The Problem of Culture-Negative Infections

G.D. Ehrlich, P.J. DeMeo, and J.W. Costerton

Abstract Because modern medicine suffers increasingly from the "silo" phenomenon, in which each specialty ponders its problems in isolation, the gradual emergence of a generalized threat to millions of patients is thus poorly countered by the disconnected efforts of small teams that address the same theme without the recognition of common ground. The recent recognition that bacteria have reverted to their natural biofilm strategy (Costerton 2007) in attacking human hosts, in response to advances in immunization (vaccines) and therapy (antibiotics), has been perceived in a piecemeal fashion that is slowly spreading amongst the silos. We respond to medical threats in relation to the immediacy of the dangers to the patient, so the first reaction was to the phenomenal resistance of biofilm infections to antibiotics and to host defense mechanisms, and the past three decades have seen a series of tactical maneuvers involving surgical resection and high-dose antibiotic therapy. While medicine reacted to this serious threat of overt bacterial infections that were not prevented by vaccination, and that persisted in spite of seemingly suitable antibiotic therapy, another equally serious biofilm problem was emerging at the bottoms of several silos. Experienced clinicians in many specialties saw cases in which they were certain that bacteria were involved, because all of the classical signs of infection were present, but the gold standard of diagnosis (culture) was negative. Some of these cases involved medical devices (Khoury et al. 1992), others involved infections of compromised tissues (Hoiby 2002), but the overall fight, conducted in isolation in many silos, was to decide on the correct antibacterial

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strategy when the bacterial etiology of many important diseases (otitis media, prostatitis) was called into question by negative cultures. Bacteria do not respect the silos created by clinicians and scientists. They have switched from an acute frontal attack by planktonic cells, to a strategy of biofilm growth and chronic attack on infected tissues, and the most serious long-term effect of this tactical change may be that they evade detection by the classic methods of Medical Microbiology.

1 Silos in Clinical Medicine

It may be instructive to examine one particular medical silo (Ear, Nose and Throat = ENT) because the biofilm concept and the most refined molecular diagnostic capability came together in the team of Chris Post and Garth Ehrlich in that specialty. Culture data from otitis media with effusion (OME) were so consistently negative that some practitioners had suggested that it was a nonspecific inflammatory condition, and the basic bacterial etiology of the disease was cast into doubt. Clusters of bacterial cells could be seen by direct microscopy in the effusions from the ears of these patients, and DNA-based PCR methods (Post et al. 1995) showed the presence of very large amounts of DNA from the three major putative pathogens that occasionally grew in culture. When questions were raised about the viability of the bacteria in the effusions from the middle ear, the team used reverse transcriptase (RT)- PCR to detect short-lived messenger RNA (Rayner et al. 1998) to prove that the bacterial pathogens were both present and alive at the time of sampling, which allayed suspicions that antibiotic therapy alone could account for the negative cultures. In an elegant "coup de grace," the team then provided direct microscopic and molecular evidence (Hall-Stoodley et al. 2006) that OME is caused by bacteria growing in biofilms in the middle ear and that culture negativity is just as much a characteristic of this biofilm disease as is resistance to antibiotics and host defenses. In another silo (chronic wounds) the expert application of modern molecular techniques (Dowd et al. 2008) has proved that cultures only detect a small proportion of the bacterial and fungal species that are actually present, and that the clinical management of these complex infections can be radically improved using this accurate and pertinent information.

The inference from these scattered examples is disturbing because, if biofilm infections are indeed significantly more difficult to detect by conventional culture methods, tens of millions of patients are at risk for missed diagnoses. The CDC and the NIH have estimated that biofilm infections now constitute 65 and 80 % (respectively) of bacterial infections treated by physicians in the developed world, and a recent publication reports (Wolcott et al. 2010) that these infections affect 14 million, and kill $>$ 400,000, Americans each year. Culture methods are the only FDA-approved and universally available technology for the detection and identification of bacterial and fungal infections in most of the developed world. In view of these well documented failure of cultures to detect such common biofilm

infections as OME in children, and chronic UTI and prostatitis in adults, we must speculate concerning whole disease categories that are presumed to be nonbacterial because of consistently negative cultures. Perhaps peptic ulcers are not the only disease whose therapy will change radically when its etiology is more accurately understood, and perhaps more individual patients will be treated more effectively when the identity of any and all bacteria in their tissues can be accurately determined. We live in the era of precise DNA-based forensics, and of whole genome sequencing, and patients will be best served when we mobilize these resources for the prompt and accurate diagnoses of bacterial disease.

