Published in final edited form as:
Clin Orthop Relat Res. 2005 August ; (437): 20–24.

Bacterial Plurality as a General Mechanism Driving Persistence in Chronic Infections

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Abstract

Classical methods for the study of bacterial pathogens have proven to be inadequate to inform with respect to chronic infections including those associated with arthroplasties. Modern methods of analysis have demonstrated that bacterial growth patterns, ecology, and intra-species heterogeneity are more complex than were envisioned by early microbiologists. Cultural methods were developed to study acute, epidemic infections, but it is now recognized that the phenotype associated with these diseases represents only a minor aspect of the bacterial life cycle, which consists of planktonic, attachment, biofilm, and dispersal phases. Over 99% of bacteria in natural populations are found in biofilms which contain multiple ecological niches and numerous phenotypes. Unfortunately, the effort to develop antibiotics has been directed solely at the planktonic minority (associated with systemic illness) which explains our inability to eradicate chronic infections. In this study we establish a new rubric, bacterial plurality, for the understanding of bacterial ecology and evolution with respect to chronic infection. The fundamental tenets of bacterial plurality are that the bacteria within an infecting population display multiple phenotypes and possess multiple genotypes. Phenotypic plurality is embodied in the biofilm paradigm and genotypic plurality is embodied in the concepts of the supra-genome and the distributed genome hypothesis. It is now clear that bacterial diversity provides bacterial populations, as a whole, the ability to persist in the face of a multi-faceted host response.

Introduction

Our existing paradigms of clonal isolation and pure culture, passed down from Robert Koch (1884) have served us well in the study and control of acute bacterial infections; however, these paradigms are insufficient to inform us about bacterial persistence in the face of traditional antimicrobial therapies and host defenses. Therefore, a new rubric is needed for the understanding of chronic infections. When metabolically active bacteria persist on a mucosal surface (8, 28) or an implanted device (24) following months of treatment it is clear that the physiological processes employed by the bacteria are unique when compared to their laboratory-cultured counterparts.

One of the basic tenets of bacteriology, taught for over a century, is that bacteria are clonal with respect to phenotype and genotype. These concepts are implicit in the paradigm of clonal

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All of the authors have received funding from Allegheny Singer Research Foundation, this work was supported in part by NIDCD grants 3R01DC02148 (GDE), 2R01DC04173 (GDE), and R01DC05659 (JCP), and the Health Services and Resources Administration (GDE).

isolation and pure culture (10). Indeed, it is the success of these concepts in the treatment of acute bacterial infections, that lead to the establishment of the paradigm in the first place. However, the continued reliance on cultural methods of analysis by medical microbiologists and clinical practitioners has resulted in what we now know, is an oversimplified picture of bacterial infections. This inadvertent straight-jacketing of the medical community's thought processes accounts for the overly long delay in the development of a model for the study of chronic bacterial infections (6). Even when confronted with an alternative understanding of bacterial growth, it has taken decades for the concept to disseminate and for the medical community to begin to embrace it (3).

The application of new diagnostic modalities to the study of clinical infections (12, 27) about a decade ago suggested that there was extensive phenotypic diversity within bacterial populations and that bacteria were protected within biofilm communities (8). These findings were reliant on advances such as the polymerase chain reaction and the confocal laser scanning microscope (13, 27,37). More recently comparative genomic studies, conducted using data from genic diversity panels and whole genome sequencing, have convincingly demonstrated that there also exists substantial genomic plasticity among bacterial strains within a population or species (30, 31). Thus, it is clear that even mono-species bacterial populations are neither phenotypically, nor genotypically clonal.

