Population-level virulence factors amongst pathogenic bacteria: relation to infection outcome

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The study of population-level virulence traits among communal bacteria represents an emerging discipline in the field of bacterial pathogenesis. It has become clear over the past decade-and-a-half that bacteria exhibit many of the hallmarks of multicellular organisms when they are growing as biofilms and communicating among each other using quorum-sensing systems. Each of these population-level behaviors provides for multiple expressions of virulence that individual free-swimming bacteria do not possess. Population-level virulence traits are largely associated with chronic or persistence infections, whereas individual bacterial virulence traits are associated with acute infections. Thus, there is a natural dichotomy between acute and chronic infectious processes which helps to explain the medical community's success in combating the former, but its utter failure in dealing with the latter. The recent recognition of multicellularity among chronic bacterial pathogens will lead the way towards new multimodality therapies.

Keywords: bacteria, biofilms, distributed genome hypothesis, horizontal-gene transfer, pathogenesis, population-level virulence factors, quorum sensing, supragenome

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The elucidation over the past quarter century that nearly all types of bacteria, whether soil microbes or human pathogens, can live as matrix-enclosed multicellular communities, in addition to living as individual free-swimming planktonic forms, has brought with it the realization that there exists population-level equivalents for all aspects of bacterial physiology, ecology, evolution and in the case of pathogens, virulence traits. The last has profound ramifications for how we think about bacterial pathogenic processes in general, and has provided the impetus for the construction of a new theoretical edifice, namely that of bacterial plurality, which more accurately informs with respect to chronic population-based microbial interactions with the host than the teachings of Robert Koch that were designed to model acute and epidemic bacterial processes [1]. Thus, there is an important dichotomy to be made between acute and chronic bacterial pathogenic mechanisms in that there is a natural affiliation between population-level virulence traits and chronic infections on one hand, and individual bacterial virulence traits and acute infections on the other. Several, broad types of population-level virulence traits have been recently recognized, and it is likely that more will be discovered as this is an infant field. The first of the population-level virulence traits recognized were biofilm-related differences in susceptibility to antimicrobial agents [2,3]. Bacteria that are exquisitely sensitive to low concentrations of antibiotics when growing planktonically can be highly metabolically resistant to the most potent, latest-generation, broad-spectrum antibiotics when living as a community within biofilms; this community survival strategy provides a mechanism for chronicity not present among single-celled bacteria. Similarly, the existence of bacterial intercellular communication systems, collectively termed quorum sensing (QS) [4–6], provides bacterial communities with the ability to coordinate toxin expression and thereby overwhelm host defenses; an example of this is *Staphylococcus aureus*-associated toxic shock syndrome [7,8]. Whilst the distributed genome hypothesis states that for all species that have horizontal-gene transfer (HGT) mechanisms there are extensive genic (as opposed to allelic) differences among the many strains of a species and that a species-level supragenome exists, therefore, that is much larger than the genome of any single strain [9–13]. This genomic plasticity combined with highly efficient inducible HGT mechanisms and polyclonal infections [14,15] provides a diversity generation mechanism that acts as counterpoint to the host's adaptive immune response providing for the evolution of new strains in response to stress [16].

Biofilms & the bacterial life cycle

The fact that the preferred growth mode for almost all bacterial species is within a matrix-enclosed community, termed a biofilm, was first recognized by Costerton and his coworkers 30 years ago [17]. This observation stood in stark contrast to the time-honored method of studying only free swimming or planktonic bacterial forms, and for some time afterwards microbiologists were working in either the biofilm camp

or planktonic camp. The medical community was particularly slow to adopt the biofilm paradigm owing to the teachings of Robert Koch, who had emphasized the importance of pure clonal culture in his eponymous postulates. Yet, van Leeuwenhoek, the first modern microbiologist, in his descriptions of the microbial world clearly observed what we would recognize as biofilms today. Careful morphological and physiological examinations spurred by the development of modern molecular imaging and molecular diagnostic techniques have revealed that many conditions, which were previously viewed as chronic inflammatory conditions, have revealed their true characters to be indolent bacterial biofilm infections. These include:

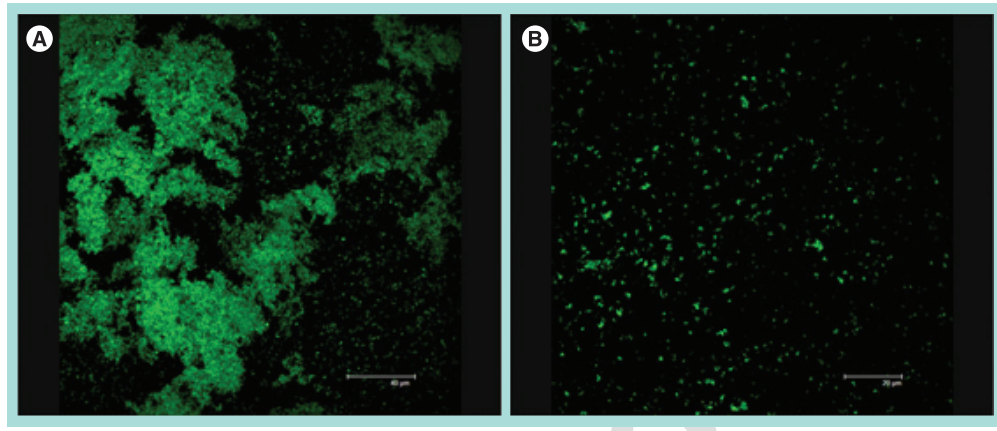
- Osteomyelitis [18,19]
- Gall bladder disease [20,21]
- Prostatitis [22]
- Otitis media with effusion, recurrent otitis media and otorrhea [2,23–25]
- Chronic rhinosinusitis [26,27]
- Cholesteatoma [28]
- Tonsillitis [29]
- Adenoiditis [30]
- Diabetic foot ulcers [31]
- Periodontitis [32–38]

Biofilms also represent the overwhelming bacterial phenotype associated with infections of implantable medical devices and indwelling catheters. These foreign body infections are nearly impossible to eradicate without removal of the device and have become the bain of many permanent and long-term interventional strategies, including arthoplasties, central lines, urinary catheriterizations, pace makers, defibrillators, ventricular-peritoneal shunts and dialysis ports (reviewed in [39]). Biofilms can be composed of a single species, however, in many environments biofilms comprise polymicrobial communities [40] with the waste of one organism being the foodstuff for another. More recently it has been realized that the biofilm (planktonic dichotomy) is in itself an oversimplification of what is in reality a complex bacterial life cycle that includes: reversible attachment; irreversible attachment, growth, effacement, stalk and tower formation; maturation, seething; and finally dispersal of planktonic cells that are free to begin the process all over again [41,42].

The bacterially-elaborated matrix associated with the final, irreversible attachment of a bacterial cell to a surface, is composed of multiple extracellular polymeric substances (EPS) including exopolysaccarides, DNA, proteins and lipids, and provides a protective physical barrier for the bacteria within. The cooperative creation of the matrix on host tissues or implantable devices by a community of bacteria is a population-level virulence trait as it provides for a community of bacteria that are collectively more difficult for the host to eradicate than individual free-swimming or attached bacteria. Once initiated, a biofilm acts like a dynamic living organism that can grow, change its physical properties in response to its environment and incorporate other pathogenic species into an integrated polymicrobial community. The biofilm provides an encasement for thousands to millions of bacteria within a single structure and provides the population of interacting organisms with an exoskeleton for shape and structure – hallmarks of higher multicellular organisms. Extending the skeletal metaphor, the biofilm matrix also plays important roles in signaling control and nutrient availablity, much as eukaryotic skeletal elements do. Recent rheological studies by Stoodley *et al.* have demonstrated that the hydrated EPS matrix is highly viscoelastic and can be rapidly remodeled in response to changes in shear stress and other environmental stressors [43–47]. Thus, in this regard it displays similar qualities to endochondral bone in that the strength of the extracellular matrix is modifiable by the cellular component in accordance with the external load. The viscoelastic properties of the EPS are highly similar to that of DNA (it is the viscoelastic properties of DNA that provide the basis for high-resolution pulse-field gel electrophoresis) and studies by Whitchurch *et al.* [48] of the *Pseudomonas aeruginosa* EPS matrix revealed that DNA was, in fact, one of the major constituents.