2 The Rational Basis of the Problem of Culture Negativity

Bacterial biofilms have now been studied very extensively, in the very well funded ($100 million to date) Center for Biofilm Engineering (CBE) and in hundreds of labs worldwide, and a clear picture of the structure and function of these very successful communities has emerged (Fig. 1). The majority of the bacterial cells in most microbial communities grow in the biofilm phenotype, and indulge in cooperative metabolic processes, as depicted in the area around the label "heterogeneity" in the middle of the figure. These cooperative processes are facilitated by physical connections between the individual cells like nanowires (Gorby et al. 2006), nanotubes (Remis et al. 2010), and temporary connection via pili and structured eDNA (Whitchurch et al. 2002; Goodman et al. 2011), and any attempt to rip one of these resolutely interactive cells from its designated bed in the biofilm would result in a group of "dazed and confused" bacterial cells that would be unable to grow in any medium. We have transferred well washed "chunks" of single species biofilms from flow cells in which they were growing, and they have failed to establish colonies on the surfaces of agar plates on which they were placed under direct microscopic observation. Pradeep Singh has recently shown (Singh P Copenhagen biofilm meeting 2011) that cells of *Pseudomonas aeruginosa* undergo a structural change in the lipopolysaccharide (LPS) of their outer membranes that makes them unable to grow on the surface of agar plates, even if the medium is otherwise ideal for the cultivation of Pseudomonads. Biofilm cells express a radically different phenotype (Sauer et al. 2002) from that of the planktonic cells that have been studied in the laboratory, for more than 150 years, and one of their salient characteristics is that they fail to grow and produce colonies when they are "ripped from their beds" in their "cozy communities" and placed on the surfaces of agar plates.

Virtually all of the cells in a mature biofilm, in vitro and during infection, operate in the biofilm phenotype, in which the pattern of gene expression may vary from the planktonic phenotype by as much as 70 % of the expressome, and this accounts for culture negativity in many cases. However, biofilms maintain a dispersal strategy to ensure that they can colonize distant sites in their ecosystems. This results in a reversal of the protective strategies in some locations of the community to produce

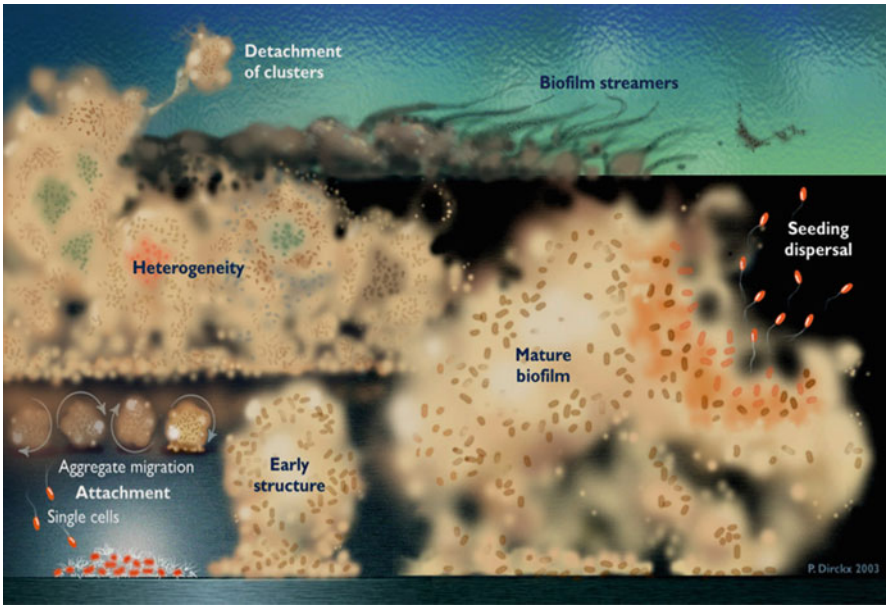

Fig. 1 Diagrammatic representation of the location and roles of planktonic and biofilm cells in a typical biofilm community

planktonic cells in a programmed manner (Sauer et al. 2004) so that significant numbers of these dispersive cells are constantly produced (Fig. 1 at 3 o'clock).