The development of a new understanding of bacterial ecology, which includes the realization that bacteria have a developmental life cycle (5, 38), along with the understanding that horizontal gene transfer is the driving force in strain evolution (30) have together provided the impetus for our development of a new over-arching description of bacterial pathogenesis. In an effort to bring together within a single rubric all of these observations regarding bacterial heterogeneity we have developed the concept of bacterial plurality as a theoretical construct to inform with respect to bacterial chronicity and persistence.(6, 7)

Bacterial Plurality

Bacterial plurality embodies the concepts of phenotypic and genotypic diversity within an infecting population of bacteria. It should be noted that phenotypic diversity can arise both from altered gene expression (based on microenvironmental differences) from genotypically identical cells, and also from the expression of different sets of genes possessed by genotypically distinct cells. Bacterial plurality also embraces the hypothesis that horizontal gene transfer among genotypically distinct bacteria acts as an evolutionary engine for the creation of new strains, some of which will have a selective advantage under the prevailing conditions of the host. In the following sections we review the experimental and conceptual data which supports these conjectures. Much of this work is recent and some of it has not yet been published.

Phenotypic diversity

One of the most important conceptual parameters to understanding bacterial persistence was the realization that the bacteria that exist within a biofilm are positioned within many different microenvironments defined by nutrient availability, pH, and oxidizing potential(1, 2, 3, 16, 29, 36). Therefore, to adapt to these myriad niches, the bacteria (even if they are all one species) show numerous phenotypes and enormous metabolic and replicative heterogeneity. (6, 7, 16, 17, 29, 36, 38, 39) This heterogeneity provides the biofilm community with great capacity to withstand challenges, whether from host defense systems or pharmaceuticals. A better way to view a biofilm is to think of the biofilm as a complex organism, with each of the individual bacteria analogous to the cells of a metazoan.

One of the most striking discoveries is that the biofilm, as an organism, has antibiotic resistance levels that are three orders of magnitude greater than those displayed by planktonic or free-living bacteria of the same strain. (4, 36). However, bacterial enumeration experiments performed on naïve biofilms treated with antibiotics shows that most of the individual bacteria are killed. Therefore, the number of truly resistant organisms within the biofilm before treatment is quite small. Importantly, the minority of bacteria that persist after treatment will very rapidly repopulate the biofilm (11, 16, 17, 29). Furthermore, subsequent antibiotic treatment of the repopulated biofilm will result in a much more modest reduction in viable bacteria, indicating that the repopulated biofilm is much more resistant than the original growth. However, after dispersion, the resultant planktonic bacteria arising from the repopulated biofilm are still antibiotic sensitive, indicating that the resistance is part of an inducible process unique to bacteria in the biofilm state.

From a clinical perspective, this means that traditional antibiotic therapy will never be successful against biofilm bacteria and that other modes of treatment, most promisingly multicomponent therapies that target biofilm-specific processes, need to be developed to prevent biofilm formation in the first place. (6) This is because even a 5 or 6 log kill is useless when there is always a nidus of infection that persists and that will immediately repopulate the biofilm with mostly phenotypically resistant bacteria. One can think of the persistent cells after antibiotic therapy as akin to the stem cells of metazoans, which permit rapid organ regrowth after damage, as in the case of the vertebrate liver.

Genotypic Diversity

Bacterial plurality also embodies the concept of genotypic diversity, which includes two separate phenomena. The first is genetic heterogeneity, which is defined as different organisms within a population or species having different alleles of the same set of genes—a widely recognized feature of natural populations. The second phenomenon is genomic plasticity, which embodies the concept that individual strains within a population each have a unique distribution of contingency genes (not alleles) from a population-based supragenome (the distributed genome hypothesis), i.e. all organisms of the same species do not have the same set of genes, and no organism contains the full complement of genes of the species (6, 7, 15, 30, 31). The phenomenon of genomic plasticity results primarily from horizontal gene transfer (HGT) processes, which provide for unidirectional (always unequal) genetic exchanges among micro-organisms. Horizontal gene transfer processes include mating, transformation, and transduction. Mating is a process in which two live bacteria temporarily are joined by a pilus (or related structure) through which one of the bacteria sends a copy of its deoxyribonucleic acid (DNA) into the other organism. Transformation is an active process through which a bacterium takes up DNA from its environment and integrates it into its own genome. Transduction results when a lysogenic bacterial virus, or phage, excises itself from the host genome and takes some of the host's genes along with its own genes and then reinserts the previous host's genes into the genome of its next host.