More recently it has also been demonstrated that the matrix of *Haemophilus influenzae* is also largely composed of very long strands of DNA [49], and our laboratory has demonstrated that a mature pneumococcal biofilm can be completely disrupted by treatment with DNAse I (**Figure 1**). These observations help to explain the efficacy of pulmozyme (recombinant human DNAse I) treatment in the cystic fibrosis (CF) lung [50,51]. Previously, it was thought that the DNA in the CF lung originated largely from lysed neutrophils and other host cells, but the realization that biofilm bacteria are elaborating high-molecular-weight DNA into the matrix as part of their normal physiology makes it likely that the pul-

Figure 1. DNA is a major constituent of the biofilm matrix.



(A) Confocal laser scanning microscopic image of a robust 96 h *Streptococcus pneumoniae* type 23 biofilm stained with Pico Green. **(B)** Identical biofilm and location following incubation with recombinant human DNAse I (Pulmozyme) for 15 min. Notice that the DNAse incubation has resulted in greater than 90% debulking of the biofilm.

mozyme treatment is also targeting the biofilm matrix. This would have at least two benefits for the patient – a reduction in sputum viscosity making it easier to clear the mucus, and conversion of some of the biofilm bacteria to planktonics, which would be easier to kill with traditional antibiotic therapy.

The biofilm provides numerous other advantageous over planktonic growth, including attachment to a surface, which assures that the biofilm bacteria will not be swept away to some less habitable locale if there is shear stress. The biofilm also provides for metabolic heterogeneity with the bacteria on the periphery, displaying much higher metabolic rates than those deeper in the biofilm as oxygen and other nutrients rapidly become limiting as distance from the bulk fluid increases [52]. Thus, the cells deep in a biofilm are almost always respiring anaerobically [53]. This metabolic heterogeneity provides the biofilm as a whole with a survival advantage over synchronized planktonic populations as enviromental challenges that negatively affect certain processes (such as DNA replication or cell division) will only affect a small percentage of the population, best encapsulated by: "in diversity lies strength".

The clinical consequence of attachment and reduced physiological output is that biofilms are nearly always associated with chronic or persistent infections as opposed to highly invasive or systemic infections, which are the hallmark of clonal planktonic infections. In other words, rapid clonal growth as planktonic populations represents only a very small percentage of the bacterial life cycle. Yet, clinical microbiologists, physicians and surgeons have largely clung to this narrow view of the microbial world in spite of significant advances over the past several decades with regard to our understanding of microbial phenotypic diversity. This is all the more puzzling as the extant models of infectious disease do not adequately explain the vast majority of infections.

Ramifications of biofilm infections

Antibiotic resistance or tolerance

Perhaps within the context of current (and past) medical practice, the metabolic resistance or tolerance of most biofilm bacteria to nearly all classes of traditional antibiotics including the aminoglycosides, β-lactams, cephalosporins, fluoroquinolones, macrolides and tetracyclines is the most important clinical ramification. This resistance of biofilms to antibiotics is also the main reason it took so long for the medical community to recognize that many chronic conditions were actually infectious in nature.

This misunderstanding stemmed from the over-riding belief that antibiotics would always kill bacteria that did not have a genetically-encoded resistance gene. Therefore, if a physician utilized antibiotics for treatment and it could be demonstrated that the antibiotic concentration in the diseased tissue or fluid compartment was high enough to kill planktonic bacteria, it was concluded that there must not have been any live bacteria present and, therefore, it was not an infectious process, but a host-inflammatory process.