In the grand Microbial Ecology design the planktonic phenotype of each species is designed to travel far from the biofilm community, often following favorable gradients, to "find a new home" and establish new communities. These streamlined planktonic cells have no cell–cell connections, their cell walls are adapted for stability in a variety of environments, and they are specially adapted to adhere to surfaces (including agar) and to form new communities, and it is these planktonic cells that culture methods were originally designed to detect. Before the development of vaccines, planktonic bacteria from an environmental source (e.g., cholera) could enter the human gut, overwhelm the local defenses, and set up a finite number of planktonic cell "factories" that would constitute a devastating infection. Diagnosis was simple, planktonic bacteria would grow on agar plates, and modern engineering was mobilized to contain the epidemic. Other epidemic diseases (e.g., typhoid and diphtheria) had human reservoirs, but the disease was still acute in that planktonic cells attacked the host and either overwhelmed its defenses in a week or less, or left the survivors immune and generally stronger for the encounter. Again, cultures gave a good indication of the presence of planktonic bacteria, and of the species that was present, and guided both preventative and therapeutic strategies.

When acute bacterial infections became much less common, because vaccines built up effective levels of immunity, and when the medical response to these diseases improved because of the development of antibiotics that killed the planktonic cells, the bacteria evoked their basic alternative strategy and began to attack as biofilms. These biofilm diseases, which were first noticed in connection with medical devices and with compromised human tissues (e.g., cystic fibrosis), initially caught our attention because they persisted in the face of active host defenses and because they are very profoundly resistant to conventional antibiotic therapy (Costerton et al. 1999). But another characteristic of biofilm infections soon began to emerge, which was that cultures were very ineffective in the detection and identification of their causative organisms. This culture-negative phenomenon is entirely logical, because cells in the biofilm phenotype grow so poorly on agar, and the problem was complicated in the case of biofilm infections, by the fact that antibiotics had often been used for extended periods of time so that the planktonic cells which might have indicated infection had been killed. These facts combined to produce a nadir in diagnoses, because the bacteria that actually cause biofilm infections are very difficult to grow in culture, and because the planktonic cells whose presence would indirectly indicate the presence of a biofilm had been killed by host defenses or by antibiotics. Various piecemeal strategies have been tried to improve detection by cultures, including the sonication of medical devices suspected of harboring biofilms (Trampuz et al. 2007), and medical laboratories with special mandates (e.g., Mayo Clinic) have explored the use of special media to grow pathogens that are recalcitrant to culture, but special culture methods travel poorly in the cash-strapped milieu of routine cultures. The problem of culture-negative biofilm infections remains complicated, it threatens the lives of millions of patients, and it constitutes an emergency because these infections now comprise the vast majority of bacterial infections and the only FDA-approved method for their detection has a sensitivity of approximately 20 % (Costerton et al. 2011).

3 Molecular Answers to the Culture-Negative Problem

Biofilm cells contain DNA in amounts approximately the same as planktonic bacterial cells and this DNA can be analyzed to determine the species identity of one or more organisms, and the presence of specific genes that cause antibiotic resistance. DNA analysis has gradually risen from a supportive role in forensics, to a legally sacrosanct basis for child support payments or release from custody for crimes of passion, so its accuracy must be said to be the "gold standard." DNA analysis forms the technical basis for the identification of all of the bacteria naturally associated with human tissues, in the new Human Microbiome Project and in virtually all studies of bacterial populations in natural ecosystems (Hugenholtz et al. 1998), but the pace of these studies is leisurely and may extend for weeks or months. The great DNA diagnostics machine whirs in mysterious ways, to the amazement of this humble morphologist, and the time necessary to

detect specific cancer markers drops from weeks to days. The immediacy mandated by the need to detect bacterial bioterror agents has stimulated the development of a new DNA analysis method that delivers accurate data on the presence of any and all bacterial and fungal species in ±6 h. This method also detects the genes responsible for methicillin and vancomycin resistance in the same time frame. This technology (the Ibis PLEX-ID) will certainly not be the only or the definitive technology that will replace cultures in the diagnosis of bacterial infections, but the message is that DNA-based technologies have the speed to be useful in the diagnosis and management of biofilm infections.