Nearly all species (prokaryotes and eukaryotes) have developed mechanisms for the exchange of DNA. The DNA exchange provides for diversity within a population as without diversity, (i.e., a clonal population) all organisms within the population will have the same level of fitness with respect to each environmental challenge. Therefore, in clonal populations, if a change affects one organism adversely, the entire population is in jeopardy; however, in a genetically diverse population, the environmental change may increase the reproductive fitness of some organisms while decreasing the fitness of others. Variability among individuals helps to ensure that the population survives during periods of environmental change. Therefore, evolutionary pressures have selected for mechanisms that generate diversity across nearly all phylogenetic

boundaries. In bacteria the primary mechanism for generating diversity is HGT, whereas in eukaryotes it is sexual reproduction

Only organisms that do not rely on sexual reproduction can evolve HGT mechanisms. The absolute need for chromosomal pairing in sexual reproduction precludes HGT mechanisms. Synapsis (the side by side pairing of homologous maternal and paternal chromosomes at the start of meiosis) would eliminate all DNA acquired through HGT processes as check point control mechanisms would arrest the cell before mitosis. It has been only during the recent post-genomic era that microbiologists have understood the relative importance of HGT mechanisms in prokaryotic evolution. It is important to understand that it is the lack of sexual reproduction, which provides microorganisms with a much greater range of options for genetic recombination.

A critically important corollary to the distributed genome hypothesis is that the population-based supragenome is far larger than the discrete genome of any single extant bacteria. Recent studies of naturally infecting populations of *Haemophilus influenzae* have evidenced that there are multiple genotypes present simultaneously during the infectious process and that over time recombinants arise with mixed parentage. (14, 23, 33–35) Shen et al. (30) recently have shown that 10% of the genes present in any given clinical isolate of *H. influenzae* are unique with respect to the sequenced laboratory strains of these species. Of even greater interest is that these authors show that no two clinical strains contain the same complement of genes. In other words, every clinical strain has a unique distribution of genes from the supragenome. These authors also have shown that it is impossible to use classical phylogenetic tree building based on allelic differences to determine the overall relatedness of strains. An exercise in tree building was done using seven different housekeeping genes for 11 clinical strains of *H. influenzae.* Surprisingly, even using housekeeping genes, that are not under selective pressure from the hosts' defense systems, the authors found that each gene gave a completely different tree with respect to the relatedness of the 11 clinical strains. (30). These observations firmly establish that HGT is the driving evolutionary force among pathogenic bacteria. The presence of multiple strains, each with a unique genomic complement, provides for the continual reassortment of genes among strains as a general pathogenic mechanism associated with chronic infections to ensure persistence.

There are several threads of evidence that can be taken to support the distributed genome hypothesis including direct comparative sequencing studies among pathogenic clinical strains of bacteria (22, 30). The first line of evidence is the evolution and maintenance of large and complex autocompetence and autotransformation systems for the uptake and chromosomal integration of foreign DNA by gram-negative and gram-positive pathogens (9, 21, 32). This observation is important for multiple reasons including the fact that the overall genome size of these pathogens is very small and the number of genes and operons involved in encoding these functions is very large. The fact that these highly complex processes are retained in the face of enormous selective pressure to minimize genome size is strong indication that there must be continuous selective pressure for bacteria to be able to take up DNA from the environment. (19, 25).

The fact that some of these organisms may use nucleic acids as a nutrient source is insufficient to explain (1) why only one strand is degraded on uptake with the second strand remaining available for transformation (26); (2) why many species have developed species-specific signal uptake sequences that preferentially ensure the DNA brought into the cell is from their own species (32); (3) why there are separate enzymatic functions encoded within the various competence operons that are not required for nucleic acid catabolism, but are absolutely required for DNA integration and transformation (7).