Originally it was thought that biofilm bacteria were antibiotic resistant owing to the fact that the antibiotics could not penetrate through the extracellular matrix. In other words, the matrix acted as a physical barrier such that the individual bacteria never actually encountered the antibiotic. This concept was challenged by Garth Ehrlich who argued, based on the penicillin selection method for *Escherichia coli* auxotrophs [54,55], that the resistance, at least in part, was due to metabolic quiescence. This was subsequently demonstrated to be true [52] and that by providing the biofilm bacteria with a fermentable substrate it was possible to upregulate the metabolic rate and simultaneously increase their susceptibility to antibiotic-mediated killing [53]. However, as in many biological systems there is additional complexity to biofilm-associated antibiotic resistance. Mah *et al.* demonstrated that biofilms of *P. aeruginosa ndvB* mutants, which are impaired in their ability to produce periplasmic glucans, displayed increased sensitivity to tobramycin and other classes of antibiotics, suggesting that binding of antibiotics within the periplasm may be an active method of biofilm resistance [56]. Additionally, Gilbert *et al.* have postulated that antibiotic resistance stems from a small subpopulation of cells, termed persisters, that – like metazoan stem cells – remain quiescent until there has been a cull of the parenchymal biofilm cells, at which point the persisters begin to divide and repopulate the biofilm [57] .

Resistance to host-defense mechanisms

In a manner similar to antibiotics, the biofilm matrix does not serve as an effective physical barrier against the host humoral and cellular defense mechanisms, nonetheless biofilm bacteria often display extraordinary resistance to these host-defense mechanisms. Leid and colleagues [58] working with *S. aureus* biofilms under physiological conditions demonstrated that host phagocytes can invade the biofilm and release cytokines, but that these actions induce little damage. This group further demonstrated that in the presence of IFN-γ treatment, mononuclear cell phagocytes could kill alginate-negative (alg^-) mutants of *P. aeruginosa*, but not their wild-type counterparts [59]. These data combined with the observation that exogenous alginate added to alg^- strains provided protection from phagocytosis strongly supports the contention that alginate, although not a key component of the biofilm matrix itself, provides a defense against host leukocyte attack. Subsequently, Cerca *et al.* [60] demonstrated antibody penetration into biofilms followed by opsonophagocytic leukocytes; however, in spite of the presence of these host defenses within the biofilm, the extracellular concentration of antigen is so high that it serves as a protective decoy for the bacterial cells themselves.

Intracellular communication systems

Many bacterial species including Gram-negative and Gram-positive pathogens have evolved complex intercellular communication systems [4–7,61] that, among many other functions, coordinate the simultaneous expression of various virulence traits by a population of bacteria which may be planktonic, biofilm or both [62]. These population-level behaviors among bacteria have been dubbed 'QS' [4]. Bacterial communities use QS systems to detect different types of environmental conditions. They then integrate this environmental and population information with their gene expression machinery to provide for population level-based coordination of gene regulation. These highly coordinated behaviors provide a selective advantage for the population as a whole that would be meaningless, and a waste of metabolic energy if carried out randomly by individuals within the population.

All Gram-negative QS systems, regardless of the particular chemistry involved, include a constitutively-produced, diffusible, autoinducer (AI) molecule – essentially a ligand. The detection of a minimal threshold stimulatory concentration of the AI leads to an alteration in gene expression by the cell. Since all cells in the population are in the same environment with respect to AI density, they will all sense a quorum, simultaneously providing for coordinated behaviors – one of the hallmarks of multicellular organisms. This coordination of expression occurs when there are a sufficient number of bacterial cells present within a given volume such that the concentration of the AI reaches a threshold that permits binding to its cognate receptor, which then signals a major metabolic shift of the bacteria. Thus, the AI is essentially an "I am here" molecule, which the bacteria count with their receptors to determine if they have a quorum before embarking on a community activity. Bacteria use QS communication circuits to regulate a host of physiological processes including: mutualistic behaviors such as production of luminescence in the light organs of deep sea fish and squid, which use the light as a lure for prey; antibacterial strategies such as antibiotic production; life-cycle regulation including motility and sporulation; and the expression of various vir-

ulence traits such as the coordinate expression of toxins, as well as those associated with other population-level virulence traits such as HGT mechanisms and biofilm formation [4,63–67].