The unblinking eye of the great DNA diagnostic machine will deliver quantitative data on the bacterial and fungal DNA present in the tissues it has analyzed, and physicians and surgeons will be projected back to the worst lectures in their least favorite class in medical school. If we walk through the process of molecular diagnosis, as it will become available during the next 2–5 years, we may be able to reduce the pain that comes when we move from seeing "through a glass darkly" to seeing all of the microbiology detail in stark bright light. If experienced surgeons simply ignore organisms they have never heard of, and concentrate on the familiar *Staphylococcus aureus* (especially MRSA), coaggulase-negative Staphylococci (CONS) and various Streptococci, we will see that these known pathogens are detected accurately and with much more sensitivity. The most pedantic amongst orthopedic surgeons may fuss about whether the very large number of MRSA in an infected prosthesis are alive or dead, which we cannot determine by the basic DNA technique, but the clinical decision to remove the prosthesis to debride extensively and to cover the wound with vancomycin can be taken with a high degree of confidence. In complex multispecies infections like diabetic foot ulcers, the detection of Candida species in a cohort of patients who had responded poorly to antibacterial antibiotics has triggered the decision to use ketoconazole, with excellent outcomes (Dowd et al. 2008). If clinicians simply stay within their comfort levels, treating organisms which they recognize as pathogens in their own specialties, and ignoring all reports of bacteria and fungi that they do not recognize, biofilm infections will be recognized earlier and treated more effectively. The universal Ibis technology that detects any and all bacterial DNAs is already "filtered" to remove any detections in which less than 3 of the 16 PCR primers did not "fire" to produce amplicons, and the system can be adjusted so that DNA present in very small amounts (e.g., $<$10 genomes/well) or DNA characteristic of known nonpathogens are simply not reported unless requested.

Physicians and surgeons who have treated biofilm infections during the era in which cultures have predominated have formed a bewildering variety of impressions of the comparative pathogenicity of various microorganisms. In a single coffee nucleus I have been told that *Staphylococcus epidermidis* is a nonpathogenic "contaminant" by one prominent surgeon, while another even more prominent surgeon said that "Staph epi" is a horrible pathogen probably worse than MRSA and that it will "eat the whole leg." We will need a prolonged period of accurate microbiology to resolve these issues. In orthopedic infections involving single organisms it will be instructive to assemble all clinical data to determine

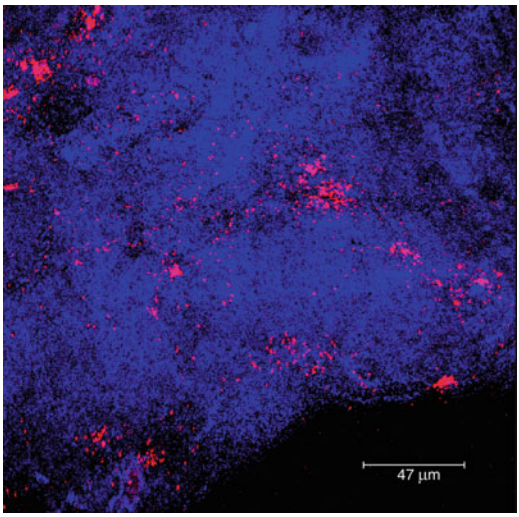

Fig. 2 Confocal micrograph of tissue from a nonunion secondary to an open fracture of the tibia stained with the species-specific 16S FISH probe for *Enterococcus faecalis*

whether *Enterococcus faecalis* can actually cause the failure of a prosthesis or the nonunion of a fracture. When we visualize cells of *E. faecalis*, in tissues adjacent to a nonunion secondary to an open fracture (Fig. 2), we see that large areas of the tissue (blue in reflected light) are permeated by these coccoid cells, and that they have formed extensive biofilms in several areas. There is no possibility of error in these direct microscopic data, because human tissues contain none of the bacterial 16S rRNA against which the probe is directed, and because the bacterial cells we see are cocci less than 1 µm in diameter that are integrated within the tissue.

These direct microscopic data tell us that cells of *E. faecalis* had occupied tissues adjacent to this nonunion, and that they were present as biofilms, but only clinical data on a cohort of similar patients infected only with this organism will gradually allow us to assess the relative pathogenic potential of this organism in orthopedic infections. If it transpires that *E. faecalis* can cause overt orthopedic infections, alone or in combination with other species, it can be added to the list of known pathogens and suitable antibiotic therapy can be used in combination with surgical debridation.