The second provocative line of circumstantial evidence is the discovery that the principal component of the extracellular matrix within the *Pseudomonas aeruginosa* biofilm is actually DNA; (40) in other words, the bacteria are essentially taking a bath in DNA. Moreover, ongoing studies within the Center for Genomic Sciences show that 10 to 15% of all genes in all pathogenic strains of *H. influenzae*, *S. pneumoniae*, and *P. aeruginosa* are unique when compared against the sequences of the laboratory type strains (6, 15, 30). Evolutionary origin studies, however, indicate that the majority of these novel genes, for any given species, have existed within that species' supra-genome for as long as most of the genes in their genomes.

Shen et al (30) and Hu (15) showed that no two clinical strains of the same species have the same set of these unique genes. Genomic plasticity exists to a high degree among the clinical strains wherein each strain receives (through a combination of vertical and horizontal transmission modalities) a unique distribution of genes from the population supragenome. This genomic plasticity combined with mechanisms (secretion and uptake of DNA) to continuously create novel gene combinations through recombination and reassortment support the distributed genome hypothesis. Authors of studies of DNA exchange have shown log-fold higher rates of transformation in biofilm bacteria than planktonic forms (20). Therefore, it would seem that bacteria in biofilms use a strategy of continuous genic reassortment to produce huge numbers of novel strains, a small percentage of which will have a selective advantage for the particular host environment in which they find themselves (6). This genomic dynamism provides a mechanism for persistence and helps to explain the chronicity of biofilm infections. Moreover, these observations shed light on why it has been so difficult to establish true models of chronic bacterial infection in animal models using clonal isolates, mainly that the input genomic diversity is too limited to provide for reassortment and adaptation to the environment.

Discussion

Bacterial plurality embodies the twin concepts that natural infecting populations use both phenotypic heterogeneity and genotypic plasticity to persist in the face of multicomponent host defense mechanisms and antimicrobial treatment. The recognition that bacteria can adopt multiple phenotypic states, including biofilms, provides us with an explanation of the mechanism of how the population as a whole has an increased level of fitness (37, 38). This is because no single environmental pressure will negatively impact all bacteria to the same degree. The recognition that bacteria have a life cycle (5) and use a strategy of functional and metabolic diversification within biofilms is akin to the phenomenon of differentiation in metazoans. These realizations also point to the fact that evolution is acting on the biofilm as a whole, not on individual bacterial cells. Horizontal gene transfer among co-infecting strains is believed to be the mechanisms by which bacteria generate genotypic diversity and we hypothesize that this genetic exchange continuously produces new bacterial strains, some of which will be better suited to persist within the host (30).

Bacterial plurality predicts that bacteria that have the ability to take up foreign DNA and be transformed would have a selective advantage over those that can not. The fact that most pathogens have autocompetence and autotransformation processes for the uptake and integration, respectively, of foreign DNA is strong circumstantial support of this hypothesis. However, direct experimental evidence demonstrating increased fitness for transformable strains versus nontransformable strains is lacking. Ongoing studies in our laboratories are designed to knock-out genes associated with transformation in bacterial pathogens. These knock-out strains will then be compared with their cognate wild-type strains for persistence during polyclonal chronic infections using an animal model. We predict that the knock-out strains will be at a selective disadvantage and will overtime disappear from the infection.

The theory of bacterial plurality provides both a rubric for the understanding of what were previously conflicting observations with regard to chronic infections; and a framework from which predictions about bacterial behavior can be made and tested. This represents a step forward in our ability to model and understand chronic bacterial pathogenesis.

Applying the paradigm of bacterial plurality to orthopedic implant infections it is easy to see that they share many of the attributes that have been rigorously associated with bacterial plurality in other chronic infections such as otitis media with effusion. Arthroplastic infections are chronic in nature and are recalcitrant to antibiotic therapy, yet rarely yield culturable bacteria. However, preliminary molecular diagnostic studies performed on specimens recovered from revisional arthroplasties have demonstrated the presence of bacterial DNA and messenger RNA (mRNA) corresponding to one or more of the staphylococcal species. Moreover, CLSM imaging have revealed the presence of cocci encased within a matrix. Taken together these findings suggest that bacterial biofilms are present on orthopedic implants. These findings would explain why it is nearly impossible to eradicate arthroplastic infections within any treatment short of removal.

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