Among the more than 75 species of Gram-negative bacteria that are known to use QS for coordinated activities, including the vibrios, enterics and pseudomonads, the AIs have been universally found to be homoserine lactones with acyl side chains varying in length between four and 18 carbons, depending on the species [61]. Some species have multiple AIs (and cognate receptors) that they use to produce integrated responses to complex environmental stimuli [68,69]. All Gram-negative species use an enzyme that builds these acylated homoserine lactones (AHLs) from adenosylmethionine and acylated acyl carrier proteins (acyl-ACP). The AHLs, which are secreted by the individual bacteria, pass freely through the bacterial cell membranes, and once in the cytoplasm at sufficient concentrations each AHL molecule binds two LuxR proteins forming a dimer that binds to regulatory DNA regions to up- or downregulate target regulons, which results in major metabolic shifts. Among Gram-positive organisms, including the staphylo- and streptococci, the AIs are short peptides, generated from longer precursor proteins, containing five to 17 amino acid residues that may be modified with thiolactone rings, lanthionines or isoprenyl groups. These peptide-based AI's are not freely diffusible across the cell membrane as are the AHLs, but instead are exported by an ATP-dependent process; once in the extracellular mileau, they then bind to transmembrane receptors and induce a second messenger cascade. Recent work has identified a common evolutionary origin for all Gram-positive QS systems [70]. Once the receptors have bound a ligand it results in their dimerization and activation of a cryptic kinase activity by the intracellular domain that triggers a phosphate- transfer cascade resulting in the creation of a master transcriptional switch for controlling gene expression. For example, the AI produced by the pathogenic staphylococci including *S. aureus* and *Staphylococcus epidermidis* is an octapeptide, termed RNA III-activating protein (RAP), which binds to a transmembrane receptor, target of RAP (TRAP). Interestingly, different species of bacteria compete by attempting to disrupt their competitor's QS systems. For example, some of the commensal staphylococci produce a different peptide, RNA III-inhibiting peptide (RIP), which binds to TRAP, but does not result in its activation [71].

Not all species and strains have fully functioning QS systems, but many of those that do not can still listen in on a second class of QS molecules, the AI2s discovered and characterized by the Bassler laboratory (Tufts University, MA, USA) [61]. The AI2 molecules (actually a family of interconverting pentanedione molecules complexed with a boron atom) are recognized by a very broad class of microbes that includes both Gram-negative and Gram-positive species, and thus serves somewhat as a *lingua franca* among the bacteria. Bacteria can recognize a variety of these AI2s allowing them to sense bacteria both of their own kind and of others, even if they do not produce the molecules themselves and which may allow invasive species to determine the strength of their foes. There is also mounting evidence that bacterial QS systems are involved in communication with their eukaryotic host organisms in both mutualistic and pathogenic symbioses [67,72].

Although the nature of the chemical signals, signal relay mechanisms and target genes controlled by bacterial QS systems differ, in every case the ability to communicate with one another allows bacteria to coordinate the gene expression, and therefore the behavior, of the entire community. Therefore, when associated with pathogenicity QS is a population-level virulence trait.

The elucidation and characterization of bacterial QS systems has provided scientists with new targets for the development of antibacterial compounds. The Kjelleberg and Givskov groups in Australia and Denmark identified factors from the red alga *Delisea pulchra* that inhibited community behaviors of bacteria [73]. They then demonstrated that this activity was due to halogenated furanones that interfered with QS [74]. Subsequently, working together with the Molin and Hoiby groups, they prepared semisynthetic derivitives of these natural compounds and demonstrated that their administration could reduce the virulence of *P. aeruginosa,* thus establishing a new avenue in medicinal chemistry [75].