Propionobacterium acnes is another bacterium whose role in orthopedic infections is sporadically invoked, even though it is a recognized pathogen in infections following neurosurgery (Nisbet et al. 2007). This gram-positive rod becomes predominant in the human skin (especially in sweat glands) just prior to sexual maturity, particularly in the axilla, and it is rarely detected by culture because it is slow-growing and requires special media and anaerobic incubation conditions. We have found *P. acnes*, often in very large and elaborate biofilms, in nonunions secondary to open fractures in which elements of the broken bone have penetrated the skin before the fracture was surgically reduced with internal fixation. However, the most extreme *P. acnes* biofilm (Fig. 3), we have seen in >200 orthopedic cases, was in the tissue removed from a painful locus adjacent to a healed fracture. The very

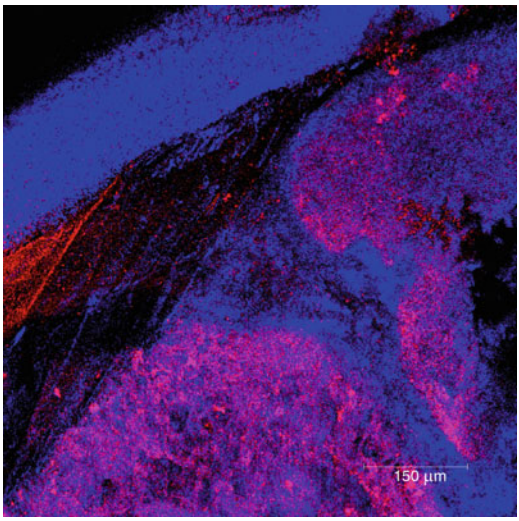

Fig. 3 Confocal micrograph of tissue removed from a painful locus adjacent to a healed fracture, and reacted with the species-specific 16S FISH probe for *P. acnes*

extensive biofilm, which is pink where the bacteria are integrated into tissue, and red where the bacterial biofilm is growing between tissue elements, has not invaded all of the available tissues (see blue region at the top of the micrograph), and its presence did not prevent the healing of the fracture.

These direct observations of the structure of *P. acnes* biofilms will be replicated in cohorts of patients in which prosthesis failure or nonunion was associated with the presence of this organism alone, and clinical data will be mobilized to determine whether *P. acnes* alone can cause these orthopedic misadventures. In the event that this organism does proved to be a *bona fide* pathogen, therapy will be simplified, because this arcane organism is still sensitive to penicillin G and the neurological lesions were readily cured by debridation and penicillin therapy (Nisbet et al. 2007).

Sensitive methods for the detection of bacteria will put an end to the archaic concept that the human body is a largely sterile edifice, into which bacteria make occasional damaging intrusions. The presence of *Helicobacter* in the human stomach was not detected until special culture techniques were developed following the discovery of its role in duodenal ulcers (Forbes et al. 1994), bacteria have been found deep in the human female reproductive tract and some have been associated with normal pregnancy (Romero et al. 2008), and it is patently clear that many organisms leave their usual lairs in the human microbiome and circulate as transient bacteremias. Most replacements of knee and hip joints are to correct advanced osteoarthritis that has persisted for decades, and molecular methods have shown that ± 30 % of the joints that are removed in primary arthroplasties show the presence of *T. denticola*, which is a spirochete whose pathological reservoir is the infected sulcus in periodontitis. We can see the spiral cells of *T. denticola* (Fig. 4) associated with the tissues of osteoarthritic joints that have been removed in primary arthroplasties, in samples stained with species-specific FISH probes, but we can only speculate concerning whether these organisms may play any important role in the arthritis.

