of their species. Only the summation of species-specific 'communal gene-pools' in a virtual supragenome can fully characterize the genome of an individual species [43]. This genetic reservoir could provide a major, previously unrecognized survival advantage at the population level.

Ehrlich *et al.* [43] are currently establishing the first supra-genome for *Haemophilus influenzae*. The genetic analysis of DNA fragments from 10 clinical strains revealed that 11% of the sequences were not represented in the genome of the reference strain *H. influenzae* Rd. On the basis of these data, the supragenome is expected to double the size of the reference strain genome.

Concluding remarks

No individual genome can truly represent a bacterial species. Thus, the interpretation of any study result must not only consider the experimental conditions, but also the genetic background of the bacterial strains used. Laboratory reference strains might have lost important pathophysiological characteristics and therefore might be inadequate to document 'real-world' pathogenesis. The close link between horizontal gene transfer and biofilm formation is increasingly recognized. The impaired biofilm formation of laboratory reference strains compared with

clinical strains might indicate further-reaching genetic alterations affecting bacterial virulence on a broader level.

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