Horizontal-gene transfer mechanisms

Evolutionary considerations for HGT

We have previously defined HGT mechanisms as supravirulence traits [76,77] because they provide for the acquisition of any other virulence trait. The ability of already resident infectious bacteria to acquire entirely new genetic traits during the infectious process in the context of an already successful pathogenic genome provides a heretofore unprecedented and unrecognized

selective advantage to those organisms possessing such mechanisms. We hypothesize that HGT mechanisms are critical for ensuring chronicity of infection and that pathogens that do not possess powerful HGT mechanisms will be relegated to the production of acute infection. The importance of these HGT mechanisms is underscored by the fact that nearly all higher taxa containing pathogenic bacterial species contain some species that possess one or more DNA uptake or transfer systems [78]. The maintenance of these systems is particularly striking when viewed from a genomic perspective. Most of the professional pathogens (those pathogenic bacterial species that do not exist naturally outside the human host), whether Gram-negative or Gram-positive maintain fairly small genome sizes – in the range of 1.5–3 megabases – even though they have access to a large species-level supragenome. Thus, they must possess very active genomic deletion mechanisms, probably to ensure that they do not maintain high percentages of nonessential genes or junk DNA, as do most eukaryotic genomes. Correspondingly, their genomes are very tidy and nearly devoid of pseudogenes and noncoding regions, yet they dedicate multiple large operons to actively support HGT. The persistence of these large regulons associated with HGT, and their widespread nature argues strongly that there is constant selective pressure for their maintenance [1,78]. Thus, by examining the global genomic architecture it can be observed that HGT plays a significant role in strain evolution.

Chronic infectious conditions possess all the elements necessary for HGT

An examination of the conditions present during colonization and chronic infectious disease processes demonstrates that all of the necessary components are present for effective HGT. First and foremost, colonization is nearly always polyclonal. This fact had been missed for over a century because of the medical community's reliance on Koch's postulates, which teach that a single clonal isolate must be obtained. If one is always purifying a single clone this is the same as having a blindfold on, because any diversity that is present will never be observed. Recently, the laboratories of Tim Murphy and Janet Gilsdorf have courageously demonstrated, by examining chronic obstructive pulmonary disease patients and the normal nasopharynx, respectively, that nearly all persons who are infected or colonized with *H. influenzae* are polyclonally infected – sometimes with more than 20 strains simultaneously [14,15,79]. Similarly, Dowson's group has observed polyclonal infection with pneumococcus [80], and the Hoiby and Molin groups in Denmark have seen polyclonal *P. aeruginosa* infections in the cystic fibrosis lung [81].

The second prerequisite for HGT is a source of DNA for transformation. As noted above, all of the biofilm EPS matrices that have been examined for molecular composition contain large amounts of DNA [Ehrlich *et al.*, Unpublished Observations] [48,49] such that the bacteria are essentially bathing in DNA. Even more interestingly, the Shi, Clavery and Havarstein laboratories have convincingly demonstrated fratricide among the streptococci. For both *Streptococcus mutans* and *Streptococcus pneumoniae*, just prior to their becoming competent (able to uptake DNA), they produce and release bacteriocins, which will kill their neighbors and thus ensure a ready supply of DNA for transformation [82–84].

The third requirement for HGT are molecular mechanisms for DNA transfer or uptake. The enterics and pseudomonads largely use pili-mediated conjugational methods where the donor DNA comes from a live bacterium, whereas most of the respiratory pathogens (both Gram-positives and Gram-negatives) use transformation systems for the uptake of DNA from the environment. Both mating and transformation have been demonstrated to be up to 10^4-fold higher in biofilms than in planktonic forms; bacteria of all types, as we have already discussed, tend to form biofilms during the chronic infectious process. The biofilm matrix in addition to containing DNA, at least in the case of *H. influenzae*, also contains very high concentrations of pili [49] that support conjugal DNA transfer [85]. Nearly all of the chronic pathogens contain inducible HGT mechanisms, with some such as *H. influenzae* maintaining multiple systems of this type. *H. influenzae, S. pneumoniae, Moraxella catarrhalis* and *Neisserial* species all possess autocompetence and autotransformation mechanisms, whereas *P. aeruginosa*, nearly all of the Enterobacteriaceae and some strains of *H. influenzae* maintain conjugal mating systems that provide for unidirectional DNA transfer from a living donor to the recipient. The fact that these systems are active and serve a non-nutritive purpose (as opposed to using the DNA simply as a food source) is actually widely recognized by the infectious disease and clinical microbiological communities, but only in a very narrow sense. Serotype switching among the streptococci has