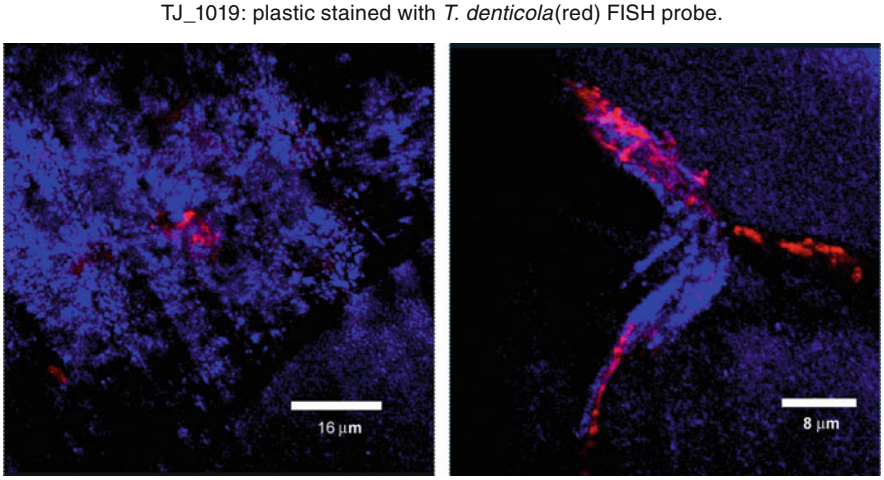

Fig. 4 Confocal micrograph of a preparation of tissue from an osteoarthritic knee, which was replaced by a primary arthroplasty, which has been stained with the species-specific 16S FISH probe for *Treponema denticola*

It may be germane to note that the elongated spiral cells of *T. denticola* seen in these preparations are seen in complex biofilm-like structures that are adjacent to the human tissues (blue diffuse), and are rarely seen to penetrate these tissues. This spatial arrangement may reflect an association, in the absence of a causal relationship. Now that we can detect the presence of any and all bacteria in orthopedic samples, we will gather clinical data to determine whether the presence of this notably motile dental pathogen has any significance when, as often happens, it is present in a total joint infection (TJI).

4 Multiple Paradigm Shifts

While the whole world of bacterial population analysis moved from cultures to DNA-based methods in the early 1980s, Medical Microbiology was prevented from making this paradigm shift, because of the necessity for rapid results to guide life-and-death decisions. This paradigm shift from cultures to DNA-based methods coincided with another major paradigm shift in Medical Microbiology, in which acute diseases that are easily detected by culture methods were gradually replaced by biofilm diseases that are inherently difficult to detect by culture but are readily detected by DNA-based methods. The result of this double paradigm dilemma has been that physicians and surgeons who treat infected patients, and infectious disease specialists who consult in this life-saving process, have been faced with increasing numbers of patients who appear by all criteria to be affected by bacterial infections, but whose cultures are negative. A double paradigm shift puts the

vulnerable human being at risk for "intellectual whiplash," but the speed of the novel DNA-based methods has now been improved such that they are now much more rapid than cultures, and the concept of biofilm infections is now widely accepted in most of the silos of modern medicine. The imminent commercialization of the Ibis system in the USA, and of the SeptiFast system in Europe, will best serve the medical community (and its patients) if we can inform the mental gymnastics necessary to accomplish this double paradigm shift in Medical Microbiology.

When a dim flickering light has barely illuminated the dangerous bacteria in each clinical area, the names of the really dangerous pathogens are well known, and the brighter light will show the same organisms and will reveal their antibiotic sensitivities in a more timely fashion. Notorious pathogens like MRSA will be detected rapidly, and the biofilm concept will be invoked to recommend that these protected bacterial communities be removed by careful surgery, and "mopped up" with specific antibiotics. This will be the practical baseline improvement provided by the double paradigm shift but, for the more perceptive, the increased sensitivity of the new molecular methods will report the presence of bacterial species whose role in pathogenicity is not yet known. Accurate microbiology can then be combined with careful clinical research to study cohorts of patients whose surgical samples contain only one putative pathogen to establish, unequivocally, that the presence of that organism can cause nonunion or implant failure. While dim lights are comfortable, and we gradually come to understand everything that we can see, wonderful things happen when we turn the light on to full intensity and see all of the bacteria that are arrayed against us: and maybe even some that are on our side!

5 Historical Origins of the Problem of Culturability

Antony van Leeuwenhoek used his primitive microscope, in 1716, to tell the world that "animalcules" less than 1 μm in size existed in the "scuff" from his non-too-immaculate teeth, and that they betrayed their viability by swimming vigorously through fluids. This curiosity went largely unremarked until, as is often the way in science, these small creatures were implicated as threats to humans survival and to the Gross National Product of France in the early to mid-1800s. The threat to the French GNP involved the death of silkworms and strange tastes that occurred in iconic wines and cheeses (mille horreurs!), and the white knight was Louis Pasteur. Louis was process-oriented, he was aware of the polymicrobial nature of most microbial ecosystems, and he bent pathogens and symbionts (alike) to his will by manipulating whole systems so that whole microbial communities obeyed human commands. The process of pasteurization and the burgeoning and vibrant Institute Pasteur, in Paris and in many other locations (including Tahiti), stand as tributes to his genius and his perceptions. Hundreds of processes, ranging from simple sewage treatment to the production of taxol by exquisitely engineered strains of microbes from the bark of the Pacific yew tree, can all trace their intellectual roots to Louis' concept of bacteria as members of integrated communities.

The response to the threat posed by bacteria to human health was equally successful but, in conceptual terms, the polar opposite. When faced with the problematical haystack of mixed species communities, Robert Koch chose to extract the "needle" and to examine it minutely, and in isolation from the neighbors and partners it would have had in the haystack. This separation of the needle from the haystack relied on a quirky but brilliant observation that certain fast-growing bacterial cells, especially those that caused acute epidemic infections, would grow to produce visible colonies when placed on the surface of an agar plate and separated from their fellows by progressive "streaking." The agar had to contain nutrients and salts similar to human blood or (in some cases) blood itself, the temperature and gas phases had to be optimal, and most important pathogens would grow to produce colonies with colors and shapes that betrayed their presence to the practiced eye. The arcane science of the detection and identification of bacteria by culture sprang up, with coffee Klatsch to exchange media and compare culture conditions, and mysterious additions like egg to encourage *Mycobacteria*, and Fisheria extract to tease *Legionella* out of the haystack and allow it to grow sufficiently to produce colonies. A prematurely wise graduate student in the CBE once observed that cultures resemble gardening, in that one "drags a rake" along the path of a mixed species English garden, then shakes the rake onto rich potting soil and observed (after a few weeks) what has grown! If a plant was reproducing at the time of sampling propagules would thrive and we would record its presence, but if it was not propagating or if its seeds did not "like" the potting soil, its presence would go unrecorded even if it dominated the ecosystem being studied. The skills necessary to recover bacteria by culture were largely experiential, and often found in people who had failed mathematics in high school (like one of us = J.W.C.), and cultures received a further fillip when the whole battery of methods to determine antibiotic sensitivity were added to the paradigm. The Koch Institut, located in a leafy suburb of Berlin, is the quintessential beautifully equipped laboratory in which a team of more than 200 brilliant microbiologists will certainly refine the DNA-based techniques that will replace the culture methods that enabled its founder to (virtually) wipe out epidemic bacterial disease and to save millions of lives.

The decision to remove bacteria from their integrated communities, by culture methods, was a very effective strategy at the time that it was adopted in the mid-1800s, and its utility has continued until the end of the twentieth century. Culture methods were successful in the detection and identification of bacterial pathogens, and the data that they provided enabled the largely complete eradication of epidemic diseases, even before the modern antibiotic era. In modern times, cultures have been pivotal in the discovery of antibiotics, and in the application of antibiotics to the virtual conquest of acute bacterial infections in uncompromised patients in the developed world. While there were no practical alternatives to culture methods, these venerable techniques served us well, and they still comprise the only FDA-approved methods for the recovery of bacteria from clinical specimens and for the determination of antibiotic sensitivity. Culture methods will, of course, continue to be the preferred methods of cultivating bacteria for study in the laboratory and for certain applications like checking strain purity, but

Microbiology is an evolving science and the utility of all widely used techniques should be evaluated by clinical microbiologists on a regular basis.

6 Two Solitudes

Many microbial processes are very important for humans and two fundamentally opposite approaches to their management developed, along parallel paths, for the last 150 years. Engineering is not tolerant of failure, so processes like wastewater treatment were managed by tweaking physical parameters, like flow and oxygen supply, until the microbial systems involved behaved properly and clean water came out of taps with monotonous regularity. If additional microbial activities were required, like the removal of phosphate, these same properties were tweaked in different ways until the whole system behaved correctly and the combination of factors that gave success was recorded and reproduced *ad nauseam*. Engineers do not like complexity or surprises, so they paid very little attention to the individual bacterial species that oxidized organic materials or bound phosphate, but they hit their performance windows by knowing how the whole ecosystem responded to physical and chemical variables. They were, without necessarily knowing it, Pasteurians. Essentially, they joined the pastoral people who, from time immemorial, have taken milk or mixtures of barley and hops and gently nudged the natural microbial ecosystem in the direction of perfection by subtle manipulations.