long been recognized, as have differences in virulence associated with serotype switching during epidemics [86], but these observations had not been widely generalized prior to 2001 when Ehrlich developed the distributed genome hypothesis (DGH). The DGH states that at the species level there is a supragenome that is far larger than the genome of any single strain within that species. Thus, the majority of genes within a species are not possessed by all strains of that species, but rather each strain contains a unique distribution of noncore genes from the species-level supragenome, as well as the species core genome (those genes that are carried by all strains of a species). These predictions, together with HGT mechansims and the polyclonality of chronic infections, provide for a setting in which new strains with unique combinations of distributed genes will be continually generated. Some of these novel strains will have improved survival characteristics under the current prevailing conditions in the host. Thus, the DGH predicts that chronic pathogens use a genomic diversity-generating mechanism as a counterpoint to the host's adaptive immune response. Our recent characterizations of the *H. influenzae* and pneumococcal supragenomes, via whole-genome sequencing of large numbers of clinical strains, has validated the DGH for these species and demonstrated that the noncore genes in each strain comprise from one fifth to one third of each of the strains' genome, and that the supragenomes are at least three- to four-times the size of the core genomes [12,13].

The genesis of the DGH was actually the original observation of transformation among the pneumococci by Griffith in 1928 [87]. In these experiments he found that there was a substance that could be extracted from killed virulent pneumococci (smooth colony phenotype) that could be used to transform avirulent (rough colony phenotype) pneumococci to virulence; thus, literally, a heritable virulence factor was obtained from dead bacteria that was able to convert avirulent strains into pathogenic strains, indicating that genes could be brought back from the dead. This work ultimately led to the characterization of DNA as the genetic material by Avery and coworkers [88], but it took another half century- plus before transformation was widely recognized as a virulence factor.

Conclusion

Population-level bacterial virulence factors are pathogenic traits that are only expressed by an organized community of bacteria and are distinct from virulence factors that individual bacterial cells display or produce, for example, endotoxins and exotoxins, enzymes that digest host intercellular matrices and immunoglobulin or host immune surveillance avoidance strategies such as siaylation of surface proteins. Currently recognized population-level virulence traits can be grouped into one of three categories: biofilm formation with its associated EPS matrix; QS and other intercellular communication systems that provide for coordinated activity; and HGT mechanisms that act as diversity generators. As a group, these traits ensure heterogeneity at many levels within a microbial population, which provides the community as a whole with an increased likelihood of survival as no single environmental stressor is likely to be effective against such a plural population. Note that the maintenance of diversity for the sake of the community is the hallmark of multicellularity, and therefore it is critical that, as scientists and physicians, we recognize that bacteria are unique in that they experience natural selection both as individual cells and as communities of multicellular organisms. It is this duality of their existence, in no small part, which makes them such excellent survivors and provides us with a continuous challenge in their management.

Future perspective

It is highly likely that as the study of multicellularity in bacteria continues, not only will additional population-level virulence factors be identified within the framework outlined in this article, but additional classes of population-level virulence factors will be discovered as well. This inevitability of additional discoveries comes because the field is currently in its infancy. Biofilms, the oldest of the population-level virulence traits, were recognized less than 30 years ago, and have only gained broad acceptance in medicine over the past decade. QS, as a generalized intercellular communication system, was recognized as such only 12 years ago, and the realization that HGT is a supravirulence trait is less than a decade old.