Another bunch of engineers joined their microbiological allies to tackle the problem of microbially influenced corrosion (MIC), which destroyed marine structures and metal pipelines at an alarming rate. Because microbial biofilms destroy metals by setting up classic "corrosion cells," in which they create cathodes by their metabolic activities and stimulate the formation of corresponding anodes from which metal is lost, engineers imposed cathodic protection currents on susceptible metals and solved the marine problem overnight. They did not culture or identify the bacterial species responsible for MIC, they did not even know the details of the metabolic activities that created the cathodes, but they found that the whole process within this damaging ecosystem could be halted by imposing an overriding DC potential and the problem was solved. Cathodic protection cannot prevent MIC in the interior lumen of pipelines, for complex physical reasons, so the same team of engineers and microbiologists used the whole system approach to detect and prevent corrosion in the millions of miles of pipes that interlace our world. They developed a test in which a steel nail is suspended in an anaerobic medium, and they mobilize their defenses when the nail and the medium turn black, when the nameless organisms set up corrosion cells. Then they regularly scrape the inner surfaces of the pipe with scrapers called "pigs," they discourage the dispersed microbial community with universal biocides, and they chalk up another victory for the Pasteurian approach.

The Kochian approach to Microbiology seems counterintuitive, in light of our current grasp of systems ecology, but it suits one human imperative, and it has saved (literally) hundreds of millions of lives. This approach, which is embodied in

Koch's principals, is perfectly suited for the management of situations in which one bacterial species causes damage to the human body or to an important human asset, by its own unique activities. The imperative is imposed by organisms that can survive in mixed species ecosystems and produce toxins and/or enzymes that kill or damage humans or human assets, and we need to only think of the strains of cholera or phyloxera that killed millions and significantly degraded the quality of life of many others. Other instances would include bacterial species that can invade normally sterile organs like the brain or the liver, by dint of special invasive mechanisms or by nosocomial routes, and whose very presence in these protected redoubts causes damage or death. When the agent of a particular damage is an individual bacterial species, the removal of the needle from the haystack yields benefits in accurate diagnosis and effective treatment, and represents the correct approach to the problem. Certain bacteria, such as *Escherichia coli O-157* and *Listeria monocytogenes*, should not be in our food and any culture or PCR method that detects them is valued highly.

The success of the Kochian approach has, paradoxically, limited its relevance in the modern world. Early in the last century the isolation of the viral and bacterial agents of acute epidemic disease facilitated the development of vaccines that converted immunologically naïve human populations to functional resistance, and allowed these threats to flicker out in the developed world. Other bacterial diseases that were readily identified by acute symptoms, and easily detected by classic culture techniques, were gradually brought under control by specific antibacterial agents that evolved from simple sulfa drugs to the most complex DNA gyrase inhibitors. In the past eight decades virtually any pathogen that had the temerity to kill or threaten humans or important human assets has been the subject of a concerted counter attack by the vaccine industry or by big pharma or big agriculture, and their elimination is not complete but is (in most cases) in progress. The specialized bacterial pathogens that represented specific needles that could be removed from the haystack by culture methods have been defined and studied and analyzed, and many of them are receding into oblivion and irrelevance. But we must be wary because even the most specialized of pathogens (e.g., *Vibrio cholera*) may retreat into the environment and emerge with full virulence, if we relax our vigilance and surveillance.

Modern microscopy, like the two-photon confocal microscope with coupled deconvolution software, has allowed us to visualize all of the bacteria on solid surfaces in natural environments and on tissue surfaces in the human body. The impact of this research was devastating to traditional microbiologists in that planktonic bacteria, which had been studied assiduously by microbiologists for 150 years, were seen to comprise <1 % of the bacterial cells in natural and pathogenic ecosystems. The vast majority of bacteria live in integrated biofilm communities (Fig. 1), and the only exceptions to this general rule are (perhaps ironically) human constructs like laboratory cultures and brewer's vats, and human and other animal bodies under attack by planktonic cells of specialized pathogens. This small Kochian cycle has now run its course, because of vaccines and antibiotics, and all of the microbial systems that influence human life are now seen in terms of Pasteurian principles in which bacteria are members of functional integrated communities which

can be manipulated and controlled as whole communities. Planktonic systems are still of some minor interest, and the culture techniques that serve them well are still valid in that limited context, but virtually all of our microbial problems now involve biofilms, and we need to find room for the Petri plates somewhere between our quill pens and our hand-wound gramophones.

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