Already, new higher (than biofilm)-order polymicrobial structures are being characterized; Schaudinn et al. have recently described the formation of rigid, repeating honeycomb-shaped structures built by certain strains of S. epidermidis that are on a scale almost unimaginable just a few years ago [89]. These caserna are tens of thousands of times the size of individual bacteria in all three dimensions and are formed by the coalescence of billions of bacteria

in a manner similar to the way endochondral bone is laid down by osteoblasts. Although the role of caserna in disease is still unknown it should be noted that the particular bacterial strains from which these structures were first observed were clinical isolates from diseased individuals. Work from the laboratories of Ken Nealson and Yuri Gorby have identified bacte-

Executive summary

Introduction

- Bacteria can live in cooperative communities in which they display many of the hallmarks of multicellular organisms.
- These bacterial communities are called biofilms and consist of both bacterial cells and bacterially-produced acellular matrices.
- Pathogenic bacteria living as multicellular organisms display multiple population-level virulence traits that are distinct from virulence traits of individual bacteria.
- Population-level virulence traits are characterized by the coordinated actions of large numbers of bacterial cells that permit the population, as a whole, to attack the host.

Biofilms & the bacterial life cycle

- The preferred mode of growth for most bacterial species is as a surface-attached biofilm that ensures the bacteria will remain in their environment of choice.
- Quantitative analyses of bacteria in numerous environments have demonstrated that the vast majority live within biofilms as opposed to free-swimming planktonic forms.
- The medical community has been particularly slow to adopt the biofilm paradigm, in contrast to other microbiological disciplines, but the vast majority of bacterial infections are biofilm related.
- Bacteria exhibit a life-cycle consisting of reversible attachment, irreversible attachment, biofilm growth, seething and escape of planktonic forms.
- The biofilm matrix consists of DNA, other polysaccharides and proteins, and can be rapidly remodeled by the bacteria in response to environmental stressors.

Ramifications of biofilm infections

- Most bacteria in biofilms are metabolically quiescent, which makes them resistant to almost all antibiotics that are designed to kill rapidly metabolizing and dividing bacteria.
- Biofilms provide protection from both the humoral and cell-based arms of the host defense systems.

Intracellular communication systems

- Nearly all pathogenic bacteria possess intercellular communication systems termed quorum sensing (QS) in which all of the bacteria within a population express an autoinducer (AI) molecule that binds to an intracellular or transmembrane receptor.
- Gram-negative species use acylated homoserine lactone AIs and Gram-positive species use modified peptide AIs. There is also a third class of AIs that serves as a *lingua franca* among many bacterial species.
- QS permits the assessment of the number of bacteria per unit volume and serves as a means to ensure the coordinated expression of virulence factors.
- An example of QS-based virulence is *Staphylococcus aureus*-based toxic shock syndrome.
- QS is also used to initiate horizontal-gene transfer (HGT), another population-level virulence factor.

Horizontal-gene transfer mechanisms

- HGT mechanisms have only recently been recognized as a virulence trait.
- HGT mechanisms are supravirulence factors as they provide for the acquisition of all other virulence traits.
- By providing for strain evolution during the infectious process, HGT mechanisms ensure persistence as they provide a counterpoint to the host's adaptive immune response.
- Chronic infections possess all of the elements necessary for HGT including: natural polyclonal infections (a source of a large number of distributed genes); a source of DNA in the biofilm matrix (extracellular DNA available for uptake); autocompetence and autotransformation systems (molecular mechanisms to uptake foreign DNA); and high rates of HGT in biofilms (biofilm bacteria have HGT rates 3–4 orders of magnitude higher than planktonic bacteria).

Future perspective

- Population-level virulence traits including biofilms, QS and HGT have only recently been discovered or recognized as such.
- It is likely, as the multicellularity of bacteria is further explored, that additional population-level virulence traits will be identified.
- Recently it has been recognized that some pathogens have the ability to build higher order, regularly repeating 3D structures termed caserna. It is likely that these structures will be found to have a pathogenic aspect.

rially produced nanowires that serve as electron transporters for bacteria in biofilms that are not surface attached, but wish to exchange electrons with a metallic surface to which their biofilm brethren are attached [90,91]. These wires then serve as action-at-a-distance devices, which permit all of the bacteria within a large 3D community to tap into scarce resources that are limited by surface area considerations. Assuming maximum parsimony, it is likely that similar structures will have been adapted by other bacterial species for the movement and transfer of all sorts of metabolites and waste products, and the further characterization of long-range bacterial structures will likely be a fruitful area of endeavor for years to come.

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